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Review

Cooperation between different variants: A unique potential for virus evolution

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ABSTRACT

RNA viruses exist as quasispecies containing many variants within their populations because of the error prone nature of viral RNA-dependent RNA polymerases. Quasispecies are not a simple collection of individual variants. Instead, internal interactions among variants provide quasispecies with unique evolvability. An example is 'cooperation' between wild-type and defective measles viruses, in which co-existence of a wild-type and a mutant genome produces a new phenotype. Such internal interactions presuppose efficient co-transmission of multiple genomes to the same cell, which is achieved by polyploid virions of some virus families or by a high multiplicity of infection. Recent studies have revealed that multiple viral genomes can also be transmitted simultaneously ('bloc transmission') by other mechanisms, strengthening the concept of internal interactions among viral quasispecies. Elucidation of the mechanisms of virus evolution, including internal interactions and bloc transmission, may provide rational strategies to solve such important problems of virus infections as drug-resistance, immune evasion, and acquisition of the new tropism and host range.

1. Introduction

RNA viruses have a unique ability to adapt readily to different environmental conditions because of the high error rates of viral RNA-dependent RNA polymerases. Accordingly, some of the difficulties in controlling RNA virus infections, such as drug-resistance, immune evasion, and expansion of the tropism and host range, result from this evolvability of causative viruses (Domingo et al., 2012). Furthermore, the error-prone nature of RNA genome replication causes virus populations to form mutant spectra (mutant clouds or 'quasispecies') (Andino and Domingo, 2015; Domingo et al., 2012; Duffy et al., 2008; Holland et al., 1982; Lauring and Andino, 2010).

Quasispecies constitute dynamic (continuously changing) repositories of genotypic and phenotypic viral variants, providing the source of viral evolvability (Domingo et al., 2012). Importantly, quasispecies are not a mere mixture of independently acting mutants. Rather, there are internal interactions among variants, which may determine the biological behavior of virus populations (Domingo et al., 2012). Three types of internal interactions, complementation, defective interference and cooperation, are observed within viral variants (Fig. 1). Among these, cooperation has eluded detailed understanding, especially in regard to its molecular mechanisms. Recently we have demonstrated that cooperation between different viral genomes can occur through the heterooligomer formation of wild-type and mutant viral proteins (Shirogane et al., 2016, 2013, 2012).

Internal interactions within a virus population presuppose co-infection of multiple virions in the same cell (Domingo et al., 2012). A high multiplicity of infection (MOI) *in vitro* could allow co-infection of multiple virions in the same cell, but this was thought to occur rarely, if at all, in natural infections *in vivo*. Recent studies have greatly changed this notion, by showing that viral spread could be mediated by 'collective infectious units' that simultaneously transmit groups of viral genomes ('bloc transmission') (Fig. 1) (Sanjuán, 2017). The collective infectious units include polyploidy virions, virion aggregation, non-viral specific structures, and direct cell-to-cell transmission. The observations suggest that collective infectious units contribute to the maintenance of viral genetic diversity and the evolution of 'social-like' virus–virus interactions (Díaz-Muñoz et al., 2017; Sanjuán, 2017).

In this review, we first briefly summarize complementation and defective interference, and next describe cooperation in detail. We then discuss how recent findings of 'bloc transmission' or 'collective infectious units' strengthen the concept of internal interactions among viral quasispecies.

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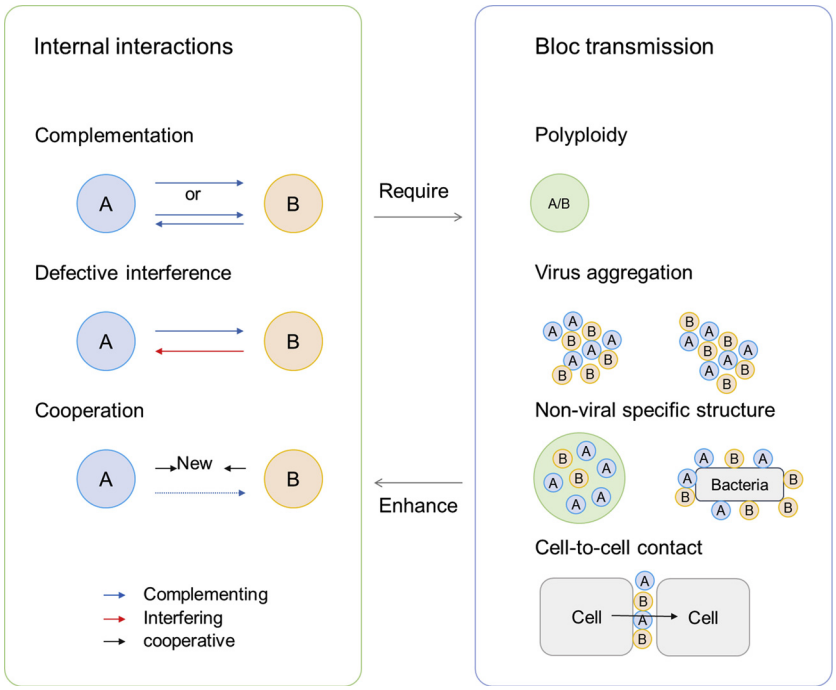

Fig. 1. Relationship between internal interactions among virus populations and bloc transmission.
Three types of internal interactions among virus populations are described in the left panel of the figure. Blue and yellow circles indicate different variants. For complementation, one variant works as a helper virus for the other. For defective interference, one variant also works as a helper virus for the other, while the other one interferes with helper virus production. For cooperation, both variants cooperate with each other to produce a new beneficial phenotype. Four types of bloc transmission are described in the right panel of the figure. A polyploid virion can include several variant genomes. Aggregation of virions, non-viral specific structures such as occlusion bodies, lipid vesicles, and bacteria, and cell-to-cell direct contact can also help co-transmission. Efficient internal interactions among variants require bloc transmission, while bloc transmission enhances evolution of internal interactions.

2. Complementation

A virus with a gene (of its genome) expressing a normal protein (the helper virus) may promote replication of another viral genome whose corresponding gene encodes a defective protein (Domingo et al., 2012). This kind of interaction is called complementation (Fig. 1). It allows defective genomes to be reproduced and maintained in virus populations as long as the relevant gene can work in-trans. Complementation is a form of internal interaction among variants, and many cases have been reported both *in vitro* and *in vivo* (natural infections) (Aaskov et al., 2006; Gelderblom et al., 2008; Moreno et al., 1997). Complementation is not necessarily a one-way phenomenon. It was reported that two defective genomes of the foot-and-mouth disease virus, which respectively have deletions in different genes, complement each other like a segmented virus (Ojosnegros et al., 2011). Another example of mutual complementation is a recombinant measles virus (MeV) with segmented genomes (Takeda et al., 2006). This virus was produced artificially by dividing its non-segmented genome into two parts whose replications rely on each other.

Defective genomes do not usually affect the growth or fitness of helper viruses. However, two other forms of interactions can occur between defective and helper viruses (Fig. 1). If co-infection of defective viruses results in lower productions of helper viruses, such defective viruses are called defective interfering (DI) particles (Huang and Baltimore, 1970). In other cases, defective genomes may cooperate with helper viruses to produce new beneficial phenotypes ('cooperation'). These are the subjects in the following sections.

3. Defective interference

Henle and Henle first reported interference with the propagation of the influenza virus by an 'inactive virus' (Henle and Henle, 1943). This was followed by the observation that serial undiluted passages of the influenza virus in embryonated eggs result in the production of virus with a decreased ratio of infectivity to hemagglutinin (von Magnus, 1951), and the phenomenon became known as the von Magnus effect. Later the term DI particle was introduced, and the four criteria for DI particles were proposed; they (1) contain a part of the viral genome, (2) contain normal viral structural proteins, (3) reproduce only in the presence of a helper virus, and (4) interfere specifically with the intracellular growth of the homologous standard virus (Huang, 1973; Huang and Baltimore, 1970). DI particles are now known to play critical roles during the course of viral infections (Rezelj et al., 2018). Because DI particles themselves are produced by complementation, defective interference is the 'mirror image' of complementation (Domingo et al., 2012).

DI particles have been demonstrated in many virus species (Cole et al., 1971; Frensing, 2015; Huang, 1973). Their genomic structures are different depending on virus species. For example, poliovirus has a non-segmented RNA genome of positive polarity, belonging to the genus *Enterovirus* of the family *Picornaviridae* (Racaniello, 2013). The open reading frame of its genome is divided into three regions, P1, P2 and P3. The P1 region encodes structural capsid proteins, and the P2 and P3 regions encode non-structural proteins including the viral RNA-dependent RNA polymerase. DI genomes of poliovirus generally have an in-frame deletion in the P1 region (Kuge et al., 1986; Lundquist et al., 1979). Therefore, non-structural proteins including its RNA-dependent RNA polymerase are translated from DI genomes, allowing DI genomes to replicate on their own. By contrast, DI genomes of negative-strand RNA viruses, such as vesicular stomatitis virus (VSV), have various structures, which are classified into four types: simple deletion, panhandle, compound and snap back (copy back) (Lazzarini et al., 1981). DI genomes of negative-strand RNA viruses do not usually replicate by themselves because their replications rely on RNA-dependent RNA polymerases from helper viruses.

Considering different genomic structures of DI genomes, there appear to be various mechanisms of defective interference. It is generally thought that shorter DI genomes replicate faster than full-length helper virus genomes in co-infected cells. DI genomes could be 'cheats' for 'public goods', such as capsid proteins and viral polymerases, provided by helper viruses (Díaz-Muñoz et al., 2017). Copy back DI RNAs form stem-loop structures and strongly induce the expression of type I interferons because double-stranded RNAs in the stem region are recognized by cytoplasmic RNA sensors such as retinoic-acid-inducible gene-I and melanoma differentiation-associated gene 5 (López, 2014). Sendai virus copy back DI RNAs were successfully used as an adjuvant with inactivated influenza vaccines because of their strong immunostimulatory activity (Martinez-Gil et al., 2013; Mercado-López

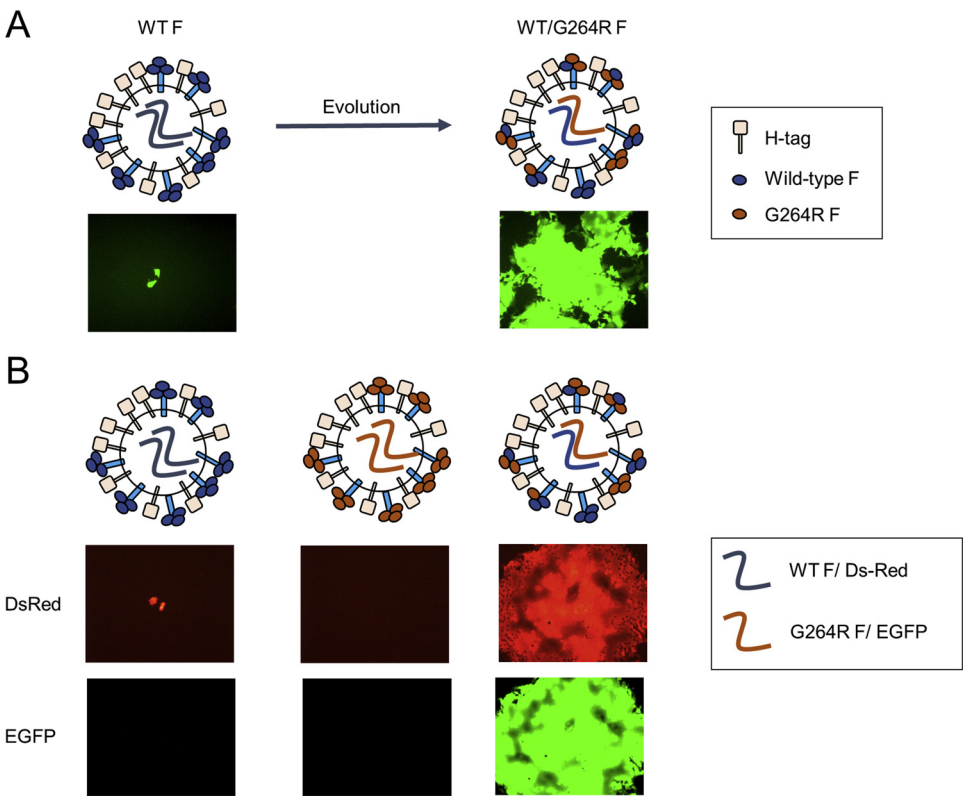

Fig. 2. Cooperation between different MeV variants. (A) A recombinant MeV expressing a tagged H protein and a wild-type (WT) F protein did not cause cell-to-cell fusion. After several passages, the virus evolved to be fusogenic by containing two types of genomes possessing WT and G264R F genes in the virus population. Co-expression of WT and G264R F proteins caused a new fusogenic phenotype, presumably because of hetero-oligomer formation of both F proteins. (B) Three types of recombinant MeVs expressing WT F, G264R F, or both F proteins were generated by a reverse genetics method. The genome expressing WT F proteins encoded the Ds-Red gene, while the genome expressing G264R F proteins encoded the EGFP gene. The recombinant MeV encoding the WT F gene did not cause cell-to-cell fusion. The recombinant MeV encoding the G264R F gene could not be rescued because the G264R F protein was defective. However, the recombinant MeV including both genomes caused cell-to-cell fusion efficiently. Mixed genomes produced a new beneficial phenotype for MeV growth. These data were modified from Shirogane et al., 2012, *Nature Communications* (Shirogane et al., 2012).

et al., 2013). However, the precise mechanisms of defective interference have not been determined for many viruses.

DI particles might have a potential as novel antiviral agents. A recent study showed that DI particles of influenza A virus protect experimental animals from lethal challenge not only with influenza A virus, but with other respiratory viruses (Dimmock and Easton, 2015). Engineered DI particles, instead of naturally occurring ones, are also being considered for therapeutic use. To optimize DI particles for that purpose, it is important to understand the mechanisms of defective interference at the cell level as well as at the host population level. A mathematical modeling of human immunodeficiency virus (HIV) evolution has provided a clue to designing engineered DI particles to make them evolutionarily robust and avoid recombination in the human population (Rouzine and Weinberger, 2013). Of course, co-infection with functional viruses in the same cell is absolutely required for the therapeutic use of DI particles to be successful.

4. Cooperation

Examples of cooperative interactions have been reported in various virus species. A mutant poliovirus with a high-fidelity RNA polymerase and reduced genomic diversity (a narrow mutant spectrum) was found to lose its neuropathogenicity, which was restored through expanding quasispecies diversity by chemical mutagenesis (Vignuzzi et al., 2006). That study indicated that cooperative interactions, rather than specific individual clones, among quasispecies contribute to neuropathogenicity. A mixed population containing two types of H3N2 influenza virus variants, which differ by a single mutation at residue 151 in the neuraminidase, grew better in cell culture than either variant alone (Xue et al., 2016). The growth advantage decreased at lower MOIs, although the mixed population grew better even at an MOI of 0.02 at later time points. The authors proposed that one variant has an advantage at cell entry, while the other variant has its advantage at cell exit. Cooperation could provide the mixed population with both efficient entry and exit. However, the variation at residue 151 in the neuraminidase was not detectable in primary clinical samples of H3N2 influenza viruses, indicating that passage in cell culture is responsible for selecting these variants (Xue et al., 2018). One study showed that the West Nile virus population may have cooperative interactions among variants because the population fitness levels exceed that of any individual variant (Ciota et al., 2012). In another study, coexistence of two naturally occurring hepatitis B virus variants improved viral replication and strongly induced host antibody and cellular immune responses, which may enhance immune-mediated liver damage (Cao et al., 2014). Adaptation of Coxsackie B3 virus to weakly permissive A549 cells resulted in group selection of minority variants, cooperation of which conferred a fitness increase in the population (Bordería et al., 2015).

It was reported that long-term transmission of defective dengue viruses in humans and Aedes mosquitoes occurs presumably through complementation (Aaskov et al., 2006). However, a recent study provided evidence that co-transmission of defective and functional dengue viruses has a higher transmission potential than transmission of functional viruses alone (Ke et al., 2013). The authors suggested two potential mechanisms of cooperation by which defective viruses could enhance transmission. First, the defective viruses may work as DI particles, causing milder disease and thereby enhancing virus dissemination. This hypothesis is notable as DI particles could provide advantage at the level of the host population. Second, the defective viruses may enhance production of the helper virus by increasing insufficient viral gene products.

Although cooperative interactions as phenomena have been well described, their exact molecular mechanisms are largely elusive. Recently, we reported a unique molecular mechanism of cooperation among different MeV genomes (Shirogane et al., 2016, 2013, 2012). MeV, a member of the *Paramyxoviridae* family, is an enveloped virus with a non-segmented RNA genome of negative polarity. MeV has two envelope glycoproteins, hemagglutinin (H) and fusion (F) proteins. The H protein binds to a cellular receptor and triggers conformational changes of the F protein from the pre-fusion to the post-fusion form, which is required to cause virus-to-cell or cell-to-cell fusion (Griffin, 2013). Unlike the wild-type MeV, a recombinant MeV with a tagged H protein did not induce syncytia in receptor-positive cells, although the

virus could grow slowly. After several passages, mutant viruses emerged with the syncytium-forming phenotype (Fig. 2A) (Shirogane et al., 2012; Watanabe et al., 2015, 2013). Importantly, a mutant virus had two types of genomes in the viral population. One genome encoded the wild-type F protein, while the other encoded the mutant F protein (G264R). The G264R F protein did not exhibit the fusogenic function, and therefore the recombinant virus encoding only the G264R F protein could not be recovered by reverse genetics. However, the defective genome was somehow included in the virus population exhibiting the syncytium-forming phenotype.

In general, defective genomes are thought to be a burden for helper viruses, but it is not the case with this mutant virus. The G264R F protein was shown to provide fusogenicity when it was expressed with the wild-type F protein (Fig. 2B). Such 'cooperation' was also observed *in vivo*. The recombinant MeV possessing both the wild-type and mutant genomes spread in hamster brains efficiently, while the wild-type virus did not, indicating that cooperation could also change MeV tissue tropism. This phenomenon is not a simple complementation between the defective genome and the wild-type genome because the former is beneficial for the latter (when the tagged H protein is used by the virus). Thus, two types of genomes cooperatively interacted with each other and produced a new phenotype (fusogenicity).

MeV F proteins form functional trimers, and therefore co-expression of the wild-type and the G264R F proteins produce the heterotrimeric F proteins. Depending on the experimental condition, appropriate stability of the F protein might be required for the conformational changes to occur upon triggering (Hashiguchi et al., 2018; Shirogane et al., 2012; Watanabe et al., 2015, 2013). The wild-type and G264R F proteins respectively have higher and lower stability than the optimal F protein, in conjunction with the tagged H protein. The high stability of the wild-type F protein might hinder the conformational change upon triggering, while the low stability of the G264R F protein might make the protein fusion-defective. The heterotrimeric F proteins could have intermediate stability, which is appropriate for inducing membrane fusion. Thus, this example clearly revealed a molecular mechanism of cooperation (Shirogane et al., 2012).

Internal interactions among virus variants, including cooperation, presuppose co-infection of multiple viruses in the same cell. How can this condition be achieved by MeV? Some viral families such as paramyxoviruses (Rager et al., 2002), birnaviruses (Luque et al., 2009) and filoviruses (Beniac et al., 2012) have multiple genomes in one virus particle, which is called 'polyploidy'. Polyploidy allows multiple genomes to infect the same cell even at low MOIs, promoting internal interactions among virus populations (Shirogane et al., 2013, 2012).

5. Internal interactions and bloc transmission of viruses

Recent studies have shown that multiple viruses could also be transmitted simultaneously as groups of viral genomes through mechanisms other than polyploidy (Sanjuán, 2017) (Fig. 1).

First, aggregation of virions can lead to co-transmission of viruses to the same cell. Single-cell isolation with ultra-deep sequencing following VSV infection revealed that pre-existing multiple variants are detected in individual infected cells, suggesting that a single infectious unit includes multiple genetically diverse viral genomes (Combe et al., 2015). Free VSV particles were shown to spontaneously aggregate, allowing the transmission of different viral variants to the same cell. Interestingly, aggregation occurs more frequently in saliva than in standard culture media. The authors propose that virus protein–lipid interactions are important for the ability to aggregate (Cuevas et al., 2017).

Second, non-viral specific structures containing multiple viral genomes can be formed. Baculoviruses are transmitted as the occlusion body containing multiple virions between host insects (Slack and Arif, 2006). Recent studies have revealed that several non-enveloped viruses can be released from infected cells as lipid vesicles containing multiple virions (Chen et al., 2015a; Feng et al., 2013; Nagashima et al., 2014; Owens et al., 2004; Robinson et al., 2014b). The origins of lipid vesicles are different depending on virus species. Hepatitis A and E virus particles are included in exosomes (Feng et al., 2013; Nagashima et al., 2014), whereas vesicles containing enteroviruses are derived from phosphatidylserine lipid-enriched autophagosome-like organelles (Chen et al., 2015b; Robinson et al., 2014b). Furthermore, poliovirus was shown to have an ability to bind the bacterial lipopolysaccharide, which promotes the environmental fitness of the virus (Robinson et al., 2014a). Certain bacterial strains increase poliovirus co-infection of mammalian cells even at low MOIs ('bacteria-mediated viral co-infection') (Erickson et al., 2018).

Third, direct cell-to-cell contacts can enhance co-transmission of multiple genomes. Syncytium-forming viruses such as MeV can transfer their genomes directly to the next cell through cell-to-cell membrane fusion. Direct cell-to-cell transfer of HIV may occur through 'virological synapses', which promote co-transmission by generating the condition of high local MOIs (Agosto et al., 2015; Jolly et al., 2004; Sherer et al., 2007). Recently, the cellular structure allowing direct cell-to-cell transmission was reported for several respiratory viruses (Cifuentes-Muñoz et al., 2018; El Najjar et al., 2016; Mateo et al., 2015; Mehedi et al., 2016; Roberts et al., 2015). Intercellular extensions produced by F-actin polymerization connect two different cells and allow direct transport of viral components, which might help efficient transmission even in the mucin-rich environment of the respiratory tract (Cifuentes-Muñoz et al., 2018).

Quasispecies are thought to be not a mere collection of mutants, but rather a mutant ensemble acting as a unit of selection, including internal interactions (Andino and Domingo, 2015). Nevertheless, it has been difficult to explain how genetically diverse variants are naturally maintained under the low MOI conditions intuitively expected *in vivo*. However, as described above, many virus species have a potential to transmit multiple genomes simultaneously with a variety of strategies. Importantly, enhanced co-transmission could promote effective internal interactions among virus quasispecies. Therefore, the observations of bloc transmission strengthen the quasispecies theory and the importance of internal interactions among virus variants.

The consensus (major) sequence of a virus does not necessarily reflect the phenotype of the virus population. Indeed, the consensus sequence of the cooperative mutant MeV described above corresponded to that of the defective genome, although the mutant was phenotypically replication-competent with a higher growth rate because of the cooperative interaction with the wild-type genome included in the virus population (Shirogane et al., 2012). Thus, careful attention should be paid to minor sequence variations when the relationship between the consensus sequence and the phenotype is investigated. Any complementation, defective interference or cooperation has potential to produce a phenotype quite different from that expected from the consensus sequence.

Bloc transmission may change the dynamics of DI particles maintained in a virus population because they need co-infection with helper viruses to reproduce themselves. Elucidation of the mechanisms causing co-transmission of DI particles and functional viruses in detail may improve the strategy for future development of DI particle-based vaccines and antivirals.

6. Conclusions

Virus quasispecies could exhibit unique evolvability through internal interactions among variants, which are necessarily supported by various types of bloc transmission. Notably, the heterooligomer formation of any viral protein could produce a new phenotype like the MeV F protein. Therefore, it is important to keep internal interactions, including cooperation, in mind when analyzing and interpreting the virus sequence information. Furthermore, we should not separately consider internal interactions and bloc transmission because there is a strong link between them (Fig. 1). If any internal interaction is found

among a virus population, there might be a specific mechanism that allows bloc transmission of multiple viruses, and *vice versa*. Exploring more examples of internal interactions may lead to better understanding of virus evolution and provide us with clues to solving important problems of virus infections such as drug-resistance, immune evasion, and expansion of the tropism and host range.

Declarations of conflict of interest

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