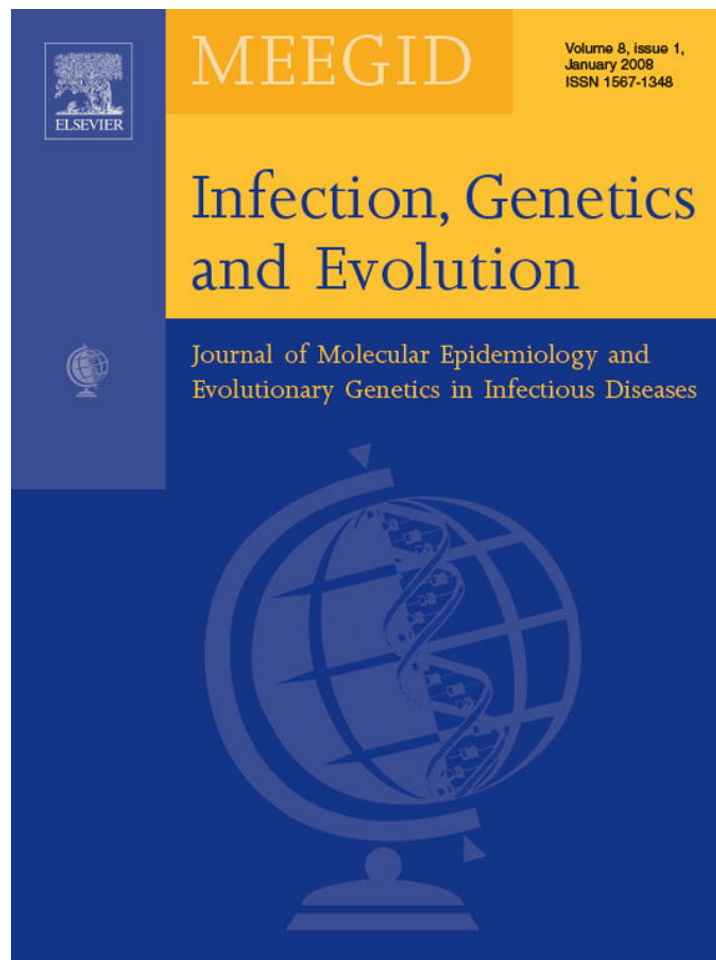


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Drug induced superinfection in HIV and the evolution of drug resistance

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Abstract

The rapid evolution of HIV drug resistance is a major cause of AIDS treatment failure. Superinfection, the infection of an already infected cell by additional virions, can be a major factor contributing to the evolution of drug resistance. However, the pattern and consequences of superinfection in HIV populations are far from fully understood. In this paper we study the implications of the fact that superinfection is regulated by HIV. We propose that superinfection is negatively associated with the success of the virus, so that more successful viruses are less likely to allow superinfection. We use computational models to investigate the effect that regulated superinfection would have on the evolution of drug resistance in HIV population. We find that regulated, fitness-associated superinfection can provide a distinct advantage to the virus in adapting to anti-HIV drugs in comparison with unregulated superinfection. Based on the results of the computational models and on current biological evidence, we suggest that the mechanism of fitness-associated regulation of coinfection in HIV is plausible, and that its investigation can lead to new ways to fight viral drug resistance.

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Keywords: HIV; Drug resistance; Computational model; Evolution; Superinfection; Coinfection

1. Introduction

The treatments to combat HIV infections have resulted in a greatly increased life expectancy for AIDS patients. However, reduced effectiveness of anti-HIV drugs due to emergence of drug resistance remains a serious problem, despite the use of a combination of anti-HIV drugs (Fumero and Podzamczar, 2003; Potter et al., 2004). HIV drug resistance evolves as a result of selection of mutations beneficial for the survival of a virus in the presence of antiviral drugs (Lengauer and Sing, 2006). With commonly used protocols for anti-HIV therapy, several mutations are required to accumulate in an initially drug-naïve virus population to make it resistant to the drug(s) (Johnson et al., 2005). Superinfection, defined as the infection of a cell by multiple virions, when combined with the high recombination rate associated with HIV replication, may play a role in the ability of HIV populations to quickly adapt to changing environments such as the presence of anti-retroviral

drugs (Potter et al., 2004; Wain-Hobson et al., 2003). Superinfection in viruses plays a similar role to sexual reproduction in higher organisms—the major mechanism allowing genetic exchange. However, previous works (Froissart et al., 2004; Bretscher et al., 2004) have demonstrated that superinfection might in fact slow the response of a viral population to selective pressures, rather than accelerate it. In this paper we use population genetic models to explore the implications of the fact that HIV can regulate the rate of its own superinfection by down-modulating CD4 and CCR4/CXCR5 receptors (Michel et al., 2005; Venzke et al., 2006). By regulating the rate of superinfection, HIV may be able to affect the degree of recombination and phenotypic mixing, as well as the fitness costs associated with superinfection (discussed below). That might have a significant effect on the ability of the virus to adapt to the presence of anti-retroviral drugs. Note that some papers use the term “superinfection” to describe the infection of already infected patient with second HIV strain. Here we use it exclusively to describe the infection of a single cell by more than one virion (as in Michel et al., 2005; Nethe et al., 2005; Venzke et al., 2006).

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An HIV virion contains two copies of a (+) strand of full length RNA surrounded by virally encoded proteins and a lipid bilayer. Since each RNA copy contains the whole HIV genome there is a built-in redundancy of genetic information enclosed in each particle similar to that found in diploid organisms. An HIV virion attaches itself to cellular receptor CD4 and then, upon rearrangement of virion surface glycoprotein, either to a chemokine receptor CXCR4 or to an alternative HIV coreceptor CCR5 (Briz et al., 2006). Attachment leads to the release of the HIV RNA and several virus-encoded proteins into the cell. HIV RNA is then reverse transcribed to DNA by HIV-encoded reverse transcriptase (RT). HIV RT has a very high error rate, producing approximately two mutations per 10^5 nucleotides (Huang and Wooley, 2005; Nowak, 1990). It is also capable of template switching or “jumping” from one RNA template to another during transcription (Nikolenko et al., 2004) (Fig. 1), which provides a major mechanism for HIV recombination. The recombination rate of HIV is orders of magnitude higher than that of eukaryotes and is estimated to be somewhere between 3 and 30 template switches per single genome replication (Levy et al., 2004; Meyerhans et al., 2003). This is a high rate even among retroviruses (Rhodes et al., 2003). However, template switching (recombination) produces new genotypes only when it occurs between two *non-identical* (+)RNA templates. Non-identical RNA's may come either from two different homozygous virions which are present in the same

cell as a result of superinfection, or from a single heterozygous virion. The formation of a heterozygous virion requires a superinfection event in the previous infection cycle. Thus, superinfection is a necessary prerequisite for productive recombination.

After reverse transcription the newly synthesized DNA is inserted into the host chromosome and serves as a template for HIV RNAs. The efficiency of HIV replication cycle at a given host environment ultimately depends on the HIV genome sequence. Presumably, most nucleotide substitutions, deletions and insertions have detrimental effects on the speed of the reproductive cycle and/or the number of HIV progeny; occasionally mutations may improve viral fitness (defined here as the ability of the virus to produce infectious offspring) for example in the case of acquired drug resistance. When more than one virion enters the cell, two or more copies of HIV genomes can be inserted into the host chromosomes. In this case there are two or more sources of each gene product. Therefore, viral genomes can “help” one another by supplying a functioning protein or RNA in place of a product of the mutated or unfit gene (Iwabu et al., 2006; Lori et al., 1992; Wilke and Novella, 2003). This phenomenon is called “phenotypic mixing”. Phenotypic mixing requires the presence of different copies of HIV genome in the same cell, i.e. superinfection. Phenotypic mixing is not always mutually useful. In particular, high rates of superinfection may lead to appearance of “cheaters”, i.e. mutants that benefit from the presence of co-infecting individuals but do not supply anything in return (Froissart et al., 2004).

Although both effective recombination and phenotypic mixing require superinfection, HIV has several mechanisms for *down*-modulation of its own receptors CD4 and co-receptors CCR5 and CXCR4, leading to decreased levels of superinfection (Venzke et al., 2006; Pastori et al., 2006; Buttica et al., 2003; Dixit and Perelson, 2005). Cells already infected by HIV are less susceptible to infection than uninfected cells. The extent of this decrease in susceptibility is termed interference.

For different viral genotypes, superinfection bears different costs and benefits. Fitter genotypes have less to benefit from recombination and phenotypic mixing caused by superinfection. Conversely, the less fit genotypes can benefit from both recombination and phenotypic mixing as long as the superinfecting viruses are not identical. There is also a productivity cost to every genotype involved in superinfection. Although the total number of virion particles made by a multiply infected cell may be higher than the number made by a single infected cell, the number of progeny virions *per genome* is likely to be lower in a multiply infected cell (Dixit and Perelson, 2005) due to competition for limited resources (Schneider and Shenk, 1987) and/or increased level of mortality of the host cell (Gilchrist and Coombs, 2006). To describe the decrease in productivity per genome we use the term “cost of competition”.

Fitness-associated superinfection may, similarly to fitness-associated recombination and sex (Hadany and Beker, 2003), give the virus an adaptive advantage by allowing it to produce environmentally advantageous (e.g., drug resistant) genetic combinations more often than to break them down. It may thus

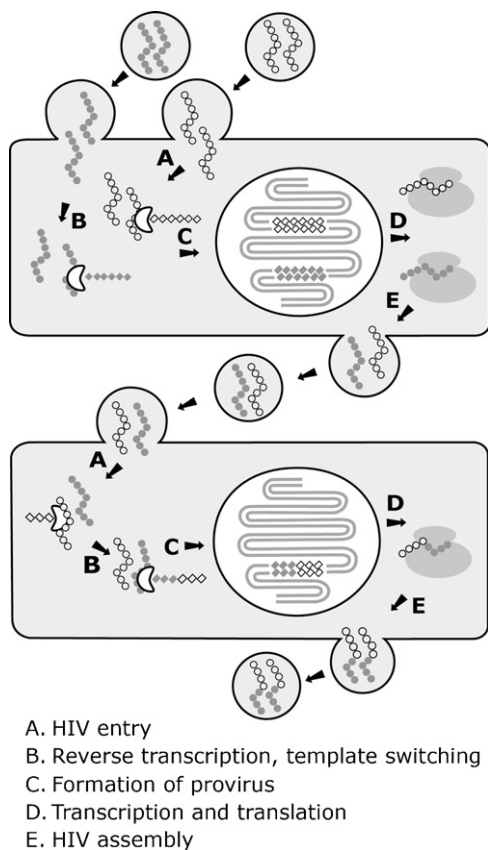


Fig. 1. The life cycle of HIV.

have a significant benefit for the ability of an HIV population to adapt to new environmental challenges such as treatment with anti-viral drugs. Fitness-associated phenotypic mixing might also create good phenotypic combinations from inferior viruses, thus allowing them to survive. The survival of more unfit genotypes would allow more rapid accumulation of deleterious mutations. However, some of these currently deleterious alleles may be advantageous under different conditions, and may be potentially beneficial in the long term by providing a wider base for adaptation.

We used computational modeling to investigate the possible implications of HIV regulation of superinfection (and thus of the probabilities of recombination and phenotypic mixing) depending on the overall fitness of the first infecting virion. We compared the evolution of drug resistance in virus populations with and without the ability to regulate superinfection. Our results clearly show that in the wide range of conditions tested, the ability to regulate the rate of superinfection brings a substantial advantage to the viral population.

2. Models

We used two types of models to study the role of regulated superinfection in the evolution of drug resistance in HIV: deterministic models and stochastic ones. In all the models we assume that in the absence of a drug, the drug-resistant genotypes have a disadvantage, because drug resistance is often achieved at the cost of enzyme efficiency (Wang et al., 2006a,b; Frankel et al., 2005).

In vivo the acquisition of multi-drug resistance by HIV is a stepwise process which requires a series of mutations (Johnson et al., 2005). In our models the HIV genome contains two drug resistance genes with two alleles each $A(a)$ and $B(b)$ (capital letters denote wild type (non-resistant) alleles and lowercase letters denote drug-resistant alleles). When drugs are introduced into the environment, the genotype ab becomes the fittest one. This genotype is produced either by two mutations $A \rightarrow a$ and $B \rightarrow b$ occurring in the same lineage, or by recombination between types Ab and aB . Given enough time after introduction of drugs, the ab genotype would spread in the population to fixation.

The fully resistant genotype ab is thus generated in the population by mutation and recombination regardless of the presence of drugs. In the presence of drugs, however, genotype ab becomes the fittest one. Consequently, the selection pressure causes it to spread through the population and become dominant. When this happens the population becomes adapted to these anti-HIV drugs. The time needed for genotype ab to spread through the population differs; shorter adaptation times mean higher danger of treatment failure. Our models were designed to compare the speed at which drug resistance evolves in the different populations when the environment is changed by adding antiviral drugs.

We assume that a single HIV entry event results in insertion of a single provirus into the genome of the host cell. The experimental evidence on multiplicity of HIV infections is

scarce. It had been shown (Jung et al., 2002) that splenocytes from HIV patients contain up to eight copies of provirus per cell with an average of 3.2 copies per cell. Copies are often genetically distinct and must be the result of multiple virions entering the same cell. For simplicity, our modeling ignores superinfection multiplicities higher than two proviruses per cell.

The viral fitness of an infected cell (i.e. its fitness as an HIV-producing “factory”) is affected both by the quantity of virions produced by the cell and by their ability to infect new cells. Both factors depend on the HIV genotype(s) the cell contains, since infectivity is determined mostly by the viral products produced and assembled in the origin cell. For simplicity we combine the quantity and the infectivity of produced virions into a single parameter, which we hereafter term ‘fitness’. The relative viral fitness of an infected cell is therefore defined as the relative probability of HIV virions produced by this cell to infect other cells in the next generation.

In the case of dual infection, phenotypic mixing allows for the rescue of mutations by another copy of the same gene from the second provirus. We tested two types of rescue: (I) complete rescue, in which the fitness of a dual-infected cell is defined by the better of the two available copies of the gene (rescue mode best available, Figs. 3–6) and (II) partial rescue, where the fitness is calculated as the average of the fitnesses of the two gene copies (rescue mode average). Changing the rescue mode from best available to average did not have significant effect on simulation results (data not shown).

2.1. A deterministic model

In this model we assume that viral population is infinite. Mutations in the drug resistance loci are bidirectional ($A \leftrightarrow a$, $B \leftrightarrow b$) and occur at rate μ , independently of drugs. The four haplotypes of proviruses in that model are denoted ‘1’ (AB), ‘2’ (Ab), ‘3’ (aB) and ‘4’ (ab). The population contains 10 different genotypes: $AB:AB$ (‘11’), $AB:Ab = Ab:AB$ (‘12’), $aB:AB = A-B:aB$ (‘13’), etc. We compare two types of populations: In the first, superinfection occurs with the same probability in all genotypes. In the second, the rate of superinfection depends on fitness, so that cells infected by fitter genotypes would have lower probabilities of superinfection.

The model considers two stages of virus infection: (1) infection of cells and formation of proviruses and (2) production of virion particles by infected cells.

2.1.1. Stage 1a: Calculation of the frequencies of potential provirus genotypes before mutation

The frequencies of the four possible haplotypes are given by

$$\begin{aligned} \tilde{p}_1 &= v_{11} + \frac{1}{2}(v_{12} + v_{13} + v_{14}(1-r) + v_{23}r), \\ \tilde{p}_2 &= v_{22} + \frac{1}{2}(v_{12} + v_{23}(1-r) + v_{24} + v_{14}r), \\ \tilde{p}_3 &= v_{33} + \frac{1}{2}(v_{13} + v_{23}(1-r) + v_{34} + v_{14}r), \\ \tilde{p}_4 &= v_{44} + \frac{1}{2}(v_{14}(1-r) + v_{24} + v_{34} + v_{23}r), \end{aligned} \quad (1)$$

where \tilde{p}_i is the frequency of haplotype i among the potential proviruses, V_{ij} the frequency of virions of genotype ij , and r is the recombination rate between loci **A** and **B** per generation.

2.1.2. Stage 1b: Frequencies of provirus after mutation

$$\begin{aligned} p_1 &= \tilde{p}_1(1 - \mu) + \mu(\tilde{p}_2 + \tilde{p}_3) + \mu^2 \tilde{p}_4, \\ p_2 &= \tilde{p}_2(1 - \mu) + \mu(\tilde{p}_1 + \tilde{p}_4) + \mu^2 \tilde{p}_3, \\ p_3 &= \tilde{p}_3(1 - \mu) + \mu(\tilde{p}_1 + \tilde{p}_4) + \mu^2 \tilde{p}_2, \\ p_4 &= \tilde{p}_4(1 - \mu) + \mu(\tilde{p}_2 + \tilde{p}_3) + \mu^2 \tilde{p}_1 \end{aligned} \quad (2)$$

2.1.3. Stage 1c: Frequencies of single and double-infected cells

The frequencies of cells infected by one or two viruses are given by

$$\begin{aligned} c_i &= \left[p_i(1 - f) + \frac{f p_i I_i (p_1 + p_2 + p_3 + p_4)}{C} \right] \\ i &\in \{1, 2, 3, 4\}, \\ c_{ij} &= \frac{p_i p_j f (1 - I_{ij})}{C} \quad i, j \in \{1, 2, 3, 4\}, \end{aligned} \quad (3)$$

where c_i is the fraction of cells infected with a single virus of genotype i , and c_{ij} the fraction of cells infected with two viruses of genotypes i and j . f is the fraction of co-infected cells in the population before interference. Eq. (3) is equivalent to first allowing primary infection in all cells, and then allowing secondary infection with probability $f(1 - I_i)$ in the cells that are already infected with provirus i . In our model f remains constant within each run, but we tested the results for a wide range of f values. I_i is the probability of a provirus of type i to interfere with superinfection by another provirus, given that it arrived first to the cell. I_{ij} is the average interference between a provirus of genotype i and a provirus of genotype j if both try to infect the same cell. I_{ij} is assumed to be the average of I_i and I_j , under the assumption that the different genotypes have identical probabilities of arriving first to the cell. I_{ij} can be either a constant value or be affected by the fitness of the two genotypes in a given environment. The effective rate of superinfection thus depends both on f and on I_{ij} . The frequencies are normalized by the factor C , which equals the sum of the numerators of Eq. (3).

2.1.4. Stage 2: Production of virions (infectious particles)

The frequencies of virions are given by

$$\begin{aligned} v_{ii} &= \frac{c_i \omega_i + c_{ii} \omega_{ii} + (1/4) \sum_{j \neq i} c_{ij} \omega_{ij}}{V} \quad i \in \{1, 2, 3, 4\}, \\ v_{ij} &= \frac{(1/2) c_{ij} \omega_{ij}}{V} \quad i \neq j, i, j = \{1, 2, 3, 4\} \end{aligned} \quad (4)$$

where V is the sum of the numerators of the right hand side of Eq. (4), ω_i the virus-producing fitness of a cell infected with a single provirus of genotype i in the current environment, and ω_{ij} is the virus-producing fitness of a cell infected with two proviruses of genotypes i and j in the current environment.

ω_i is determined by the genotype i and by the environment, i.e. presence or absence of drugs, so that

$$\begin{aligned} \omega_1 &= 1, \omega_4 = 1 - s \quad (\text{in the absence of drugs}) \quad \text{or} \\ \omega_1 &= 1 - s, \omega_4 = 1 \quad (\text{in the presence of drugs}), \\ \omega_2 &= \omega_3 = \sqrt{1 - s - E} \end{aligned} \quad (5)$$

where s is the fitness difference between the fittest and the least fit genotype in a given environment. In the case where each drug resistance gene contributes to the overall fitness equally and independently, the fitness of **aB** and **Ab** genotypes would be equal to $\sqrt{1 - s}$. However, in reality the effects of mutations on fitness are rarely independent and some level of epistasis (interaction between genes) usually takes place. We represent it by the single parameter E , measuring the degree of synergistic or antagonistic fitness interactions between the two loci.

We considered two definitions of ω_{ij} :

$$\begin{aligned} \omega_{ij} &= \frac{1}{2} (\omega_i + \omega_j) \quad (\text{rescue mode average}) \quad \text{or} \\ \omega_{ij} &= \max(\omega_i, \omega_j) \quad (\text{rescue mode best available}) \end{aligned}$$

The recursion Eqs. (1)–(4) are repeated until the change in the frequency of the most common genotype from one generation to the next falls below 10^{-8} for 50 generations. At that point all genotypes exist in the population, and a “drug cocktail” consisting of two drugs is introduced. The fitness values of all genotypes immediately change and the double drug-resistant genotype becomes the fittest one. The speed of spread of the drug-resistant genotype is monitored and used as a measure of adaptability of the HIV population.

2.2. Stochastic models

It has been shown in many cases that population size might play a role in the effect of sex and recombination (Barton and Otto, 2005; Muller, 1964). We complemented our deterministic model with stochastic simulations using different population sizes—from 5000 to 50,000.

In the stochastic models the population is finite and the viral genomes and infected cells are represented explicitly. The genome is represented by two drug resistance loci as in part A.

Each simulation run starts with a population of infected cells, each carrying a single HIV provirus. Infected cells produce virions (not represented explicitly), which are used to infect the cells in the next infection cycle. The computational steps performed at each infection cycle are described in more detail below:

- Calculating fitness:** First, the virus-producing fitness (ω_i) of each infected cell is determined. In the case of a single infection the fitness of the infected cell equals the fitness of the infecting provirus, determined by the drug resistance loci **A/a** and **B/b**. In the case of double-infected cells, the fitness depends on the fitness of both contained proviruses and the rescue mode (see above).
- Primary infection:** For each infected cell in the next cycle, an infecting virus is chosen in the following way: a ‘parent’

cell where the infecting virus is produced is chosen at random with probability proportional to the fitness of the parent cell. An infecting virus is then chosen at random from the three possible forms of progeny viruses (heterozygous and two homozygous) produced by that cell according to their expected frequencies. If the cell contains only one virus, this virus is chosen for infection.

(c) *Secondary infection*: The choice of the cells being infected by a second virus is at the core of our model. The superinfected cells were either chosen at random, with all cells having equal chance of second infection (non-regulated superinfection), or chosen according to their fitness, where cells that produce viral products less efficiently were more likely to be superinfected (regulated superinfection).

The probability of a cell infected with provirus of genotype g to be infected by an additional virus was equal to $f(1 - I_g)$, where f is the fraction of cells that would have been superinfected had no interference been occurring at all, and I_g is the level of interference generated by a provirus of genotype g . I_g is constant in non-regulated superinfection, and dependent on the fitness of the first infecting virus in regulated superinfection models, so that in the latter case:

$$I = \frac{\omega - \omega_{\min}}{\omega_{\max} - \omega_{\min}},$$

where ω_{\min} is the lowest possible fitness under the given parameters, and ω_{\max} is the highest one.

As an additional control we tested the case where the average rate of superinfection in each population is kept constant (Fig. 4). This is straightforward in the non-regulated cases, but in the cases of regulated superinfection the level of interference was dynamically determined so that the fraction of double-infected cells remains constant. This was done by sorting the population according to fitness and then choosing the constant fraction of least fit individuals for the secondary infection:

(d) *Mutation and recombination*: Mutation and recombination occur randomly during reverse transcription. The number of mutation events is sampled from Poisson distribution with expectation λ_m . The number of template switching (recombination) events per genome per generation is sampled from Poisson distribution with expectation $\lambda_r = 0, 0.2, \text{ or } 0.4$, depending on the specific simulation run.

Each simulation run starts with a population of wild type viruses not containing drug resistance alleles. Mutations are allowed to accumulate until the population is close to equilibrium between mutation and selection pressures, defined as the time t when the frequency p of the most common genotype satisfies the condition: $\sum_{i=t-50}^t \bar{p} - p_i < \varepsilon$, where the summation is performed over the previous 50 generations, \bar{p} is the average frequency of the most common genotype during that period, p_i the frequency of the most common genotype in generation i , and $\varepsilon = 0.005$. At that point the vast majority of the

population carries non-resistant wild type alleles due to the fitness cost of resistance, and drugs are introduced. In the presence of drugs the fitness of the different genotypes changes, according to (5). The double-resistant genotype becomes the fittest one, while wild type genotype becomes the least fit one. The rate of spread of the double resistant genotype is monitored and used as a measure of adaptability of the population.

All the simulations used in this work were written in standard C++ and are available upon request. BOOST C++ libraries (www.boost.org) were used for generation of random numbers.

3. Results

We tested the effect of regulated superinfection (where the fittest virus tends to inhibit the consequent infection of the cell it occupies, whereas less fit viruses are more likely to allow secondary infections) on the ability of the population as a whole to adapt to changes in the environment such as introduction of the multi-drug anti-HIV “cocktail”. Both the deterministic model and the stochastic simulations show that when superinfection is fitness-associated the adaptation of the viral population to drugs ($AB \rightarrow ab$) happens considerably faster compared with the scenario where the rate of cellular superinfection is constant.

Fig. 2 shows how the fraction of double-resistant mutants (ab) in the HIV population changes with time after introduction of drugs under different superinfection strategies. To obtain a quantitative measure of the adaptability of HIV populations, we

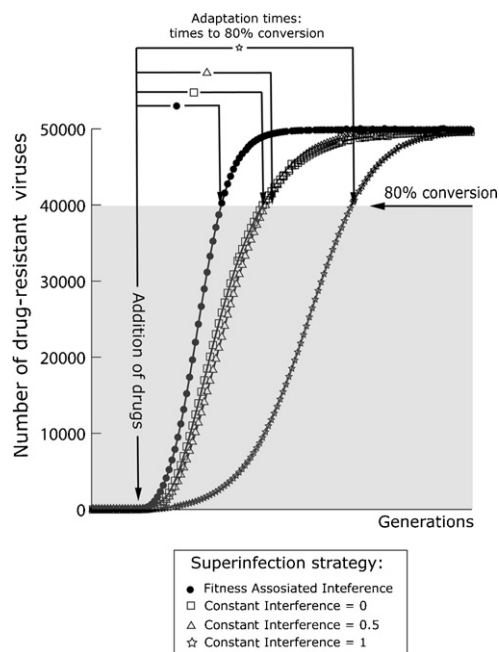


Fig. 2. Evolution of drug-resistant genotype after addition of drugs. Adaptation time is defined as the time between addition of drugs and the time when the fittest genotype comprises 80% of population. Under these parameters fitness-associated interference (circles) resulted in shorter adaptation time in comparison with either no interference (squares), full interference (stars), or intermediate unregulated interference (triangles). Model parameters: $\lambda_m = 10^{-3}$, $s = 0.2$, $f = 0.3$, rescue mode = best available.

measure the time interval between the addition of drugs and the occurrence of drug resistance in 80% of the viral population. This time interval is termed here the “adaptation time”, and is represented by the horizontal distance between the arrows in Fig. 2. We see that under these parameters, adaptation time is shorter for fitness-associated interference (where the less fit viruses interfere less) than for any of the constant interference strategies tested.

In Figs. 3 and 4 we present the relative conversion times for populations with either regulated coinfection or constant rates of coinfection. The Y-axis shows the relative shortening of adaptation time T_{rel} calculated as $T_{rel} = (T_0 - T_x)/T_0$, where T_0 is the adaptation time of the control population without superinfection, T_x the adaptation time of the population plotted, and T_{rel} the relative adaptation time of the population plotted. T_{rel} is always positive for populations with regulated super-

infection demonstrating that these populations have shorter adaptation times than populations with no superinfection ($T_0 - T_x > 0$). This means that HIV populations benefit from regulated superinfection when adaptation to new conditions (such as drugs) is needed. For populations with unregulated superinfection, T_{rel} is often negative, with the exception of negative. In all cases, adaptation times of populations with regulated superinfection are considerably shorter than those of populations with uniform superinfection.

We tested our models on populations of different sizes (including infinite population size using the numerical model) with different combinations of mutation and recombination rates. At lower mutation rate drug resistance mutations appear less frequently. As expected, the evolution of drug resistance indeed takes longer for lower mutation rates, regardless of the regulation of superinfection. Another important factor is

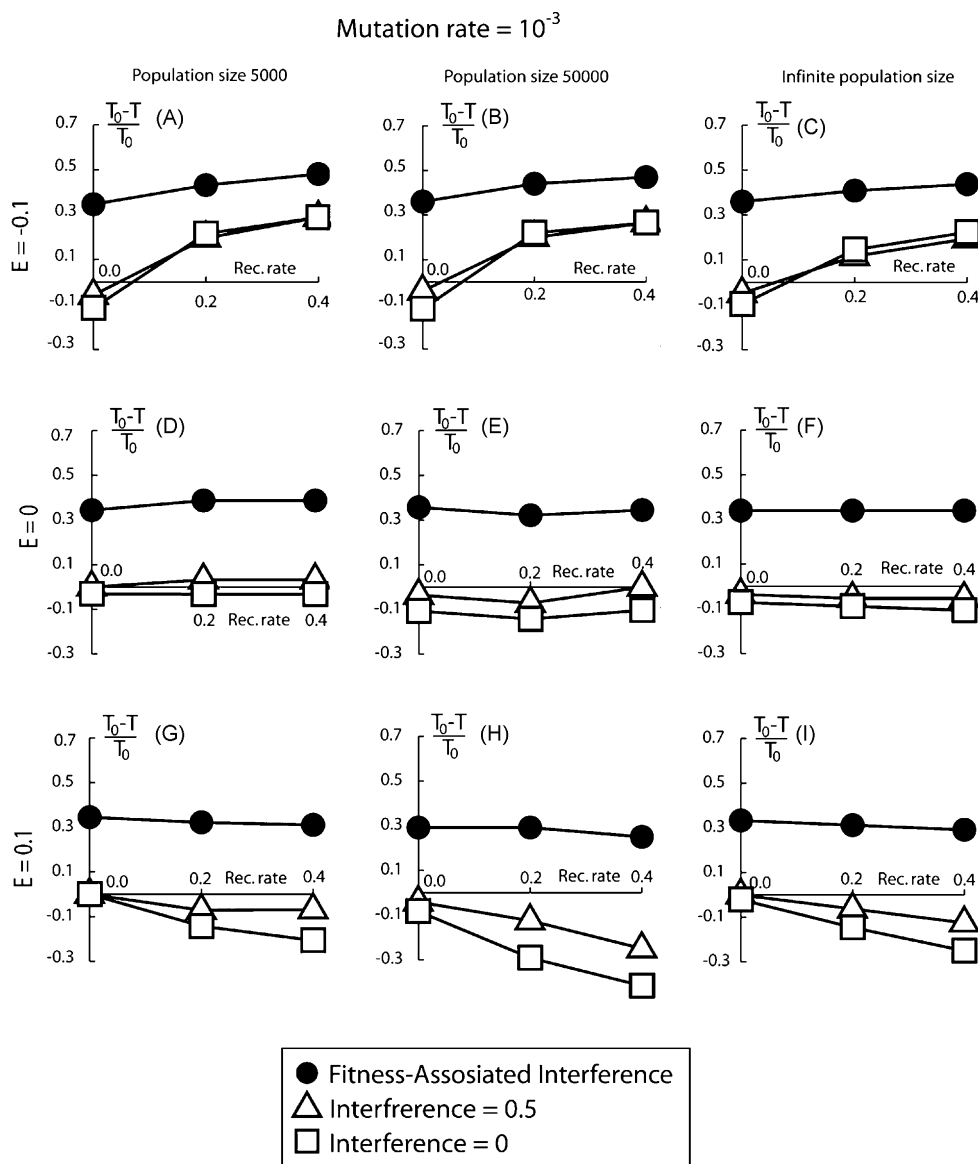


Fig. 3. Shortening of adaptation time due to superinfection in simulations with intermediate (5000, stochastic model), large (50,000, stochastic model) and infinite (deterministic model) population sizes. Simulation parameters: $\lambda_m = 10^{-3}$, $s = 0.2$, $f = 0.3$, rescue mode = best available. (X-axis) Recombination rate. (Y-axis) The shortening of adaptation time relative to a non-superinfecting population.

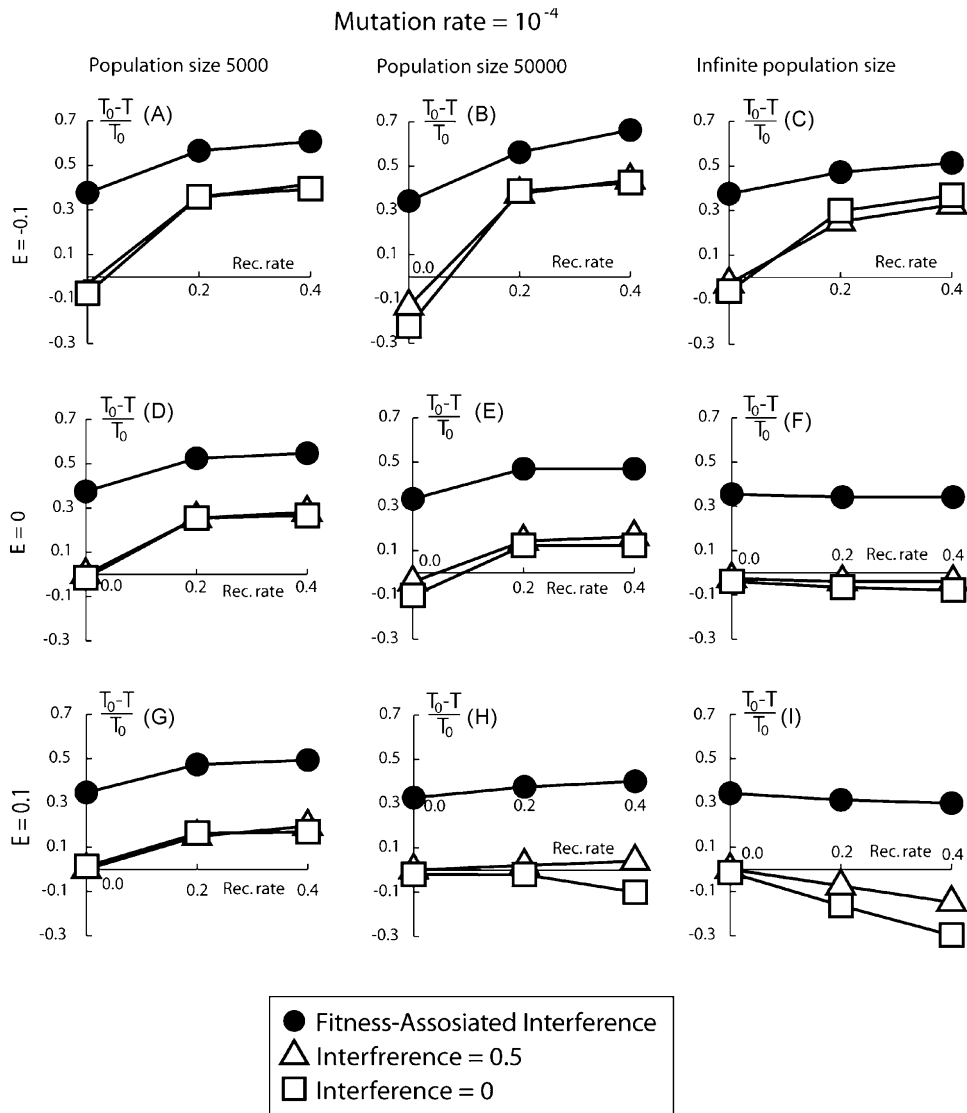


Fig. 4. Shortening of adaptation time due to superinfection in simulations with intermediate (5000, stochastic model), large (50,000, stochastic model) and infinite (deterministic model) population sizes. Simulation parameters: $\lambda_m = 10^{-4}$, $s = 0.2$, $f = 0.3$, rescue mode = best available. (X-axis) Recombination rate. (Y-axis) The shortening of adaptation time relative to a non-superinfecting population.

epistasis—the fitness interaction between the loci. Epistasis is defined as negative when the combined effect of multiple mutations is smaller than the sum of their individual effects and positive when the situation is reversed. Consistently with previous works (Bretscher et al., 2004) superinfection that involves recombination resulted in faster adaptation when epistasis was negative and slower adaptation when epistasis was positive and the mutation rate was high (10^{-3}). For positive epistasis and a low mutation rate (10^{-4} Fig. 4), population size had a significant effect: In the infinite model (where all genotypes are present at all times, Fig. 4I) recombination indeed slowed the rate of adaptation. In contrast, when the population was finite recombination was advantageous (pop. size 5000, Fig. 4G) or nearly neutral (pop. size 50,000, Fig. 4H) even with positive epistasis. However, the effect of epistasis was weaker when superinfection was regulated. The overall effect of regulated superinfection, even in the case of high

recombination and positive epistasis, was shortening of waiting time.

Despite inherent differences between deterministic and stochastic models, there is a good agreement between the results obtained by both models (Figs. 3 and 4), and in particular between the large finite population and the infinite one, suggesting that the results may be valid for very large populations. Current estimates of the effective population size range from 500 to 10^6 (Althaus and Bonhoeffer, 2005). We have tested three population sizes (5000, 50,000, and infinite population size), and within the tested range the advantage of regulated superinfection for the virus was clear in both models.

The overall superinfection rate has an effect on the adaptability of the population, regardless of whether superinfection is regulated or not. Therefore, we performed a series of control simulations in which we kept the average

superinfection rate in the populations at the same level, f_{fixed} . In populations with regulated superinfection the $f_{\text{fixed}}N$ least fit cells were chosen for superinfection; in the control (unregulated) population the same number of cells was chosen at random, regardless of fitness. Result from our simulations show that regulated superinfection remains advantageous (Fig. 5) The advantage of regulated superinfection is therefore not due to the difference in the average superinfection rate between regulated and unregulated populations, but due to the fact that superinfection is regulated.

There are three possible factors that could make regulated superinfection, rather than unregulated superinfection, advantageous to the adaptation of HIV to drugs. These include: (1) superinfection increases the rate of productive recombination; (2) it provides the possibility of phenotypic mixing; and (3) there is a negative effect of superinfection on fitness (the cost of competition, see Section 1). Since regulated superinfection is advantageous even in the absence of recombination (Figs. 3–5), it is unlikely that recombination is the main “driving force” behind the advantage seen here. Furthermore, phenotypic mixing actually decreases the advantage, by weakening the effectiveness of selection (data not shown; see also Froissart et al., 2004). Therefore, the

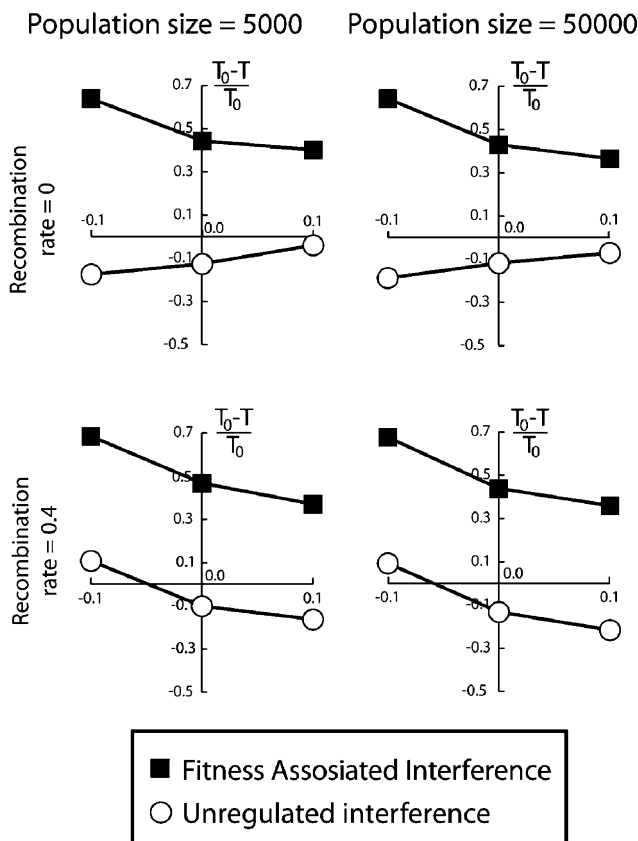


Fig. 5. Shortening of adaptation time due to superinfection in stochastic simulations with fixed overall rate of coinfection for intermediate (5000) and large (50,000) population size. Each point represents the average conversion time in 100 runs. Confidence intervals are smaller than markers. Simulation parameters: rescue mode = best available, $s = 0.2$, $f_{\text{fixed}} = 0.3$, $\lambda_m = 10^{-3}$. (X-axis) Epistasis. (Y-axis) The shortening of adaptation time in comparison with a non-superinfecting population.

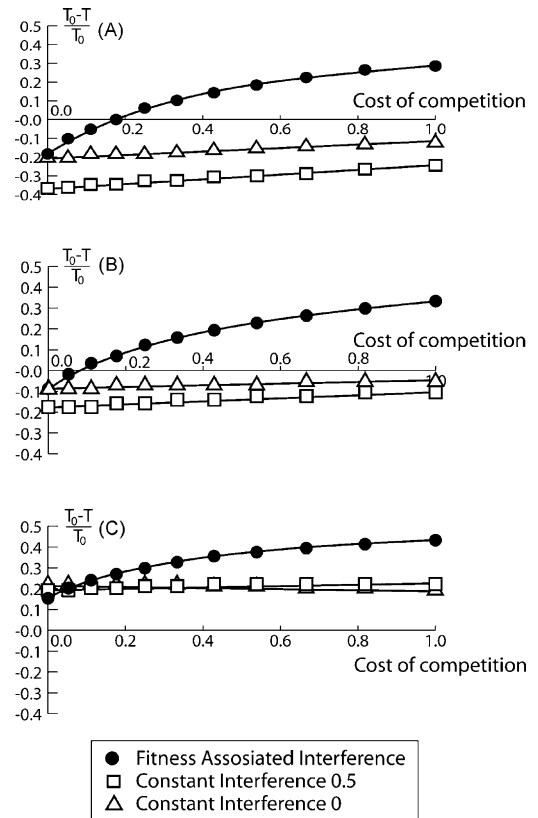


Fig. 6. Shortening of adaptation time due to superinfection in deterministic simulations (infinite population size) as a function of the cost of competition. Simulation parameters: $s = 0.2$, $f_{\text{fixed}} = 0.3$, $\lambda_m = 10^{-3}$, $\lambda_r = 0.4$, rescue mode = best available. (X-axis) Cost of competition. (Y-axis) The shortening of adaptation time in comparison with a non-superinfecting population. (A) positive epistasis, (B) no epistasis, (C) negative epistasis.

likely factor behind the positive effect of regulated superinfection is the cost of competition.

In order to test this possible explanation for regulated superinfection we varied the cost of competition from no cost (where double-infected cells produce double the number of virions in comparison with single infected cells) to “full cost” (where double-infected cells and single infected cells produce the same number of virions). The average superinfection rate was kept constant. We found that the cost of competition has a significant effect on the advantage of regulated superinfection. For most of the possible cost values, regulated superinfection is indeed advantageous, and the extent of the advantage increases with the cost (Fig. 6). However, when the cost of competition is very low, unregulated superinfection may lead to faster adaptation at some parameter combinations (such as high mutation and recombination rates combined with negative epistasis). In the unlikely event that competition has no cost, unregulated superinfection is in many cases a better adaptation strategy.

4. Discussion

In this paper we investigated a model in which the probability of HIV superinfection depends on the overall fitness of the first infecting virus so that less fit viruses are more

likely to allow superinfection. We examined the effect of such fitness-associated superinfection on the evolution of drug resistance in viral populations. In all but few of the cases tested, we found that when the level of superinfection is regulated in that way, the ability of the virus population to adapt to new conditions is greater than that of a population lacking the ability to regulate the rate of superinfection. Adaptation to drug resistance was chosen because of its practical importance, but the results apply to the evolution of any genes with significant effect on viral fitness.

Some viruses possess mechanisms to regulate the level of superinfection (Nethe et al., 2005; Singh et al., 1997). In particular, HIV goes to extraordinary lengths to down modulate its receptors on the surface of HIV infected cells. However, the biological significance of receptor downregulation is not fully understood. Down-modulation of receptors has been shown to increase the overall fitness of HIV (Hanna et al., 2006; Tanaka et al., 2003). Loss of receptors from the surface has other consequences as well, such as increase of viral budding from lipid rafts (Zheng et al., 2001), preventing incorporation of CD4 into viral particles (Tanaka et al., 2003), and the modulation of the immune response (Marodon, 2001). Superinfection also causes the accumulation of unintegrated HIV DNA (Pauza et al., 1990), which can lead to programmed cell death. (Daniel et al., 1999; Wildum et al., 2006) (Maury et al., 2003). However, regardless of other consequences, the removal of receptors results in the suppression of HIV entry. Down-regulation of CCR4/CXCR5 protects 293T cells from HIV infection (Anderson and Akkina, 2005). It had been shown (Butticaz et al., 2003) that even a modest decrease in the density of CCR5 coreceptors can lead to dramatic decrease of HIV entry. Long-term HIV non-progressors often have antibodies that induce CXCR5 internalization (Pastori et al., 2006).

The HIV-encoded proteins Nef-1, Env and Vpu are involved in receptor down-regulation. Nef-1 interacts with residues on the cytoplasmic tail of CD4 (Aiken et al., 1994; Benson et al., 1993; Grzesiek et al., 1996; Stoddart et al., 2003). It uses the clathrin-mediated endocytic pathway to induce removal of CD4, CCR4 and CXCR5 and from the plasma membrane to endosomes (Burtey et al., 2006; Michel et al., 2005; Venzke et al., 2006). In contrast, both the Env and Vpu interfere with trafficking of newly synthesized CD4 to the cell surface (Chen et al., 1993; Willey et al., 1992a; Willey et al., 1992b). Regardless of mechanism, HIV carrying wild type NEF-1 strongly inhibits superinfection in Jurkat T cells (Wildum et al., 2006). While the significance of CD4/CCR5/CXCR4 down-regulation remains the subject of controversy (Lama, 2003), the downregulation itself is well documented. In this work we focused on the effect of HIV-induced downregulation of HIV receptors on the rate of superinfection and the possible consequences of regulated superinfection.

Our data suggests that the effect of recombination on the advantage of regulated superinfection is minor (Figs. 3 and 4), and that the major driving force behind its success is redistribution of the cost of competition (Fig. 6). In addition to the usual cost of competition resulting from sharing of cellular resources, enhanced programmed cell death in super-

infected cells (Wildum et al., 2006) could further increase the selection burden on superinfecting virions. However, more detailed models including multiple loci with small effects might yield a greater advantage to recombination, due to increased probability of fixation of advantageous mutations (Hill and Robertson, 1966; Peck, 1994), or reduced probability of fixation of deleterious alleles during the evolution of drug resistance, typically involving strong selection (Hadany and Feldman, 2005).

Our results may help to address the issue of HIV recombination. The analysis by (Althaus and Bonhoeffer, 2005; Bretscher et al., 2004; Fraser, 2005) shows that under a constant rate of superinfection and positive epistasis recombination is likely to slow down the adaptation of HIV to new conditions rather than accelerate it. Epistasis in case of HIV drug resistance has been shown to be positive in many cases (Bonhoeffer et al., 2004), although other authors question this conclusion (Wang et al., 2006b). A negative effect of recombination with positive epistasis, echoing the classical puzzle regarding the advantage of recombination in other organisms (Charlesworth and Barton, 1996; Feldman et al., 1996) is especially difficult to comprehend given the fact that HIV, a pathogen particularly notorious for its rapid adaptation, is recombining at a very high rate. We found that even when superinfection is unregulated the effect of epistasis depends on the size of the population and on the mutation rate: for low mutation rates and small to intermediate population sizes, recombination is advantageous even with positive epistasis (Fig. 4).

It had been shown that in haploid organisms a gene regulating the recombination rate based on fitness will spread through the population to fixation (Agrawal et al., 2005; Hadany and Beker, 2003), as would one regulating the tendency to reproduce sexually in diploid organism (Hadany and Otto, 2007). We obtained similar results for a gene regulating the rate of superinfection in an HIV model, showing that such a gene, once it appears, will spread through the population to fixation (Leontiev and Hadany, manuscript in preparation).

The proposed fitness-associated superinfection does not require a complex regulatory mechanism. We can hypothesize that the “window of opportunity” for superinfection, i.e. the time between the first infection and the expression of Nef1 and then Env and Vpu is shorter for fitter, faster reproducing viruses and longer for unfit viruses. Furthermore, unfit viruses may have deficiency in their mechanism of receptor downregulation. Down-regulation of receptors may be delayed or not happen at all, and the window of opportunity for a secondary infection would be even wider. Michel et al. (2005) suggest that primate lentiviruses have evolved time windows – after infection and prior to early HIV gene expression – during which the permission or prevention of superinfection is regulated by gene expression. If a fitter virus will reach the stage of early HIV gene expression faster than a less fit one, the rate of superinfection will be governed by fitness, or at least by the component of the fitness that depend on the early HIV genes. This will constitute a simple mechanism of fitness-associated superinfection.

At the same time, the existence of a simple mechanism does not preclude the possibility of more elaborated pathways of fitness-associated regulation of superinfection. Chen et al. (2005) demonstrated that the rate of double HIV infection *in vitro* is higher than the rate one would expect had double infection been occurring at random. They suggest several explanations, the simplest of which is that some cells are more susceptible to HIV entry than others. Since we already know that HIV can down-modulate coinfection it seems possible that such difference in susceptibility may be “orchestrated” by HIV rather than merely be the result of natural variability in cell population. Although we do not base our model on this assumption, one can allow the possibility that HIV can up-regulate as well as down-regulate its superinfection rate.

It is worth mentioning that the rate of recombination can also be subject to regulation as proposed by (Hwang et al., 2001; Nikolenko et al., 2004). The authors describe the mechanism of dynamic copy choice. According to this mechanism a faster reverse transcriptase will commit fewer template switches than a slower one. This, along with regulated superinfection, can be another mechanism decreasing the recombination frequency of the fittest HIV genomes compared to less fit ones. Last, we can speculate that a mechanism directly regulating the level of interference as a function of viral fitness can evolve (Leontiev and Hadany, manuscript in preparation).

Our results show that viral populations that are able to regulate superinfection based on the fitness of individual genomes might be able to adapt to environmental changes, including drugs, considerably faster than populations of viruses that lack such ability. The existence of the mechanism of regulation of superinfection may have several practical implications. First, drug regimens may be adjusted taking into account the propensity of HIV to regulate the multiplicity of infection. This especially concerns an emerging class of drugs that target HIV entry. If our model is correct such drugs will be particularly useful at early stages of infection and at points when treatment failure demands the switch to the new drugs. It is likely that in early stages of infection or after a change of treatment the HIV population would have low average fitness (since it has not yet adapted to the new host environment), resulting in more common superinfection. As soon as well-adapted virions arrive at the scene, their fitter genomes would tend to down-modulate superinfection more effectively. To the extent that this factor is significant, it might be beneficial for both the patient and the human population that the therapeutic effort at early stages of infection (or soon after recurrence of the disease) be directed in part against superinfection. Development of new classes of drugs may help to counter the ability of HIV to increase its adaptation rate by regulating the rate of superinfection.

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References

- Agrawal, A.F., Hadany, L., Otto, S.P., 2005. The evolution of plastic recombination. *Genetics* 171 (2), 803–812.
- Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E., Trono, D., 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* 76 (5), 853–864.
- Althaus, C.L., Bonhoeffer, S., 2005. Stochastic interplay between mutation and recombination during the acquisition of drug resistance mutations in human immunodeficiency virus type 1. *J. Virol.* 79 (21), 13572–13578.
- Anderson, J., Akkina, R., 2005. HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. *AIDS Res. Ther.* 2 (1), 1.
- Barton, N.H., Otto, S.P., 2005. Evolution of recombination due to random drift. *Genetics* 169 (4), 2353–2370.
- Benson, R.E., Sanfridson, A., Ottinger, J.S., Doyle, C., Cullen, B.R., 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.* 177 (6), 1561–1566.
- Bonhoeffer, S., Chappey, C., Parkin, N.T., Whitcomb, J.M., Petropoulos, C.J., 2004. Evidence for positive epistasis in HIV-1. *Science* 306 (5701), 1547–1550.
- Bretscher, M.T., Althaus, C.L., Muller, V., Bonhoeffer, S., 2004. Recombination in HIV and the evolution of drug resistance: for better or for worse? *Bioessays* 26 (2), 180–188.
- Briz, V., Poveda, E., Soriano, V., 2006. HIV entry inhibitors: mechanisms of action and resistance pathways. *J. Antimicrob. Chemother.* 57 (4), 619–627.
- Burtey, A., Rappoport, J.Z., Bouchet, J., Basmaciogullari, S., Guatelli, J., Simon, S.M., Benichou, S., Benmerah, A., 2006. Dynamic interaction of HIV-1 Nef with the clathrin-mediated endocytic pathway at the plasma membrane. *Traffic* 8 (1), 61–76.
- Butticaz, C., Ciuffi, A., Munoz, M., Thomas, J., Bridge, A., Pebernard, S., Iggo, R., Meylan, P., Telenti, A., 2003. Protection from HIV-1 infection of primary CD4 T cells by CCR5 silencing is effective for the full spectrum of CCR5 expression. *Antivir. Ther.* 8 (5), 373–377.
- Charlesworth, B., Barton, N.H., 1996. Recombination load associated with selection for increased recombination. *Genet. Res.* 67 (1), 27–41.
- Chen, M.Y., Maldarelli, F., Karczewski, M.K., Willey, R.L., Strebel, K., 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of CD4 *in vitro*: the cytoplasmic domain of CD4 contributes to Vpu sensitivity. *J. Virol.* 67 (7), 3877–3884.
- Chen, J., Dang, Q., Unutmaz, D., Pathak, V.K., Maldarelli, F., Powell, D., Hu, W.S., 2005. Mechanisms of nonrandom human immunodeficiency virus type 1 infection and double infection: preference in virus entry is important but is not the sole factor. *J. Virol.* 79 (7), 4140–4149.
- Daniel, R., Katz, R.A., Skalka, A.M., 1999. A role for DNA-PK in retroviral DNA integration. *Science* 284 (5414), 644–647.
- Dixit, N.M., Perelson, A.S., 2005. HIV dynamics with multiple infections of target cells. *Proc. Natl. Acad. Sci. U.S.A.* 102 (23), 8198–8203.
- Feldman, M.W., Otto, S.P., Christiansen, F.B., 1996. Population genetic perspectives on the evolution of recombination. *Annu. Rev. Genet.* 30, 261–295.
- Frankel, F.A., Marchand, B., Turner, D., Gotte, M., Wainberg, M.A., 2005. Impaired rescue of chain-terminated DNA synthesis associated with the L74V mutation in human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* 49 (7), 2657–2664.
- Fraser, C., 2005. HIV recombination: what is the impact on antiretroviral therapy? *J. R. Soc. Interface* 2 (5), 489–503.
- Froissart, R., Wilke, C.O., Montville, R., Remold, S.K., Chao, L., Turner, P.E., 2004. Co-infection weakens selection against epistatic mutations in RNA viruses. *Genetics* 168 (1), 9–19.
- Fumero, E., Podzamczak, D., 2003. New patterns of HIV-1 resistance during HAART. *Clin. Microbiol. Infect.* 9 (11), 1077–1084.
- Gilchrist, M.A., Coombs, D., 2006. Evolution of virulence: interdependence, constraints, and selection using nested models. *Theor. Popul. Biol.* 69 (2), 145–153.
- Grzesiek, S., Stahl, S.J., Wingfield, P.T., Bax, A., 1996. The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry* 35 (32), 10256–10261.

- Hadany, L., Beker, T., 2003. On the evolutionary advantage of fitness-associated recombination. *Genetics* 165 (4), 2167–2179.
- Hadany, L., Feldman, M.W., 2005. Evolutionary traction: the cost of adaptation and the evolution of sex. *J. Evol. Biol.* 18 (2), 309–314.
- Hadany, L., Otto, S.P., 2007. The evolution of condition-dependent sex in the face of high costs. *Genetics* 176 (3), 1713–1727.
- Hanna, Z., Priceputu, E., Hu, C., Vincent, P., Jolicoeur, P., 2006. HIV-1 Nef mutations abrogating downregulation of CD4 affect other Nef functions and show reduced pathogenicity in transgenic mice. *Virology* 346 (1), 40–52.
- Hill, W.G., Robertson, A., 1966. The effect of linkage on limits to artificial selection. *Genet. Res.* 8 (3), 269–294.
- Huang, K.J., Wooley, D.P., 2005. A new cell-based assay for measuring the forward mutation rate of HIV-1. *J. Virol. Methods* 124 (1/2), 95–104.
- Hwang, C.K., Svarovskaia, E.S., Pathak, V.K., 2001. Dynamic copy choice: steady state between murine leukemia virus polymerase and polymerase-dependent RNase H activity determines frequency of in vivo template switching. *Proc. Natl. Acad. Sci. U.S.A.* 98 (21), 12209–12214.
- Iwabu, Y., Goto, T., Tsuji, S., Warachit, J., Li, G.M., Shoji, S., Kameoka, M., Ikuta, K., 2006. Superinfection of human immunodeficiency virus type 1 (HIV-1) to cell clone persistently infected with defective virus induces production of highly cytopathogenic HIV-1. *Microbes Infect.* 8 (7), 1773–1782.
- Johnson, V.A., Brun-Vezinet, F., Clotet, B., Conway, B., Kuritzkes, D.R., Pillay, D., Schapiro, J.M., Telenti, A., Richman, D.D., 2005. Update of the drug resistance mutations in HIV-1: Fall 2005. *Top HIV Med* 13 (4), 125–131.
- Jung, A., Maier, R., Vartanian, J.P., Bocharov, G., Jung, V., Fischer, U., Meese, E., Wain-Hobson, S., Meyerhans, A., 2002. Multiply infected spleen cells in HIV patients. *Nature* 418 (6894), 144.
- Lama, J., 2003. The physiological relevance of CD4 receptor down-modulation during HIV infection. *Curr. HIV Res.* 1 (2), 167–184.
- Lengauer, T., Sing, T., 2006. Bioinformatics-assisted anti-HIV therapy. *Nat. Rev. Microbiol.* 4 (10), 790–797.
- Levy, D.N., Aldrovandi, G.M., Kutsch, O., Shaw, G.M., 2004. Dynamics of HIV-1 recombination in its natural target cells. *Proc. Natl. Acad. Sci. U.S.A.* 101 (12), 4204–4209.
- Lori, F., Hall, L., Lusso, P., Popovic, M., Markham, P., Franchini, G., Reitz Jr., M.S., 1992. Effect of reciprocal complementation of two defective human immunodeficiency virus type 1 (HIV-1) molecular clones on HIV-1 cell tropism and virulence. *J. Virol.* 66 (9), 5553–5560.
- Marodon, G., 2001. CD4 down modulation on T-cells: an 'immune' checkpoint for HIV. *Immunol. Lett.* 79 (3), 165–168.
- Maury, W., Wright, P.J., Bradley, S., 2003. Characterization of a cytolytic strain of equine infectious anemia virus. *J. Virol.* 77 (4), 2385–2399.
- Meyerhans, A., Jung, A., Maier, R., Vartanian, J.P., Bocharov, G., Wain-Hobson, S., 2003. The non-clonal and transitory nature of HIV in vivo. *Swiss Med. Weekly* 133 (33/34), 451–454.
- Michel, N., Allespach, I., Venzke, S., Fackler, O.T., Keppler, O.T., 2005. The Nef protein of human immunodeficiency virus establishes superinfection immunity by a dual strategy to downregulate cell-surface CCR5 and CD4. *Curr. Biol.* 15 (8), 714–723.
- Muller, H.J., 1964. The relation of recombination to mutational advance. *Mutat. Res.* 106, 2–9.
- Nethe, M., Berkhout, B., van der Kuyl, A.C., 2005. Retroviral superinfection resistance. *Retrovirology* 2, 52.
- Nikolenko, G.N., Svarovskaia, E.S., Delviks, K.A., Pathak, V.K., 2004. Anti-retroviral drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase increase template-switching frequency. *J. Virol.* 78 (16), 8761–8770.
- Nowak, M., 1990. HIV mutation rate. *Nature* 347 (6293), 522.
- Pastori, C., Weiser, B., Barassi, C., Uberti-Foppa, C., Ghezzi, S., Longhi, R., Calori, G., Burger, H., Kemal, K., Poli, G., Lazzarin, A., Lopalco, L., 2006. Long-lasting CCR5 internalization by antibodies in a subset of long-term nonprogressors: a possible protective effect against disease progression. *Blood* 107 (12), 4825–4833.
- Pauza, C.D., Galindo, J.E., Richman, D.D., 1990. Reinfection results in accumulation of unintegrated viral DNA in cytopathic and persistent human immunodeficiency virus type 1 infection of CEM cells. *J. Exp. Med.* 172 (4), 1035–1042.
- Peck, J.R., 1994. A ruby in the rubbish: beneficial mutations, deleterious mutations and the evolution of sex. *Genetics* 137 (2), 597–606.
- Potter, S.J., Chew, C.B., Steain, M., Dwyer, D.E., Saksena, N.K., 2004. Obstacles to successful antiretroviral treatment of HIV-1 infection: problems and perspectives. *Indian J. Med. Res.* 119 (6), 217–237.
- Rhodes, T., Wargo, H., Hu, W.S., 2003. High rates of human immunodeficiency virus type 1 recombination: near-random segregation of markers one kilobase apart in one round of viral replication. *J. Virol.* 77 (20), 11193–11200.
- Schneider, R.J., Shenk, T., 1987. Impact of virus infection on host cell protein synthesis. *Annu. Rev. Biochem.* 56, 317–332.
- Singh, I.R., Suomalainen, M., Varadarajan, S., Garoff, H., Helenius, A., 1997. Multiple mechanisms for the inhibition of entry and uncoating of superinfecting Semliki Forest virus. *Virology* 231 (1), 59–71.
- Stoddart, C.A., Gelezianas, R., Ferrell, S., Linquist-Stepps, V., Moreno, M.E., Bare, C., Xu, W., Yonemoto, W., Bresnahan, P.A., McCune, J.M., Greene, W.C., 2003. Human immunodeficiency virus type 1 Nef-mediated downregulation of CD4 correlates with Nef enhancement of viral pathogenesis. *J. Virol.* 77 (3), 2124–2133.
- Tanaka, M., Ueno, T., Nakahara, T., Sasaki, K., Ishimoto, A., Sakai, H., 2003. Downregulation of CD4 is required for maintenance of viral infectivity of HIV-1. *Virology* 311 (2), 316–325.
- Venzke, S., Michel, N., Allespach, I., Fackler, O.T., Keppler, O.T., 2006. Expression of Nef downregulates CXCR4, the major coreceptor of human immunodeficiency virus. From the surface of target cells and thereby enhances resistance to superinfection. *J. Virol.* 80 (22), 11141–11152.
- Wain-Hobson, S., Renoux-Elbe, C., Vartanian, J.P., Meyerhans, A., 2003. Network analysis of human and simian immunodeficiency virus sequence sets reveals massive recombination resulting in shorter pathways. *J. Gen. Virol.* 84 (Pt 4), 885–895.
- Wang, J., Dykes, C., Domaoal, R.A., Koval, C.E., Bambara, R.A., Demeter, L.M., 2006a. The HIV-1 reverse transcriptase mutants G190S and G190A, which confer resistance to non-nucleoside reverse transcriptase inhibitors, demonstrate reductions in RNase H activity and DNA synthesis from tRNA(Lys, 3) that correlate with reductions in replication efficiency. *Virology* 348 (2), 462–474.
- Wang, K., Mittler, J.E., Samudrala, R., 2006b. Comment on evidence for positive epistasis in HIV-1. *Science* 312 (5775), 848 author reply, 848.
- Wildum, S., Schindler, M., Munch, J., Kirchhoff, F., 2006. Contribution of Vpu, Env, and Nef to CD4 down-modulation and resistance of human immunodeficiency virus type 1-infected T cells to superinfection. *J. Virol.* 80 (16), 8047–8059.
- Wilke, C.O., Novella, I.S., 2003. Phenotypic mixing and hiding may contribute to memory in viral quasispecies. *BMC Microbiol.* 3, 11.
- Willey, R.L., Maldarelli, F., Martin, M.A., Strebel, K., 1992a. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J. Virol.* 66 (12), 7193–7200.
- Willey, R.L., Maldarelli, F., Martin, M.A., Strebel, K., 1992b. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *J. Virol.* 66 (1), 226–234.
- Zheng, Y.H., Plemenitas, A., Linnemann, T., Fackler, O.T., Peterlin, B.M., 2001. Nef increases infectivity of HIV via lipid rafts. *Curr. Biol.* 11 (11), 875–879.