Review

Quasispecies of dengue virus

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Abstract: Pathogenic viruses have RNA genomes that cause acute and chronic infections. These viruses replicate with high mutation rates and exhibit significant genetic diversity, so-called viral quasispecies. Viral quasispecies play an important role in chronic infectious diseases, but little is known about their involvement in acute infectious diseases such as dengue virus (DENV) infection. DENV, the most important human arbovirus, is a causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF). Accumulating observations suggest that DENV exists as an extremely diverse virus population, but its biological significance is unclear. In other virus diseases, quasispecies affect the therapeutic strategies using drugs and vaccines. Here, I describe the quasispecies of DENV and discuss the possible role of quasispecies in the pathogenesis of and therapeutic strategy against DENV infection in comparison with other viruses such as Hepatitis C virus, human immunodeficiency virus type 1, and poliovirus.
Key words: Primary infection, Secondary infection, immune response, antibody-dependent enhancement, Neutralization, Serotype

CONTENTS

1. INTRODUCTION

The genome of RNA viruses, including dengue virus, rapidly accumulates mutations because of the error-prone nature of RNA polymerase [1, 2], resulting in variations of the virus genome, so-called quasispecies. This diversification leads to fitness or loss due to the deleterious mutation or rapid and unpredictable fitness gains [3]. Although this genetic variation is produced by mutation, it could reflect the process of natural selection. In quasispecies of virus infections, the two key terms are "random mutation" and "selection." Mutations are introduced by random mutation, and the produced virus population is influenced by the host environment, which causes the selection of virus.

Quasispecies play a critical role in the progression of chronic infectious diseases such as Hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) [4–6]. With respect to acute infectious diseases such as dengue virus or influenza virus infections, the biological relevance of quasispecies in pathogenesis remains unclear although there is accumulating circumstantial evidence showing that these viruses also exist as quasispecies. One of the reasons is the difficulty in monitoring the diversity of viruses during such a short course of acute infection.

In this review, I discuss the virus quasispecies of DENV with reference to other RNA viruses because little information is available on quasispecies of DENV, while, on the contrary, several studies on other viruses demonstrated the biological relevance of quasispecies, for example, escape of HIV-1 and HCV from immune pressure and drug treatment, and their effects on poliovirus pathogenesis [7].

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2. DENV INFECTION

DENV, the most important arbovirus for humans, is the pathogen of dengue fever (DF) and dengue hemorrhagic fever (DHF), the latter being the more severe form of the disease [8]. It is estimated that there are 50–100 million cases of infections and more than 2.5 billion people are at risk [9]. DENV is highly restricted in its natural host range transmitted between humans and mosquitoes [10].

DENV belongs to the genus Flavivirus of the family *Flaviviridae*, which are positive-sense, single-strand RNA viruses. The viral genome is approximately 10,700 bases in length. Its single polyprotein is produced from one long open reading frame, from which individual proteins are produced by cleavage of cellular and viral proteases [11]. The genome encodes three structural proteins (capsid [C], premembrane [preM/M], and envelope [Env]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). There are four serotypes (DENV 1–4). All four DENVs are responsible for severe disease. Infection with one serotype of DENV leads to life-long protection against homologous serotypes of DENV but only temporary protection against heterologous infection with a different serotype [12]. Epidemiological observations indicate that 90% of DHF cases occur during secondary heterologous DENV infection and that the risk of DHF is highly increased in secondary infection [13]. The unique feature of DENV infection is the antibody-dependent enhancement (ADE) of virus infection [14], which explains why most severe cases occur in secondary infection. The pre-existing non-neutralizing antibodies against different serotypes of DENV, which were obtained in previous infection with other serotypes of DENV, bind to heterologous DENV and enhance viral entry into FcR-bearing target cells such as monocytes [15].

3. QUASISPECIES OF RNA VIRUSES

Viruses with RNA genome are the most abundant group of human, animal and plant pathogens. Unlike DNA polymerase, RNA viruses lack proofreading activity, resulting in an error rate during replication that is estimated to be 10^{-3} to 10^{-5} mutations per nucleotide per replication cycle[16, 17]. An acutely infected organism harbors 10^9 to 10^{12} viral population at any given time. With an RNA virus genome length of approximately 10^4 nucletides, every possible single mutant and many double mutants are likely to occur. Although most mutant viruses are probably defective, the produced virus population potentially contains diverse viruses. RNA viruses cause both acute and chronic infections. It is understandable that viral quasispecies play

an important role in chronic infectious disease, because the course of these diseases is long, sometimes lasting for decades. When confronting the host immune system, these viruses change their genomic information, and as a result, enhance the possibility of producing mutant viruses. On the other hand, viruses causing acute infections have less chance to produce mutant viruses. However, recent reports suggest the importance of quasispecies in acute viral infectious diseases [7, 18]. Moreover, it was recently reported that quasispecies at the early stage of disease were a determinant of severity even for chronic infectious diseases such as HIV-1 and HCV [4, 19–21]. Studies on acute infectious diseases from this aspect have been just started, even though the concept of quasispecies was proposed more than thirty years ago [22].

4. THE ROLE OF VIRAL QUASISPECIES IN THE ESCAPE FROM IMMUNE RESPONSE, THERAPEUTICS, AND VACCINES

4-1. The role of viral quasispecies in the escape from the host immune response

There are accumulating reports demonstrating the important role of quasispecies of other RNA viruses in the progression of disease. Two well-studied examples are HCV and HIV-1 [4–6]. HCV, the cause of hepatitis C in humans, belongs to the genus Hepacivirus within the family of Flaviviridae and is also known to exist as quasispecies. It causes a chronic infection, but the viral quasispecies of HCV at the acute phase of infection determine the course of disease progression [4, 19, 20]. The specific humoral and cellular immune response clear HCV until all the variants are effectively eliminated from the patient's body. However, when the host immune system fails to reduce the genetic diversity of HCV quasispecies, HCV continuously escapes from the immune response [23]. In case of infection with HIV-1, the quasispecies of HIV-1 play an important role in the escape from the host immune response [21]. The control of the genetic diversity of these viruses during the first few months after infection determines the progression of disease [4, 21, 24, 25]. Even in chronic infection, a key factor is the increase of the virus population size at the acute phase of infection. Otherwise, these viruses would fail to escape from the immune response during the chronic phase.

On the other hand, it remains unclear whether viral quasispecies play an important role in acute infections. DENV infection, however, is not simply an acute infectious disease, because it has four serotypes and the infection is enhanced by pre-existing non-neutralizing antibodies as described above. In the case of secondary infection, some pre-existing antibodies partially inhibit viral infection, but

A. Diversities of pre-existing antibodies and DENV

Diverse antibody population   Diverse DENV population

VS.

B. Neutralization vs. ADE

Blocking of infection
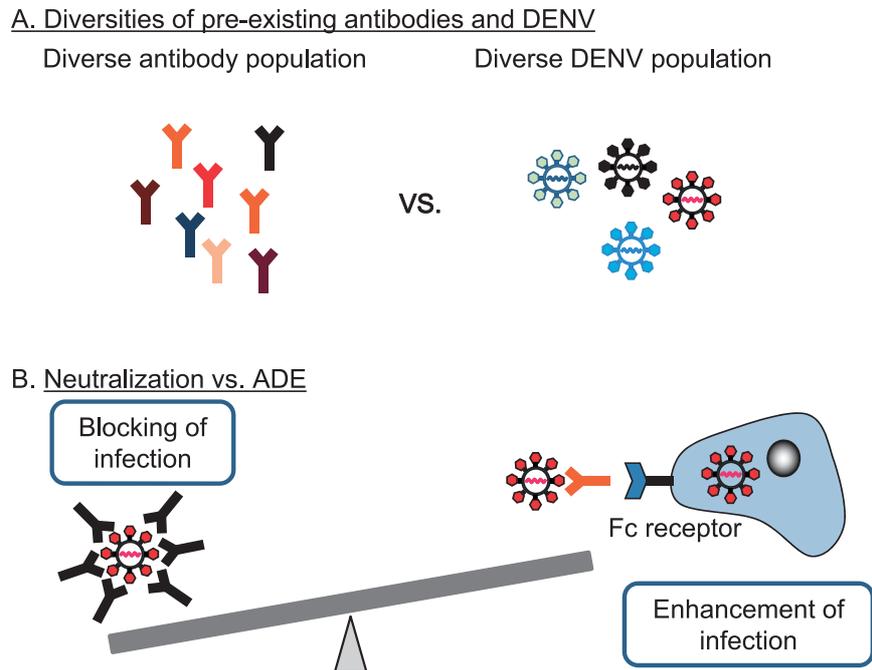
Fc receptor

Enhancement of infection

Fig. 1.   A) Diversity of pre-existing antibodies and DENV. In DENV-infected patients with secondary infection, the diverse antibody population and the diverse DENV population exist at a very early stage of infection. B) Pre-existing neutralizing antibodies block DENV infection. On the other hand, non-neutralizing antibodies enhance DENV infection through ADE. DENV infection is positively and negatively regulated by pre-existing antibodies.

other antibodies may enhance infection through ADE. The balance between neutralization and ADE is probably a key factor determining severity by controlling the production of DENV (Fig. 1). We observed that DENV-2 derived from patients with primary infection were heterogenous, while on the other hand, DENV-2 derived from patients with secondary infection were homogenous (unpublished observation). This result suggests that the homogenous population of DENV-2 may be the result of selection by ADE (Fig. 2). That is, the DENV-2 may be positively selected by non-neutralizing pre-existing antibodies derived from previous infection with other serotypes of DENV. Otherwise, this homogenous virus might be able to success-fully escape from the immune system. Although the mecha-nism of selection is unknown, our observation suggests that DENV quasispecies affects the progression of disease, especially in secondary infection.

4-2. Effects of viral quasispeceis on drugs

Antiretroviral therapy, known as "highly active anti-retroviral therapy" (HAART), which use a cocktail of three or more drugs working together to fight HIV, has led to a dramatic reduction in mortality and significant improve-ment in the quality of life of HIV-1-infected patients [26–29]. However, side effects, the requirement for strict

Secondary infection
Heterogenous DENV population

ADE

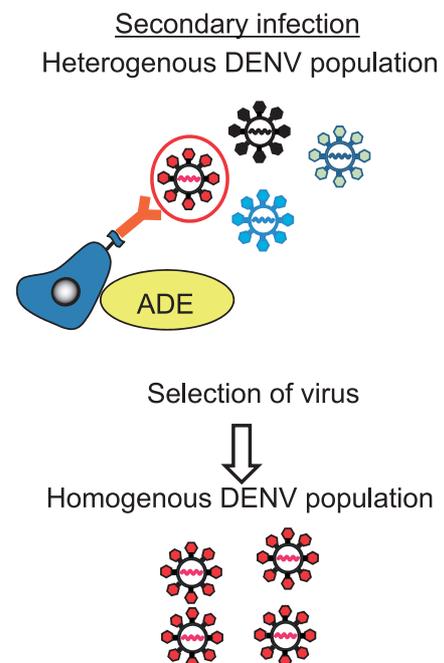Selection of virus

Homogenous DENV population

Fig. 2.   Hypothetical mechanism of DENV infection. DENV present as a heterogenous population at an early phase of infection. The parts of DENV are positively selected by non-neutralizing/enhancing antibodies. Homogenous DENV population is predominantly produced at the peak of infection.

adherence and the fact that HAART does not completely inhibit residual replication of HIV-1, can lead to therapy failure. It is extremely important that patients take medicines and do not miss doses. If they do not, the uncontrolled usage of drugs allows the development of a drug-resistant mutant virus due to quasispecies of HIV-1. A number of studies have reported the presence of homogeneous HIV-1 quasispecies during primary infection [6, 30–32], while others have suggested that a heterogeneous viral population may predominate during the early period [11, 33, 34]. In recent years, most researchers believe that HIV-1 produces a heterologous virus population at the early phase of infection. The disparity among these reports is probably due to the different detection methods or sensitivity of each protocol. Although the fittest virus rapidly outgrows other populations during the early stages of acute infection and goes on to establish a homogenous virus quasispecies HIV-1, it is clear that drug-resistant viruses sooner or later emerge by selection after starting medication. Subsequently, the drug-resistant mutant virus becomes the major presence in the virus population and causes the death of the patient. More importantly, drug resistant viruses have the potential to infect other HIV naïve persons because they are already drug-resistant at the outset of infection. In this case, the drug-resistant mutant progressively produces viruses from the beginning of anti-virus therapy.

Another drug-resistant pathogen is the antibiotic resistant bacterium. Antibiotic resistance poses a significant problem. The prevalence of antibiotic-resistant bacteria is the result of antibiotic use. As resistance becomes more common, there is a greater need for alternative treatments. However, the development and approval of new drugs does not easily catch up with the mutant bacterium. Even for acute viral infectious disease, the appearance of drug-resistant mutants is a serious problem, as shown by the antiviral-resistant influenza virus.

Although an antiviral drug 'to combat' DENV has not been developed yet, these examples suggest a need to develop several types of anti-DENV drugs. Also, patients should be treated with several drugs at the same time in order to prevent the emergence of drug-resistant mutant viruses.

4-3. Effects of viral quasispecies on the development of vaccines

A similar situation may occur when vaccines are developed to combat viruses. Because of their high mutation rates, these viruses frequently develop a resistance to vaccines similar to that seen in the application of antiviral drugs [35–39].

There are several ongoing vaccine projects, but no DENV vaccine has been licensed to date. The tetravalent live-attenuated chimeric vaccine seems to be the most promising. These vaccines mainly target the surface molecules of DENV particles, pre-membrane (prM) and envelope (Env) proteins of the four serotypes of DENV. They are based on one strain of each serotype of DENV. If DENV is genetically stable, the vaccine will exert its maximum effect. However, DENV quasispecies found in mosquitoes and some substitutions potentially change the conformation of Env protein [40], indicating the possible escape from the host immune system. If this is the case, it may be difficult to develop an effective DENV vaccine because a vaccine against limited strains may be unable to fight a diverse DENV population. Efforts to develop effective vaccines must overcome the complex evolutionary dynamics in DENV-infected individuals and within affected populations. Quasispecies of DENV have to be more clearly investigated for effective vaccine development, and the candidate vaccines need to be studied from this point of view.

Also, quasispecies exert an effect on the vaccine itself. The most promising DENV vaccine is a chimeric vaccine constructed using the live-attenuate yellow fever virus vaccine YFV 17D strain [41, 42]. It should be noted that this vaccine carries the risk of producing a revertant virulent virus because of the error-prone nature of RNA polymerase in the live-attenuate vaccine. Originally, most live-attenuated vaccines were established from virulent viruses by passage in cell culture through the introduction of mutation. It is not be surprising that they might revert to a virulent state in the future, even though their genomes are relatively stable compared with wild-type virus. Increasing the fidelity of genome replication may paradoxically attenuate the virus, but there is no report focusing on the fidelity of DENV polymerase. Further detailed studies are needed for the development of a safe and stable vaccine.

5. THE ROLE OF VIRAL QUASISPECIES IN PATHOGENESIS

Quasispecies play a positive role in the escape from immunological pressure and from the pressure of drug treatment as discussed above. They may be also involved in the progression of disease and pathogenesis. Since effect of the quasispecies of DENV on pathogenesis has not been reported yet, I will discuss the effect of the quasispecies of other viruses on pathogenesis. A report on the pathogenesis of poliovirus using a mouse model system indicates that quasispecies play an important role in the progression of the disease [7]. Poliovirus, the causative agent of paralytic poliomyelitis, starts by infecting lymphoid tissues of the pharynx and gut, leading to a viremia that may result in an

invasion of the central nervous system (CNS) [43]. In the CNS, poliovirus replicates in motor neurons within the anterior horn of the spinal cord, the brain stem, and the motor cortex. By destroying these cells, it produces the characteristic paralysis in approximately 1%–2% of all infections [44]. Within the infected individual, viral replication occurs in a limited number of cells and tissues. This host range restriction is determined at the level of the cell receptor [43]. Vignuzzi et al. first found a mutant poliovirus, which was resistant to the anti-viral drug ribavirin and carried "super-accurate" high-fidelity polymerase [7]. Wild-type poliovirus lethally infects mice by intramuscular injection. In the final disease stage, the virus invades the brain and causes mortal infection. Although, the super-accurate mutant poliovirus showed similar replication kinetics to the wild-type virus, the "super-accurate," poliovirus lost its lethality. Next, the authors created an artificially expanded quasispecies from the super-accurate mutant poliovirus by treating virus stock with chemical mutagens. Interestingly, this virus population reached the mouse brain and caused death by intramuscular injection. This observation is predictable because a diverse virus population has a better chance to overcome the blood-brain barrier (BBB). A mutation may confer a different cell (tissue) tropism on viruses and enable them to invade the BBB and replicate in the brain. If this is the case, the brain-adapted viruses would be found in the mouse brain. However, the above authors found a major population of virus, namely the super-accurate poliovirus. More importantly, when the virus isolated from the brain was injected into mice, it lost its virulence. Their observation provides direct evidence for complementation between members in the quasispecies. It also indicates that selection indeed occurs at the population level rather than on individual variants. Although the detailed mechanism of invasion by poliovirus into the CNS is still unclear, the complexity of the viral quasispecies enabled the virus population to spread systemically and successfully access the CNS, perhaps through the complementary function of different subpopulations.

Taken together, these data support a concept in quasispecies theory, namely that the successful colonization of an infected host occurs through the cooperation of different virus variants. This type of positive cooperation may also occur during coinfection of a given host with different viruses, which leads to profound consequences in the pathogenic outcome of an infection. In the case of DENV infection, is likely that coinfection occurs very often [45]. Next, I will discuss the coinfection of DENV.

6. WHAT SUPPORTS QUASISPECIES?

6-1. Coinfection

To deeply understand the concept of DENV quasispecies, we need to understand how quasispecies are maintainedin virus population. A coinfection of different viruses carrying different genetic information is one of the important factors. Two different or even several types of DENV possibly infect into a single cell. It is thought that a large number of defective viruses are produced in a single host since most mutations are deleterious. In addition, the diverse virus population also contains low-fitness intact viruses, which may be able to show higher fitness in different environments. These viruses could survive by coinfection with high-fitness viruses even if they have a disadvantage in viral replication in the current environment. The long-term transmission of defective DENV by coinfection has been proven. Aaskov et al. reported that a defective virus, after acquiring a stop-codon mutation in the surface Env protein gene, was transmitted from Myanmer to New Caledonia and Singapore within a few years. Moreover, this defective virus was found in both humans and mosquitoes, suggesting epidemic circulation. Coinfection provides the capacity to maintain a broad range of diversity in the virus population.

6-2. Memory of quasispecies

Another important concept is the memory genomes of RNA viruses. A viral quasispecies is defined by a master sequence and a mutant spectrum. The master sequence is the dominant nucleotide sequence in the genomic distribution. It may coincide with the consensus or average sequence of the virus population. It is usually transmitted to other hosts and plays a major role in the evolution of viruses. On the other hand, there is no explanation as to the survival or transmission of minor virus populations such as the small population of quasispecies. However, Ruiz-Jarabo et al., provided direct evidence for the presence of minor populations among major virus population, which was thought to carry a homogenous single virus genome using foot-and-mouth disease virus [46]. They also showed later that the minority memory genome of virus potentially influenced the evolution of multidrug-resistant HIV-1 in patients [47]. This is an important observation, suggesting as it does two points regarding quasispeceis. Firstly, the authors demonstrated that viruses could carry the minority memory genome in their virus population at the intrahost level as well as the interhost level, although this was maintained at a level undetectable by conventional protocols. Secondly, their observations suggested that the virus population, as quasispecies, carries the historical background, that is to say, molecular memory genome. For example, a virus popula-

tion which experienced anti-viral drug treatment may maintain the drug-resistant virus even in the absence of the anti-viral drug.

There is a report which may be related to the memory genome of DENV. Kinoshita et al. isolated two different DENVs with different cell tropisms from a single patient, suggesting the co-circulation of these viruses [48]. One isolate was B-cell tropic, even though B-cell is not thought to be a target of DENV. DENV showing B-cell tropism may be an example of the memory genome of DENV. At some point, this DENV population may have encountered an environment which provided an advantage to the B-cell tropic minor population of DENV. We have not completely determined what organ can be a target of DENV, although monocytes/macrophages are thought to be the main targets. This observation suggests that the DENV "population" replicates in B-cells.

In this section, coinfection and memory of quasispecies are discussed. Both are necessary to maintain the quasispecies of a virus population. It remains unclear how the memory genome of minor population is maintained, but coinfection is probably one of the mechanisms by which viral quasispecies are maintained. The concept of molecular memory genome is important. If the genome of a minor population of DENV is maintained through infection in several hosts including both mosquitoes and humans, the DENV population may contain various minor populations. When persons who lack a normal immune system, such as infants and AIDS patients, are infected with DENV, the DENV population may come to carry unique genomes, because the immune system is also a factor affecting tissue/cell-tropisms in addition to virus receptor on host cells. An intensive study of DENV quasispecies may reveal the history of the DENV population and provide a new vision for the understanding of virus infection and transmission.

7. QUASISPECIES OF DENV

Studies on quasispecies of DENV by several groups have revealed that DENV is a swarm of mutant viruses [40, 49–52]. These studies analyzed intrahost sequence diversity of the Capside, Env, and NS2B, but none identified any particular amino acid substitution associated with disease severity. This is understandable because the virulence of DENV is still unknown. One reason why none of the studies has clarified the DENV gene associated with virulence is that the outcome of disease, that is, severity, is due to the interaction or battle between DENV and the host immune system. Although the interaction between pathogen and host is always important, it is particularly important in DENV. Severe symptoms have been observed when the host eliminates DENV, suggesting that these symptoms are due to the protective host reaction. In terms of disease severity, most researchers have assumed that high virus titer is associated with severity [53]. Nevertheless, it remains unclear what viral genome among quasispecies contributes to the higher viral titer in patients. Even though no evidence showing that quasispecies of DENV affect pathogenesis, is currently available, there is logical evidence. DENV from primary infection and from secondary infection presumably have different features of intrahost sequence diversity because DENVs are subjected to different types and levels of immune pressure between primary and secondary infections. In addition, the DENV population may be under positive selection through ADE in secondary infection. That is, pre-existing antibodies negatively and positively regulated the production of DENV in secondary infection. Like other groups, we observed the various Env gene sequences of DENV from single patient (unpublished observation), suggesting their involvement in the interaction between virus particles and antibodies.

One important question is whether only one major population of DENV is transmitted between humans and mosquitoes [10] The quasispecies of DENV is maintained during transmission because a diverse DENV population was observed in both mosquitoes and humans [40]. DENV presumably always exists as quasispecies and gradually changes its population during transmission to other hosts.

8. METHODOLOGICAL PROBLEMS ENCOUNTERED IN STUDIES ON QUASISPECEIS OF DENV

Although the quasispecies concept was proposed by Eigen 30 years ago [22], no intensive study has been conducted on viral quasispecies, especially acute infectious diseases such as DENV. This is partly due to methodological problems. As described above, the minor virus population is thought to account for a very low percentage of the DENV population. To detect the minor virus population, the genes have to be analyzed by gene cloning, which requires much more time-consuming work than direct sequencing. If the minor population accounts for one percent of the virus population, at least 100 clones have to be analyzed. Another problem is selection or adaptation of virus to cultured cells during virus isolation. Thus, methodological problems are obstacles for the detailed analysis of quasispecies. However, a recent technological improvement in sequencing may allow a breakthrough in studies on quasispecies. The ultra-deep method facilitated by 454 Sequencing and other new systems offers dramatically improved sensitivity in detecting low-frequency viral variants, which means minor populations of viral quasispecies, compared to traditional

population-based sequencing methods. Further studies using thge new technologies are necessary to shed light on the physiological relevance of DENV quasispecies.

9. CONCLUSION

Studies on quasispecies of viruses causing acute infectious diseases such as DENV have only just started. It is now clear that DENV exists as quasispecies. What is less clear is whether individual populations contain viruses that differ in important phenotypic properties and what proportion of DENV genomes are defective. Detailed studies on quasispecies of DENV will assist the development of anti-DENV strategies such as antiviral drugs and vaccines.

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