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ORIGINAL ARTICLE

Coinfection rates in ϕ6 bacteriophage are enhanced by virus-induced changes in host cells

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Abstract

Two or more viruses infecting the same host cell can interact in ways that profoundly affect disease dynamics and control, yet the factors determining coinfection rates are incompletely understood. Previous studies have focused on the mechanisms that viruses use to suppress coinfection, but recently the phenomenon of enhanced coinfection has also been documented. In the experiments described here, we explore the hypothesis that enhanced coinfection rates in the bacteriophage ϕ6 are achieved by virus-induced upregulation of the ϕ6 receptor, which is the bacterial pilus. First, we confirmed that coinfection enhancement in ϕ6 is virus-mediated by showing that ϕ6 attaches significantly faster to infected cells than to uninfected cells. Second, we explored the hypothesis that coinfection enhancement in ϕ6 depends upon changes in the expression of an inducible receptor. Consistent with this hypothesis, the closely related phage, ϕ12, that uses constitutively expressed lipopolysaccharide as its receptor, attaches to infected and uninfected cells at the same rate. Our results, along with the previous finding that coinfection in ϕ6 is limited to two virions, suggest that viruses may closely regulate rates of coinfection through mechanisms for both coinfection enhancement and exclusion.

Introduction

Coinfection of single host cells by multiple virions is common in many viruses (ϕ6: Turner et al. 1999; hepatitis B: Bollyky et al. 1996; HIV: Dang et al. 2004; Jetzt et al. 2000; Levy et al. 2004; Robertson et al. 1995; norovirus: Rohayem et al. 2005; measles: Schierup et al. 2005; VEEV: Smith et al. 2008) and can affect disease outcome in numerous ways. Perhaps the most significant consequences of coinfection have been the recombinant viral genomes that are produced when a single host is infected by two very different viral genotypes. Reassortment between human and animal influenza has repeatedly given rise to influenza pandemics (Cox and Subbarao 2000), recombinant HIV virions have proven capable of evading drug treatments (Kellam and Larder 1995; Moutouh et al. 1996) and immune responses (Streeck et al. 2008), and recombination between wild enteroviruses and polio vaccine strains has given rise to polio outbreaks (reviewed by Kew et al. 2005). In addition to recombination, coinfection allows functional interactions between virus genotypes within the same cell, and these interactions often lead to a direct or evolved increase in virulence. For example, host cells persistently infected with defective HIV virions can become highly pathogenic upon coinfection with a competent virus (Iwabu et al. 2006). These potentially extreme consequences of coinfection for viral disease and evolution merit a closer examination of the conditions under which coinfection is likely to occur.

From the perspective of the virus, coinfection generates both costs and benefits (Turner and Duffy 2008). Coinfection imposes a direct cost in the loss of shared host resources (Delbrück 1945; Doermann 1948), as well as an indirect cost in the evolution of phenotypes that increase success during coinfection but decrease fitness in single infections (Turner and Chao 1998). Coinfection also

enables genetic exchange and complementation, each of which may enhance fitness. Genetic exchange can increase the speed of adaptation by bringing adaptive gene complexes together (Fisher 1930; Muller 1932) and halting the deleterious action of Muller's ratchet (Muller 1964), while complementation can mask the fitness effects of deleterious mutations (Mindich et al. 1985; Cicin-Sain et al. 2005).

Given that the costs of coinfection increase more rapidly than the benefits as number of coinfecting partners increases, it is not surprising that viruses have evolved mechanisms that limit the number of viruses that coinfect a cell. These superinfection exclusion mechanisms have been observed in multiple viruses, including the T-even bacteriophages (Dulbecco 1952; Anderson and Eigner 1971), phiX174 (Vanderavoort et al. 1984), lambda (Ptashne 2004) and HIV (Michel et al. 2005), and may be mediated by virus-induced changes in host cells that inhibit injection (Lesley et al. 1951), cause degradation (Hershey et al. 1954), or repress replication (Vanderavoort et al. 1984; Ptashne 2004) of the genomes of superinfecting viruses, or that reduce the expression of virus receptors on the cell surface (Marschall et al. 1997; Breiner 2001; Michel et al. 2005). One important feature of superinfection exclusion is that it is not manifested instantaneously, so that viruses that completely block superinfection may still undergo coinfection if the interval between entry of coinfecting partners is sufficiently small (Christen et al. 1990). As a result, when virus densities are high (i.e. the interval between infections is small), coinfection should be common in spite of superinfection exclusion.

In contrast, when coinfection rates are low, as expected when virus densities in the environment or in a multicellular host are low, the ability to enhance coinfection may confer a selective advantage. It has recently been observed in HIV (Dang et al. 2004), Venezuelan equine encephalitis (Smith et al. 2008), human cytomegalovirus (Cicin-Sain et al. 2005), and the bacteriophage ϕ6 (Turner et al. 1999), that the frequency of individual host cells coinfected with two marked genotypes is higher than expected if viruses infect cells at random. These studies suggest that a subpopulation of host cells are particularly susceptible to virus infection, however, they do not distinguish the mechanism by which variation in host cell susceptibility arises. Differences in susceptibility could result from inherent physiological differences among host cells that existed prior to infection, or from physiological differences that are induced by virus infection. Distinguishing between these mechanisms would shed light on the potential for virus-mediated coinfection enhancement.

The current study was motivated by Turner et al.'s (Turner et al. 1999) anecdotal finding that coinfection frequencies are enhanced in ϕ6 bacteriophage. We investigated the mechanism for this enhancement, focusing on the hypothesis that infection triggers increased surface-expression of pilus (the ϕ6 receptor), thereby increasing the likelihood of coinfection. This hypothesis seemed plausible because surface-expression of pilus is facultative and known to change in response to environmental cues (Merz et al. 2000). Specifically, we tested two predictions of this hypothesis: (i) ϕ6 infection or attachment will result in enhanced attachment of subsequent viral particles and (ii) enhanced attachment will not occur in phage that utilize constitutively expressed lipopolysaccharide as a receptor. As described below, both predictions were supported, suggesting that ϕ6 coinfection is enhanced by upregulation of pilus expression in infected bacterial hosts.

Materials and methods

Strains and cultures conditions

The double-stranded RNA bacteriophages ϕ6 (Vidaver et al. 1973) and ϕ12 (Mindich et al. 1999) were used in this study. ϕ6 and ϕ12 use as their receptor the host pilus (Vidaver et al. 1973; Bamford et al. 1976, 1987), and lipopolysaccharide (LPS) (Mindich et al. 1999), respectively. The ϕ6 strain used in this study was reconstructed from cloned genome segments (Gottlieb et al. 1992) of a wildtype ϕ6 isolate (Vidaver et al. 1973). Leonard Mindich (Public Health Research Institute of New Jersey Medical School) generously supplied the plasmids and bacteria used to assemble ϕ6 and supplied an isolate of ϕ12.

Bacteriophages and their hosts were cultured and titered in standard LC media (5 g yeast extract, 5 g NaCl, and 10 g Bacto-tryptone per liter H_2O) (Burch and Chao 1999). Phages were grown on plates by overlaying a mixture of phage, bacteria, and top agar (LC + 0.7% agar) onto solid media (LC + 1.5% agar). ϕ6 bacteriophage were cultured with their standard host *Pseudomonas syringae* pathovar phaseolicola strain HB10Y, which was obtained from the American Type Culture Collection (ATCC no. 21781). ϕ12 bacteriophage were cultured on LM2333 bacteria obtained from Leonard Mindich. LM2333 is a mutant of HB10Y and is thought to produce rough LPS which facilitates ϕ12 binding (Mindich et al. 1999).

Attachment rate

We performed three experiments using the following general protocol to measure the rate that bacteriophage attach to host bacteria. First, bacteriophage were mixed with exponentially growing bacteria that had achieved a

density of approximately 1×10^8 cells/mL. Initial phage (P_0) and bacteria (N_0) densities were determined by plating. Phage and bacteria were then mixed and incubated for $t = 55$ or 60 min at 25°C with continuous shaking. At the end of the incubation period, the density of free phage (P_t) was again determined by plating and attachment rate (k) was calculated as:

$$k = \ln(P_0/P_t)/(N_0 t) \quad (1)$$

Statistical analyses

Standard analyses were performed using JMP statistical software (version 6.0.3, SAS Institute, Cary, NC) and the model selection analysis was performed using R statistical software (version 2.6.2).

Results

Preferential attachment to infected bacteria

The previous finding that coinfection rates in ϕ6 are higher than expected if hosts are equally susceptible to infection (Turner et al. 1999) can be explained by two different scenarios: (i) uninfected bacteria differ in their susceptibility to infection, or (ii) an initial infection increases the susceptibility of a bacterium to subsequent infection. We first investigated whether attachment rate was affected by the infection status of the bacteria. If infection modifies host cells in ways that cause other viruses to attach to them more quickly, then we predict that attachment rates will be the highest in populations containing a high proportion of infected host cells and the lowest in populations with few infected cells.

We tested this hypothesis by measuring attachment rates in populations with different ratios of viruses to hosts (i.e. different multiplicities of infection or MOIs) and, therefore, different proportions of infected cells (Fig. 1A). This was achieved by incubating 43 densities of ϕ6 bacteriophage, ranging from 2.8×10^6 to 1.8×10^9 ϕ6/ mL, with HB10Y bacteria at a density of 7.7×10^7 ($\pm 3.8 \times 10^7$) cells/ mL for 60 min. The results of these experiments (Fig. 1A) indicate that attachment rate depends on MOI, but they do not show the highest attachment rates in the highest MOI populations. Rather, the data were best described by a second order polynomial (AIC score of second order polynomial = −2014 and AIC score of linear model = −2002.8), with maximum attachment rates in populations with intermediate MOI values (Fig. 1A; $R^2 = 0.34$, $F_{2,40} = 10.28$, $P = 0.0003$).

The second order polynomial that gave the best fit to the data supported a model of enhancement by showing that attachment rates were higher at intermediate MOIs than at low MOIs. However, the observed curvilinearity resulted primarily from the drop in attachment rates at high MOIs. In fact, the decrease in attachment rate at high MOIs would have been enough to generate a similar polynomial even if attachment rate was similar among low and intermediate MOIs. We confirmed that attachment rate is higher at intermediate than at low MOI by measuring attachment rates with a high level of replication at one low MOI and one intermediate MOI. In the low MOI assays, 12 replicates containing 1×10^7 ϕ6 bacteriophage were incubated with 4.5×10^8 cells of HB10Y in 1 mL LC for 55 min (log MOI = −1.65, proportion of infected cells = 4%) and in the intermediate MOI assays, 12 replicates of 5.5×10^8 ϕ6 bacteriophage were incubated with 2.7×10^8 cells of HB10Y for the same time period (log MOI = 0.3, proportion of infected cells = 63%). Consistent with our initial predictions, the intermediate MOI populations had a significantly higher mean

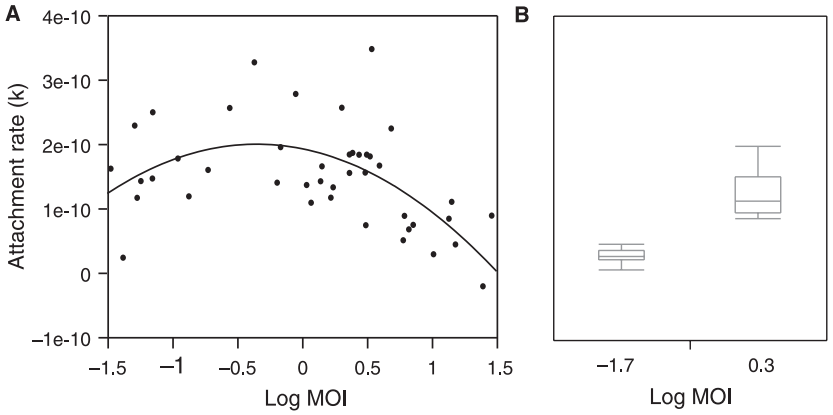

Figure 1 Preferential attachment to infected cells. (A) The relationship between the rate that ϕ6 attach to their bacterial host and log MOI as estimated from forty-three attachment assays. This relationship is described by a second order polynomial ($R^2 = 0.34$; $P = 0.0003$). (B) Attachment rate estimates based on twelve attachment assays performed at each of two log MOIs. Attachment rates are significantly higher in intermediate MOI populations where infection is common than in low MOI populations where infection is rare (*t*-test; $P < 0.0001$).

attachment rate than the low MOI populations (Fig. 1B; $F_{1,22} = 345.6$, $P < 0.0001$) .

In these attachment rate experiments we used MOI as a proxy for the proportion of infected cells. In order to make the relationship between these values more explicit, we calculated the proportion of infected cells in the 43 replicate populations examined in the first attachment rate experiment. The proportion of infected cells can be expressed as the Poisson probability that a cell is infected by one or more bacteriophage, given the total number of bound phages (P_{bound}) and the total number of bacteria (N_0):

$$\Pr(\text{infected}) = 1 - e^{-P_{\text{bound}}/N_0} \quad (2)$$

P_{bound} can then be calculated as a theoretical expectation, given a particular attachment rate k, phage density P_0, and bacterial density N_0:

$$P_{\text{bound}} = P_0(1 - e^{-kN_0 t}) \quad (3)$$

Or, P_{bound} can be determined empirically by subtracting the measured number of free (unbound) phages at 60 min from the initial measure at 0 min:

$$P_{\text{bound}} = P_{60} - P_0. \quad (4)$$

We used the average bacterial density measured in this experiment (7.7×10^7 bacteria/ mL) and the attachment rate measured at log(MOI) = 0.3 ($k = 1.4 \times 10^{-10}$) to determine the theoretical expectation. We then show both the expectation and the experimentally determined relationship between MOI and the proportion of infected cells in Fig. 2. The empirically determined probabilities show an excellent fit to the expectation, deviating mainly because of measurement error in our estimates of bacterial and phage densities.

There was good correspondence between the range of MOIs over which the proportion of infected cells shifted from few to many and the range of MOIs over which attachment rates shifted from increasing to decreasing.

Pilus-mediated coinfection enhancement

We further examined the relationship between coinfection enhancement in ϕ6 and the use of host pilus as the phage receptor by comparing the effect of infection on attachment rate in ϕ6 and its close relative ϕ12. We made this comparison because ϕ6 uses the facultatively expressed (Roine et al. 1996) host pilus as its receptor (Bamford et al. 1987; Gottlieb et al. 1988), while ϕ12 binds to host LPS (Mindich et al. 1999) which is constitutively expressed (Whitfield and Valvano 1993). If the ultimate cause of coinfection enhancement is upregulation of bacteriophage receptor, then viruses such as ϕ6 – whose receptor is inducible – will be capable of enhancement, while viruses such as ϕ12 – whose receptor is constitutively expressed – will be incapable of enhancement.

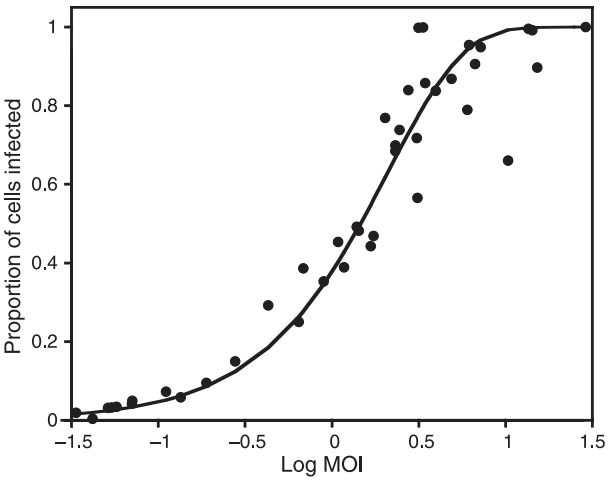

Figure 2 Proportion of host cells infected. The expected relationship between the proportion of infected host cells and log MOI is estimated using equations 2 and 3 (shown as a line). Each point is the experimentally determined proportion of infected cells in one of 43 replicate attachment assays (Fig. 1A), calculated using equations 2 and 4. The action of a superinfection exclusion mechanism in ϕ6 is suggested by the correspondence between the range of MOIs over which the proportion of infected cells shifted from few to many and the range of MOIs over which attachment rates shifted from increasing to decreasing (Fig. 1A).

We supported this hypothesis using 32 assays examining the rate that ϕ12 attach to their bacterial host over a range of MOIs. These attachment assays were performed by incubating 32 densities of ϕ12 bacteriophage, ranging from 6.7×10^6 to 8.7×10^8 ϕ12/ mL, with LM2333 bacteria at a density of 1.2×10^8 ($\pm 8.5 \times 10^7$) cells/ mL for 60 min. The results of these assays indicate that there is no relationship between attachment rate and log MOI in ϕ12 (Fig. 3; $R^2 = 0.02$, $F_{1,30} = 0.72$, $P = 0.40$) . Therefore, the rate that ϕ12 phage attach to host cells does not depend on the proportion of cells that are infected.

Discussion

Turner et al.'s (Turner et al. 1999) finding that coinfection rates are enhanced in ϕ6 strongly suggests that host cells differ in their susceptibility to infection – with the most susceptible cells being infected by multiple virions. In the current study we test this possibility and examine whether differences in host susceptibility arise from inherent physiological differences that existed prior to infection or physiological differences that are induced by virus

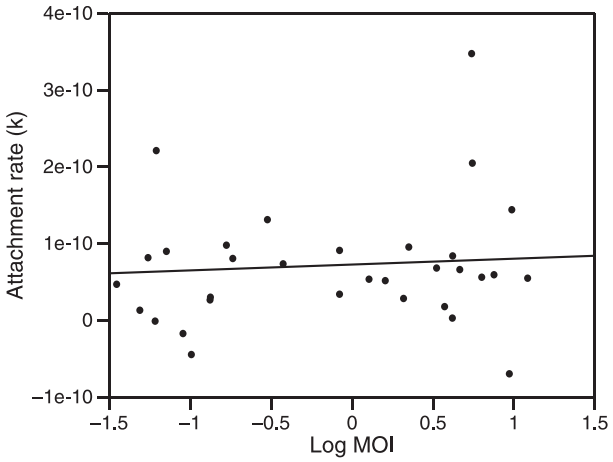

Figure 3 Pilus-mediated coinfection enhancement. Thirty-two assays were performed to estimate the rate that ϕ12 bacteriophage attach to their bacterial host over different MOIs. There was no significant relationship between attachment rate and log MOI ($R^2 = 0.01$; $P = 0.47$), indicating that ϕ12 bacteriophage do not enhance coinfection rates by preferentially attaching to infected bacteria.

attachment or infection. By demonstrating that ϕ6 bacteriophages attach significantly faster in host populations containing a larger proportion of infected hosts, we confirm that hosts can differ in their susceptibility to infection and that these susceptibility differences are induced by virus attachment or infection. These results represent the first demonstration that coinfection enhancement can be virus mediated.

We also investigated the mechanism by which ϕ6 enhances the likelihood of coinfection. Viruses that suppress superinfection frequently do so by downregulating the host receptor (Marschall et al. 1997; Breiner 2001; Michel et al. 2005); we therefore reasoned that upregulation of the ϕ6 receptor – the host pilus (Vidaver et al. 1973; Bamford et al. 1976, 1987; Roine et al. 1996) – could enhance coinfection by making host cells more susceptible to infection. We also reasoned that a related bacteriophage, ϕ12, whose receptor (LPS (Mindich et al. 1999)) is constitutively expressed (Whitfield and Valvano 1993) should be incapable of enhancing coinfection. Our observation that ϕ6, but not ϕ12, bacteriophage attach at a higher rate in populations with a higher proportion of infected hosts is consistent with these predictions and suggests the following model of coinfection enhancement in ϕ6. (i) A ϕ6 bacteriophage attaches to a host pilus and infects the host cell. (ii) Events during phage attachment or infection cause pilus extension to be favored over pilus retraction. (iii) Pilus extension exposes additional phage attachment sites, thus increasing the rate that bacteriophage attach to infected hosts. This model is consistent with the major observations of our study, and provides direction for future investigations into the timing and type of modifications made to host cells.

It is worth noting that the data cannot be fully explained by the simplest version of this model in which pilus retraction is simply turned off. Although turning off pilus retraction could result in higher attachment rates, it could not result in enhanced coinfection because pilus retraction is a required step for phage infection of the host cell. In this simple model, the only remaining explanation for the previous observation of enhanced coinfection in ϕ6 (Turner et al. 1999) is that a substantial subset of host cells are uninfectable. However, in our hands >99.5% of hosts become infected in the high MOI conditions used here and in Turner et al. (Turner et al. 1999) (data not shown). Thus, our observations of enhanced coinfection and infection-induced increases in attachment rate are best explained by a more complex model in which phage infection shifts the balance in favor of pilus extension, but does not completely prevent pilus retraction.

Although we did not investigate the molecular mechanism responsible for virus-mediated increases in attachment rate in ϕ6, recent studies of pilus-dependent motility in *Neisseria meningitides* and *N. gonorrhoeae* suggest multiple ways to alter the balance between pilus extension (which exposes attachment sites) and retraction (which hides attachment sites). In *Neisseria*, 15 proteins are required for pilus extension and retraction (Carbonnelle et al. 2005), some of which must be actively synthesized during those processes (Merz et al. 2000). Further, both processes require ATP binding and hydrolysis (Jakovljevic et al. 2008). These results suggest that bacteriophage attachment rates may fluctuate due to changes in the expression of proteins mediating pilus extension and retraction or due to changes in bacterial kinetics that make one of these processes more energetically favorable than the other.

One observation that our model of coinfection enhancement does not predict is the decline in attachment rate that we observed at high MOIs (Fig. 1A). One possible explanation is that at very high MOIs phage binding sites on the pilus become saturated with phage. However, attachment rate begins to decline at an MOI of around two phages per host cell – too few to achieve saturation. A more likely explanation for the decline in attachment rate may be the engagement of a pilus-mediated superinfection exclusion mechanism that prevents more than two phages from infecting the cell. Indeed, we already know that coinfection in ϕ6 is limited to two phages per cell (Turner et al. 1999). Although speculative, this explanation is intriguing because it would require that pilus expression is under sufficiently fine control that infection by one phage favors pilus extension, whereas

infection by two phages favors pilus retraction. Furthermore, by acting together to generate host cells infected by exactly two phages, coinfection enhancement and superinfection exclusion would maximize the net benefits of coinfection.

Causes of variation in virus coinfection rates

The finding of coinfection enhancement in only a few viruses (HIV (Dang et al. 2004), VEEV (Smith et al. 2008), HCMV (Cicin-Sain et al. 2005), and ϕ6 (Turner et al. 1999)) in comparison to the near ubiquity of mechanisms for superinfection exclusion (Delbrück and Luria 1942; Dulbecco 1952; Anderson and Eigner 1971) leads one to question what aspects of virus biology sets coinfection enhancers apart from other viruses? Although the apparent rarity of coinfection enhancement may have resulted simply because this phenomenon is more difficult to observe than superinfection exclusion, there are both mechanistic and evolutionary reasons to expect viruses to differ in the use and strength of enhancement versus exclusion mechanisms.

Mechanistically, the relative abundance of superinfection exclusion, as compared to enhancement, may occur because there are a greater number of mechanisms by which viruses can achieve superinfection exclusion. While individual virus species may experience constraints that prevent the use of any particular exclusion mechanism, they are likely to possess the ability to evolve at least one mechanism. In contrast, enhancement has been observed to occur only via an increase in expression of virus receptor on the host cell surface (Chen et al. 2005) or via a cell-mediated pathway in which dendritic cells or monocytes/macrophages capture and transmit multiple virions to a target cell (Chen et al. 2005; Cicin-Sain et al. 2005). These results suggest that viruses may differ in their ability to enhance coinfection simply based on the type of receptor they use or whether they have cell-mediated infection.

Alternatively, populations could differ in the costs and benefits associated with coinfection, causing natural selection to favor different responses to infection and coinfection in different populations. First, viruses may differ intrinsically in ways that affect the benefits (or costs) of coinfection. For example, some viruses, such as HIV and ϕ6, have mechanisms like polymerase switching (Coffin 1979) and segmentation (Vidaver et al. 1973) that allow them to frequently recombine/reassort during coinfection, whereas other viruses, such as some flaviviruses rarely, if ever, recombine (Monath et al. 2005). If the primary advantage of coinfection is recombination/reassortment then viruses lacking such mechanisms will receive little benefit from coinfection. Second, virus and host ecology will have a direct effect on the realized costs and benefits of coinfection. Phage in environments with higher ratios of phages to hosts should exhibit less coinfection enhancement. In such environments, there is no need to enhance coinfection, and moreover, enhancement should be disfavored if it tends to elevate the number of coinfecting viruses above two (costs would increase, but benefits would not).

Returning to our comparison of ϕ6 and ϕ12, we proposed the following mechanism for their differences in phage-mediated increases in attachment rate – the use of a constitutively expressed receptor prevents ϕ12 from evolving coinfection enhancement. However, our data do not rule out the possibility that ecological differences in the natural environment of these phages favored coinfection enhancement in ϕ6 but not in ϕ12. The nature of the difference between these two phages raises the intriguing possibility of distinguishing between these mechanistic and evolutionary hypotheses in future laboratory evolution experiments that manipulate host and virus ecology to impose selection either for coinfection enhancement or coinfection inhibition.

Implications of coinfection enhancement for disease management

Coinfection enhancement poses a challenge to disease management due to its ability to generate recombination in populations where virus densities are kept low by suppression mechanisms. In any scenario in which two (or more) mutations are required for escape from suppression, the small size of suppressed virus populations makes it unlikely that an escape double mutant will evolve de novo or be generated by recombination between viruses carrying different escape mutations. This type of recombination event would be possible, however, if the virus was capable of coinfection enhancement. Here we discuss how coinfection enhancement may promote such rare recombination events.

We can think of three scenarios in which viral densities are suppressed to the point where coinfection is unlikely and where two or more mutations are needed to escape from suppression. In the first scenario, virus titers are suppressed by antiviral drugs, and the use of multiple drug therapies ensures that viruses need to simultaneously acquire multiple drug resistance mutations. Under these conditions, coinfection enhancement could speed the evolution of multi-drug resistance by facilitating recombination between viruses carrying different resistance alleles. Similarly, virus titers could be kept low by partial host immunity, either via natural immunity or imperfect vaccines (Gandon et al. 2001). In this second scenario, coinfection enhancement has the potential to facilitate

immune escape if escape requires multiple mutations. In the third scenario, hosts are vaccinated with live, attenuated viruses whose densities remain too low to cause disease. This last scenario could be particularly problematic because many vaccines are multivalent, containing multiple virus strains. Attenuated vaccine viruses are thought to be safe because multiple mutations are required to make them pathogenic, however, if the strains contained in a multivalent vaccine are attenuated via different suites of mutations, then recombination could produce pathogenic viruses by combining mutation-free genome regions from multiple strains. To minimize this risk, multivalent vaccines are often comprised of multiple virus strains attenuated by common mutations. If coinfection enhancement is a general characteristic of viral pathogens, then strategies like this one may be critical to the success of antiviral treatments.

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