Review

Issues Related to Recent Dengue Vaccine Development

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Abstract: Dengue fever (DF) and dengue hemorrhagic fever (DHF) are mosquito-transmitted diseases of global importance. Despite significant research efforts, no approved vaccines or antiviral drugs against these diseases are currently available. This brief article reviews the status of dengue vaccine development, with particular emphasis on the vaccine strategies in more advanced stages of evaluation; these include traditional attenuation, chimerization and engineered attenuation. Several aspects of these vaccine design strategies, including concerns about vaccine candidates inducing infection-enhancing antibodies, are also presented.
Key words: Dengue, vaccine, neutralizing antibody, enhancing antibody

INTRODUCTION

Diseases caused by dengue virus infections are an increasing problem worldwide because of current globalization trends [1, 2]. Despite intense research efforts over more than 30 years, no dengue vaccine is commercially available [3–10]. Approved antiviral drugs are also unavailable to treat dengue diseases. Currently, management of dengue virus infections relies on control measures targeting mosquito vectors. However, the emergence of mutant vector strains acquiring resistance against insecticides is an ongoing problem [11–13].

This paper briefly reviews efforts to develop a dengue vaccine. Currently, three vaccine strategies, namely traditional attenuation, chimerization and engineered attenuation, are in advanced stages of evaluation. After giving an overview on virus and gene structure, epidemiology, transmission and pathogenesis, several aspects of these vaccine strategies are presented. Other vaccine strategies, including virus-vectored, pseudo-infectious virus, DNA, inactivated and subunit vaccines developed for dengue viruses, have been described elsewhere [3–10].

DENGUE VIRUS STRUCTURE AND GENOME

Four types of dengue virus (dengue type 1 to 4 viruses; DENV1 to DENV4) belong to the genus *Flavivirus* of the family *Flaviviridae* [14]. The flavivirus virion consists of a nucleocapsid structure surrounded by a lipid bilayer containing an envelope (E) glycoprotein and a non-glycosylated membrane (M) protein. The nucleocapsid is composed of a capsid (C) protein and a single strand of positive-sense RNA. The E protein is the major surface protein with a role in receptor binding and membrane fusion, and it is known to constitute a major immunogen during flavivirus infection. Specifically, E protein contains most of the sites that react with neutralizing antibodies as well as many protective epitopes. The M protein is found in infected cells as a glycosylated precursor, premembrane (prM) protein. Dengue viral proteins, including these three structural proteins, are encoded by a single long translational open reading frame present in the genomic RNA. These viral proteins are synthesized in the order of C, prM, E, followed by non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The open reading frame is flanked by untranslated regions, the 5'-UTR and the 3'-UTR.

The similarity in antigenic structure among the four

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types of DENV is closely related to the characteristic features of the manifestations of dengue diseases. Most members of the genus *Flavivirus* can be grouped into eight antigenic complexes and four dengue viruses belong to the dengue virus serocomplex. These four dengue viruses are antigenically cross-reactive. Homology in the amino acid sequence of the E protein is approximately 70% among DENV1–4 [15].

EPIDEMIOLOGY

DENV1–4 are responsible for dengue fever (DF) and dengue hemorrhagic fever (DHF). These diseases occur throughout most of the tropical and subtropical areas of the world, with an estimated 50–100 million cases of DF and 250–500 thousand cases of DHF reported annually [1, 2]. DF and DHF are endemic in at least 100 countries and >2.5 billion people are at risk of infection.

In non-endemic areas, dengue infections may result from imported infectious cases [16]. An individual who has traveled and acquired an infection in an endemic area may return to their home country (non-endemic area) within an intrinsic period and then manifest symptoms. For example, DENVs do not currently circulate in Japan but approximately 1.7 million people travel overseas every year, increasing the risk of imported dengue infections [17]. According to a report from the National Institute of Infectious Diseases, around 100 virologically confirmed cases of dengue virus infection have been detected annually in recent years: however in 2010, 215 cases have been reported until the end of October [18]. This raises concerns that there are a large number of viremic patients in Japan and that these infecting viruses may be transmitted to domestic mosquitoes via mosquito bites during the summer season.

Phylogenetic analyses of the nucleotide sequences of the E coding region in the genome of isolated viruses demonstrate that several genotypes exist within each of the DENV types, DENV1–4 [19]. In addition to evolving within a particular environment, viruses may be transported from other areas and introduced into new environments because of the frequent movement of human hosts, both domestically and internationally. If the novel virus is better adapted to survive and propagate in its new environment, this virus may dominate over previously circulating viruses in the area. The replacement of a lineage, genotype or even a virus type has been reported in several areas [20–26].

TRANSMISSION

DENV exists in a transmission cycle between monkeys/humans and mosquito vectors. In urban settings, humans

have a role in the amplification of the viruses and their transmission cycles [27]. Although *Aedes aegypti* and *Aedes albopictus* are the major vectors for dengue virus transmission, the former is the more important vector because it has adapted to inhabit human dwellings. Patients can show serum virus titers up to 7 $\log_{10}$ PFU/ml [28, 29], which is high enough to infect mosquitoes when they ingest a blood meal (approximately 2 μl). In sylvatic settings, monkeys are considered an amplification host, transmitting the virus to mosquitoes.

Transovarial transmission is another mechanism by which the virus is maintained in nature. In susceptible mosquitoes, the first organ to allow virus replication is the midgut. The viral offspring released from the cells of the midgut into the body lumen may disseminate to most organs/tissues of the mosquito, including the salivary gland, allowing direct transmission of the virus to humans. The virus may also be disseminated to the ovary of the mosquito, allowing transmission of the virus to their eggs. Therefore, the next generation may possess the virus without bloodsucking and may potentially be competent to transmit the virus to humans at first bite. This transmission mechanism has been demonstrated in the laboratory [30, 31] and in the field [32–35].

PATHOGENESIS

Most infections with dengue virus are asymptomatic. Clinical cases usually take a benign form (DF) and occasionally a severe form (DHF) [1, 2]. DF patients develop high fever, headache and muscle and joint pain, from which almost all cases recover, whereas DHF patients develop mainly plasma leakage and hemorrhagic manifestations, which may lead to shock. The case-fatality rate of DHF can exceed 20% without proper treatment, and a large proportion of hospitalized patients are children [36].

Several hypotheses have been proposed regarding the mechanism of increased disease severity from DF to DHF, which include both host and viral factors [1, 2, 37, 38]. As described above, the four DENVs are antigenically cross-reactive. Many host factors are involved in the immune response after initial infection. Cross-reactive memory T cells are closely related to increased disease severity, and increased levels of cytokines and chemokines are also associated with the secondary infection. The genetic background of the host has also been proposed to be a factor involved in disease severity. Viral factors are attributed to the nucleotide sequence differences between viruses isolated from mild (DF) and severe (DHF) forms of the disease.

Although several mechanisms have been proposed for dengue pathogenesis, it is generally accepted that higher levels of viremia correspond to increased disease severity

[39–41]. One of the mechanisms increasing the viremia level is antibody-dependent enhancement (ADE) of infection [42, 43], which is mediated by Fc gamma receptors (FcγRs) on monocytes/macrophages in the presence of cross-reactive non-neutralizing (enhancing) antibodies [44]. In contrast, neutralizing antibodies are widely believed to reduce viremia levels [45, 46]. Thus, the viremia level depends on the balance of both neutralizing and enhancing antibodies and may determine the outcome of the disease; that is, by providing protection when neutralizing activities are higher than enhancing activities and conferring deterioration when enhancing activities are higher than neutralizing activities.

VACCINES

In the preclinical stages, the effectiveness of vaccine candidates has been evaluated by their ability to induce neutralizing antibodies in experimental animals and to reduce viremia levels in monkey models. Reduction of viremia is associated not only with reduced disease severity at an individual level but also with reduced efficiency of virus transmission to vector mosquitoes, thus contributing to reduced infection rates at the population level.

Currently, there are no commercially available dengue vaccines. However, several types of vaccine have been developed. Vaccines are considered the most effective preventive measure and, in addition to their potential contribution to reducing disease in endemic areas, vaccines are useful for protecting travelers from non-endemic to endemic countries.

Several lines of epidemiological evidence indicate that once an individual becomes infected with one type of DENV they are usually protected from subsequent infection with the same type of DENV (hereafter referred to as homotypic infection) [47, 48]. Therefore, humoral and cellular immune responses to homotypic viral antigens are considered responsible for protection from subsequent infections and are therefore the target for induction by protective vaccines.

Viral proteins required for inducing protective immunity

In flavivirus infections, the prM, E and NS1 proteins are considered important to induce protective immunity. In a mouse model, immunization with purified E, or passive administration with monoclonal antibodies to E, induces protection from lethal infection by the homologous virus, and the protection correlated with *in vitro* neutralizing activity [49–53]. Protective immunity was also rendered in mice by transferring monoclonal antibodies against prM [54] or NS1 [55, 56]: antibodies to prM have neutralizing activity *in vitro*, but antibodies to NS1 protect mice by a non-neutralizing mechanism. Based on this knowledge, the flavivirus prM, E, and/or NS1 genes have been used for developing genetically engineered dengue vaccines.

Tetravalent formulation

Immunization with a single type of dengue virus (monovalent vaccine) may present a risk of increased disease severity upon exposure to later infection with a different type of dengue virus in endemic areas where more than one dengue virus type exists, because non-neutralizing cross-reactive antibodies and cross-reactive memory T lymphocytes are potential mechanisms to cause dengue hemorrhagic fever. By contrast, people once infected with a certain type of dengue virus are usually protected from a subsequent homotypic infection [47, 48]. Therefore, a combination of vaccines that can induce immune responses against all four types of dengue virus (tetravalent vaccine) would be highly desirable for developing a safe and effective dengue vaccine [3–10].

Vaccine development

Traditional attenuation: Live attenuated vaccines are considered the most economical strategy and are therefore affordable in endemic areas comprised mainly of developing countries. Two tetravalent vaccine candidates have been separately developed; at the Mahidol University of Thailand and at the Walter Reed Army Institute of Research in the USA. Both vaccine viruses were developed by sequential passage through primary dog kidney cells, primary green monkey kidney cells or fetal rhesus lung cells. The vaccine candidate developed in Thailand has been licensed to Aventis Pasteur and that developed in the United States to GlaxoSmithKline.

Both attenuated vaccines have produced high seroconversion rates to all four serotypes after two or three doses in clinical trials [57–61], but concerns have been raised about the interference in virus replication, which is a potential problem that may occur when infectious vaccines are combined. Such interference is of particular concern in the development of dengue tetravalent vaccines, since imbalanced immune responses may cause increased disease severity [62]. Therefore, dosage formulations and/or vaccine schedules are considered important to adjust the immunogenicity of the four different live vaccine components [63, 64]. Because of this problem, and issues relating to reactogenicity, further clinical trials of the Aventis Pasteur vaccine candidate have been halted. The phase II trial for evaluating the GlaxoSmithKline vaccine using a protocol involving "formulation17" has demonstrated less reactivity in volunteers [65]. Tetravalent neutralizing antibody responses were achieved in 63% of volunteers after two doses, and this

vaccine candidate will proceed to a phase IIb trial.

Chimera: Advances in gene engineering technology have enabled the construction of chimeric viruses in which specific proteins from one virus are substituted for those of another virus. For dengue vaccine candidates, chimeric viruses have been constructed by exchanging the prM/E genes of each of DENV1–4 for homologous genes of the yellow fever virus (YFV) strain 17D [66], or the DENV2 vaccine strain developed by Mahidol University included in the live-attenuated vaccine described above [67]. The former was licensed by Sanofi Pasteur and the latter by InViragen.

The chimeric tetravalent vaccine using YFV strain 17D as a backbone virus was well tolerated and produced high levels of neutralizing antibodies against DENV1–4 and/or viremia protection following challenge in preclinical evaluations using non-human primates [68–70] and a phase I clinical trial [71]. Phase II trials are currently underway in several countries and a phase III trial has started in Australia. Another chimeric tetravalent vaccine using a DENV2 vaccine strain as a backbone was also immunogenic and protective in AG129 mice [72]. A phase I trial is ongoing for this vaccine strain. Although the chimerization strategy appears to produce an ideal vaccine, the possibility of genetic recombination with virulent viruses remains a concern [62, 73].

Attenuation by deletion at the 3'-UTR: The 3'-UTR is critical for RNA replication. A 30-nucleotide deletion at the 3' site (nucleotides 172–143) resulted in attenuation but retained immunogenicity of DENV4 in monkeys [74]. This DENV4Δ30 vaccine was also well tolerated and immunogenic in humans [75, 76]. The same strategy was successful in attenuating DENV1 [77, 78], but not DENV2 [79] or DENV3 [80]. For DENV2 and DENV3, a chimerization strategy similar to that described above was implemented: DENV4Δ30 was used as the backbone virus and the prM and E genes of DENV2 and DENV3 were replaced with those of DENV4Δ30. Experiments in monkeys indicated that the tetravalent formulation composed of these genetically engineered viruses was safe and induced balanced immune responses [81]. For DENV3, another strategy using a full-length infectious clone containing two deletions in the 3'-UTR or the entire 3'-UTR derived from DENV4Δ30 was implemented [82]. Moreover, DENV4Δ30 was further attenuated and proven to be safe and immunogenic in phase I clinical evaluations [83, 84]. These vaccine candidates were licensed by Panacea Biotec Ltd. and Biological E. Ltd. in India, Vabiotech in Vietnam and the Butantan Foundation in Brazil.

Viremia levels were low or undetectable in vaccinated volunteers, and the virus was not transmitted to mosquitoes.

This engineered attenuated vaccine candidate also showed less propagation in mosquitoes, potentially reducing transmission efficiency. However, a potential concern remained that immunocompromised individuals, who may be included in a large population of vaccinees, could experience high-level viremia and that different geographical strains of mosquitoes may have different vector competences.

Concerns for inducing enhancing antibodies

Enhancing antibodies are one mechanism for increasing disease severity. *In vitro* studies have demonstrated that neutralizing antibodies that show no enhancing activity at low serum concentration show enhancing activities at high concentration [85]. The explanation for this phenomenon is that neutralizing activities overcome enhancing ones at low concentrations, although enhancing activities still exist. Specifically, neutralizing antibodies are usually measured by conventional neutralization tests using cells such as Vero or BHK cells. Since these cells do not have FcγRs, these tests only reflect the neutralizing activity in the serum samples. On the other hand, conventional ADE assays using FcγR-bearing cells represent enhancing activities in a range of serum dilutions where the effect of neutralizing activity is negligible (subneutralizing doses).

This phenomenon is observed with immune sera and most monoclonal antibodies [86, 87]. Serum contains polyclonal antibodies comprising different antibody species. Some antibody species may have neutralizing activities, while others may have enhancing activities. Thus, the balance of neutralizing and enhancing activities is important for understanding the immune status of the host. However, conventional neutralization tests using cells without FcγRs can only detect neutralizing activities included in the serum sample. The evaluation of vaccine candidates has mostly been performed using neutralization tests rather than ADE assays. However, there are concerns that the current vaccine candidates capable of inducing neutralizing antibodies can also induce enhancing antibodies. It is highly probable that neutralizing antibodies constitute an immunological correlate against DENV, although this is still a subject of debate [45].

This balance of neutralizing and enhancing activities can be evaluated in a system that uses a BHK-21 cell line engineered to express FcγR [88] or a K562 cell line adapted to adhere to a plastic surface [89]. These cells can be used in an assay system similar to the conventional neutralization test. However, these cells can detect the enhancing, as well as the neutralizing, activities in the serum samples because of the FcγR on their surface. The balance of neutralizing and enhancing activities in the serum samples can also be measured *in vivo*, in a suitable animal model producing viremia following challenge.

Animal model

Intracerebral inoculation of DENV has been the conventional method for evaluating the efficacy of candidate dengue vaccines in mice. In this method, the clinical symptoms related to encephalitis, and death, were monitored [90]. Adult mice are not usually susceptible to peripheral challenge with DENV, and therefore, fail to develop viremia following challenge [91–93]. A/J mice developed paralysis following intravenous infection with high-titer DENV2, but viremia was limited on day 2 after infection, as determined by the detection of viral RNA [94]. Severe combined immunodeficient mice engrafted with human cells susceptible to DENV infection produced viremia that could be detected by infectivity [95–98]. Other immunodeficient mouse models [99–107] have been developed, including AG129 mice deficient in interferon (IFN)-α/β and IFN-γ receptors [108]. However, the major problem with these models is the lack of a normal immune response, making vaccine evaluation difficult, although they are useful for investigating the pathogenesis of dengue disease and for developing antiviral agents [109, 110].

Non-human primates are the most reliable animal models in which to evaluate the efficacy of candidate dengue vaccines, although they only develop low levels of viremia for short periods compared with the viremia that occurs in humans. The induction of neutralizing antibodies and the reduction in viremia levels can be used as indicators of vaccine efficacy, but monkeys exhibit only some of the symptoms of dengue disease observed in humans. Nevertheless, non-human primate models are still being developed and optimized as preclinical dengue vaccine evaluation systems [111, 112].

CONCLUSION

Dengue vaccine candidates that can induce neutralizing antibodies have been developed based on several strategies. Some of these vaccines are currently undergoing clinical trials. However, since neutralizing antibodies display enhancing activities at subneutralizing doses *in vitro*, there is a concern that dengue vaccine candidates that induce neutralizing antibodies may also induce enhancing activities. Thus, careful evaluation of candidate dengue vaccines is essential to monitor potential enhancing activities. Further optimization may be required to reduce such unwanted activities.

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