Challenges for the formulation of a universal vaccine against dengue

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Abstract

Dengue is rapidly becoming a disease of an escalating global public health concern. The disease is a vector-borne disease, transmitted by the bite of an *Aedes* spp. mosquito. Dynamic clinical manifestations, ranging from asymptomatic, flu-like febrile illness, dengue fever (DF) to dengue hemorrhagic fever (DHF) with or without dengue shock syndrome (DSS), make the disease one of the most challenging to diagnose and treat. DF is a self-limited illness, while DHF/DSS, characterized by plasma leakage resulting from an increased vascular permeability, can have severe consequences, including death. The pathogenesis of dengue virus infection remains poorly understood, mainly due to the lack of a suitable animal model that can recapitulate the cardinal features of human dengue diseases. Currently, there is no specific treatment or antiviral therapy available for dengue virus infection and supportive care with vigilant monitoring is the principle course of treatment. Since vector control programs have been largely unsuccessful in preventing outbreaks, vaccination seems to be the most viable option for prevention. There are four dengue viral serotypes and each one of them is capable of causing severe dengue. Although immunity induced by infection by one serotype is effective in protection against the homologous viral serotype, it only has a transient protective effect against infection with the other three serotypes. The meager cross protective immunity generated wanes over time and may even induce a harmful effect at the time of subsequent secondary infection. Thus, it is imperative to have a vaccine that can elicit equal and long-lasting immunity to all four serotypes simultaneously. Numerous tetravalent vaccines are currently either in the pipeline for clinical trials or under development. For those frontrunner tetravalent vaccines in clinical trials, despite good safety and immunogenicity profiles registered, issues such as imbalanced immune responses between serotypes and questions with regard to whether the optimum formulation have been identified remain unresolved. This review centers on these issues and offers strategies that may improve the tetravalent vaccine formulation.

Keywords: flavivirus, dengue fever, DHF, dengue vaccine

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Introduction

Dengue virus has been recognized as a cause of illness in human beings for more than two centuries. The disease is the most important mosquito-borne illness in tropical and subtropical zones. It has been reasoned that advances in transportation has currently led to its presence worldwide including the USA.[1] The disease by nature is an acute febrile illness, and in the predominant number of cases self-limiting. Due to the similarities in clinical presentation to a number of other common febrile illnesses, dengue cannot be easily diagnosed by attending physicians during the early stages of disease; additionally, the patients may not seek professional help soon enough, arriving at the hospital during the later stages of the infection which in some cases rapidly progresses within a few days to a serious condition or death.[2] Currently, there are no antiviral or other specific therapeutic modalities or effective vaccines to treat or prevent the disease. Supportive care with vigilant monitoring is the principle form of management.

The pathogenesis of the dengue virus infection is not fully understood in spite of many decades of intensive

investigation.[3,4] Lack of a suitable animal model that can capture the cardinal features of human dengue infection and disease is the major hurdle in understanding the detailed pathogenic causes of dengue.[5]

The etiology of dengue has been known for more than seven decades.[6,7] Dengue virus belongs to the Flavivirus family. Dynamic viral properties with distinct features while resident in the vector and the human host have made the virus very complicated and difficult to study. There are four well-known dengue serotypes: DENV1, DENV2, DENV3 and DENV4. Each one of these serotypes is capable of causing dengue disease.[8] An immune response generated against one serotype appears to confer a lifelong protection to homologous dengue strains while it offers only a transient if any protection to the other dengue serotypes.[9] These transient cross-protective effects to other serotypes wane over time and have been hypothesized to potentially induce a harmful and detrimental outcome in the affected hosts including hemorrhagic fever, shock and death.[10] This view that a virus-specific immune response against one serotype can enhance severity of disease against the other serotypes has served as the basis for the concept that an effective vaccine against dengue needs to elicit significant disease protective immune responses to all four serotypes simultaneously and equally.[11] The current candidate vaccines include inactivated dengue strains, recombinant subunit dengue proteins, DNA and live-attenuated tetravalent formulations. The live-attenuated vaccine candidates include intertypic dengue chimeric strains, yellow fever 17D vaccine-dengue chimeras and flavivirus–non-flavivirus recombinant vaccine vectors.[4,12,13] To date, several of these vaccine candidates are in the pipelines for clinical trials. Some or at least one of them, the chimeric tetravalent vaccine based on the Yellow Fever 17D vector, will be available for public use within a few years should the results of the clinical trials in progress warrant its widespread use. However, despite the potential success, there are many issues and concerns relevant to the formulation of the tetravalent vaccines. This review article does not deal with the technical aspects of vaccine development and details with regard to the conductance of appropriate clinical trials since there are currently many recent reviews on this subject.[13–21]

Epidemiology

The geographic distribution of dengue virus was primarily assumed to be restricted to the tropical regions during the past century. However in recent years, with climate changes, frequent human migration, unplanned urbanization, failure in instituting effective mosquito control measures and lack of public health awareness, the disease has spread to nearly every corner of the world.[1] According to the World Health Organization (WHO), more than 100 countries are currently affected and about two-fifths of the world's population living in tropical and the continuous temperate zone regions are at risk of infection. An estimated 50–100 million annual infections with 500,000 cases of DHF/DSS and approximately 25,000 deaths are registered. The economic burden of the potential spread of dengue from the regional endemic countries to non-endemic countries such as the USA and Europe is not only alarming but also carries with it the potential to stress public health control systems globally.[22,23] Thus, a preventive vaccine to dengue has been a priority for the WHO agenda for several decades.

Diagnosis

The dynamic clinical manifestations of dengue and its similarity to other illnesses specially during the early febrile phase, have made the disease one of the most difficult to recognize and may be misdiagnosed until the disease progresses to more severe stage. There are two major aspects of dengue diagnosis. These include clinical and laboratory diagnosis.

Clinical diagnosis

Clinical presentations of dengue virus infection varies from person to person, and perhaps from region to region.[1] Four major categories are recognized; asymptomatic, mild undifferentiated fever, classical dengue fever (DF) and dengue hemorrhagic fever (DHF). According to the WHO case classification,[24] DHF is classified as grades 1–4, depending on the severity. The initial clinical presentations of dengue virus infection that results in mild DF as compared with the more severe form of DHF/DSS can be very similar, making it difficult to identify the patients at risk for severe disease and manage them appropriately. One of the readily recognizable features that differentiate DHF from DF is capillary leakage. Patients with two or more of the following clinical symptoms are designated as DF: headache, retro-orbital pain, myalgia, arthralgia/joint pain, rash, bleeding tendency with petechiae and positive tourniquet test, and leukopenia with WBC less than 5000 cells/mL. In addition to the DF criteria, the more severe DHF cases are required to meet all four of the following parameters: high continuous fever for 2–7 days, hemorrhagic manifestations, platelet count below 100,000 cells/mL and evidence of plasma leakage, such as rising hematocrit (Hct) more than 20% above baseline, pleural effusion and ascites. This WHO classification has been mostly adequate and used for many decades; however, there have been occasional difficulties in classifying patients who present with unusual manifestations, some of which have severe disease but do not fit the classical DHF definition. These include patients with shock or severe bleeding who do not have evidence of leakage or sometimes other DHF criteria; unusual manifestations, particularly older patients with chronic conditions, do not always meet the DHF definition. Additionally, patients diagnosed with DF occasionally display with very severe bleeding or organ involvement. In summary, many patients cannot be easily classified at the initial stage due to the extensive overlap in criteria that have been established for DF and DHF syndrome diagnosis.

In 2009, WHO published another case classification system as a guide for the diagnosis and management of dengue with the aim to better distinguish severe dengue

cases during the early stage.[1] This new classification includes dengue without warning signs, dengue with warning signs and severe dengue, with improved sensitivity for detection of severe cases but less specific for dengue.[25,26] In 2011, WHO South-eastern Asia Regional Office (SEARO) also published an expanded case definition based on the previous definition of DF/DHF (WHO 1997[8]) with the inclusion of unusual clinical manifestations[24] that were previously not covered by the WHO 1997 classification. Both WHO guidelines 2009 and SEARO 2011 are in use in several countries. However, the available case definition systems are still non-specific and overlap with other acute febrile illnesses, therefore making laboratory-based tests a requirement for confirmed diagnosis for dengue virus infection.

Laboratory diagnosis

The clinical criteria for dengue based simply on observation have been and continue to be valuable for clinical decision-making and for empirical patient management. In order to confirm the etiology of the causative agent, a number of dengue tests have been applied.[3,27] These include biological methods, which include serological tests for the presence of dengue specific IgM and IgG by enzyme-linked immunosorbent assay, functional assays for dengue virus neutralization and hemagglutination inhibition (HAI), and the detection of dengue viral genome by polymerase chain reaction (PCR). The isolation of virus is direct confirmation, which can be achieved by mosquito inoculation and cell culture. However, these assays have many pros and cons, such as the timing of sample collection after the onset of the clinical symptoms, which dictates the sensitivity of the confirmatory test. Dengue viremia and antigenemia occur briefly in the plasma during the febrile stage and disappear around the onset of the critical stage, while the antibody positivity appears somewhat later after the acute early stage, primarily during convalescence. This pattern makes the sensitivity of viral or antigen detection tests decrease after 1–2 days of fever, and the sensitivity of antibody detection insufficient before days 4–5 of illness. Moreover, the tests for viral detection are expensive and not available in most primary care facilities.

Management

Since there has been no specific treatment or antiviral chemotherapy approved for dengue, supportive care with close monitoring for shock and bleeding is the principle of management at present. Early treatment with appropriate intravenous fluids is the key to obtaining successful outcomes. The severity of DHF outcomes is therefore dependent upon the experience of the attending physicians and the supportive staff and monitoring facilities. Severe disease progresses very rapidly, but usually ceases within 48 h after the critical stage. With the widely utilized techniques for volume replacement that are successful in most instances, there are only 25,000 deaths per year reported globally. To further reduction of mortality, other intervention or specific treatment may need to be implemented.

Dynamic biology of dengue virus

Dengue is a vector-borne virus that is contracted through the bite of an infected female *Aedes* spp. mosquito. It is often the case with vector-borne parasites that the infectious agent takes on a different structural form to accomplish infection in diverse species; a well known example can be found with the plasmodium malaria.[28] The literature suggests that the virions of dengue virus may have dynamic morphology upon infecting different hosts or cells even though the in vivo compelling evidence is lacking for the time being.

Historical observations

On microscopic examination of fresh dengue specimens, Ardati[29] observed mosaic-shaped bodies moving freely in the blood corpuscles, and these bodies, with the size of one-fifth to one-third of a normal erythrocyte, stain purple to blue in color after staining with Giemsa Romanowsky; this description is consistent with the structure of platelets. This lead to the concept that dengue was caused by parasites. Ardati also observed that leukocytes appeared to contain small granules and cellular debris, suggesting that these presumptive parasites or platelet-like particles were taken up by the white corpuscles. The observation that WBCs bind platelets during dengue virus infection has been verified recently.[30,31]

Modern technology investigation

Morphological analysis of virions derived from tissue culture by electron microscopy suggests that the classic viral particle contains an RNA genome wrapped up within the capsid core that is encapsulated with a lipid bilayer consisting of envelope and membrane proteins.[32] However the morphology of the dengue viral particles is dependent upon the source of infected cells. For instance, two types of viral particles, classical and capsidless, have been reported in the supernatant of infected C6/36 mosquito cells.[33] Interestingly, the morphology of virions in the saliva of infected mosquitoes and *in vivo* has been less clear since no classical viral particles are observed from specimens prepared from saliva of infected mosquitoes or from patients with high levels of viremia.[34] This may partially explain why the epitopes recognized by human dengue monoclonal antibodies are different from that of mouse monoclonal antibodies raised against virions produced from *in vitro* cell culture systems.[35–37] This evidence suggests that an alternate form of viral particle may exist and circulate *in vivo*,[28] a significant implication to the field of dengue vaccine development.

Reservoir of dengue virus *in vivo*

Despite the dynamic range of cells that are permissive for dengue virus infection *in vitro*,[38] the evidence that these cells become infected *in vivo* has not been convincingly substantiated. Viral antigens and viral genomes have been detected in cells of the reticulo-endothelial system,[39]

although this has been a rare finding from biopsy or autopsy samples. Some studies advocate that these cells with phagocytic properties, such as macrophages and dendritic cells, are likely the target cells for dengue virus infection.[40,41] However only very low titers can be observed in these lineage of cells. The presumption that the virus infects phagocytes may be true for other flaviviruses, such as Japanese encephalitis virus and West Nile virus, in which humans are the dead-end host. But for viruses in which humans are the natural host and high viremia is a major feature occurring during the acute febrile stage, such as with dengue virus, the scenario may be different. Interestingly, encephalitis is often seen in patients who are infected with pathogens that have been identified to infect macrophages.[42–46] However, encephalitis is hardly seen in acute dengue-infected patients, which makes the case of macrophages as the primary and/or only targets of the dengue virus subject to question. This suggests that an alternate cell lineage must be permissive for dengue virus infection. Furthermore, recent reports on the presence of dengue viral particles inside platelets isolated from acute dengue patients may guide some to advocate for an alternate cell lineage as being the target of dengue virus.[47] Infection of platelets or platelet progenitors may partially explain platelet dysfunction and thrombocytopenia characteristically observed in dengue patients.

Dengue patients can suffer excruciating bone pain. Thus the term 'break-bone fever' was coined to describe DF in the early literature. This evidence also suggests the involvement of the bone marrow during the course of dengue virus infection. In fact, early literature suggested that bone marrow suppression, or reduced numbers of total bone marrow cells, is an early clinical finding in dengue patients, especially prior to the onset of clinical symptoms.[48] Also, cells of the megakaryocytic lineage were the most significantly altered, as far as cell number and distribution throughout the body. Numerous factors may account for these abnormalities. Studies with bone marrow biopsies have suggested that the progenitor cells, megakaryocytes, are a likely target of the virus.[48] Other recent reports also support the scenario.[49–51] The transient bone marrow suppression may contribute to the transient immunosuppression as suggested by others.[52,53] However, whether this results in and is associated with the clinical complications observed in dengue patients remains to be further investigated.

Pathogenesis

The majority of patients infected by dengue virus are asymptomatic, but a certain percentage of the infected individuals may proceed to DF or DHF and/or associated with dengue shock syndrome (DSS). Understanding the mechanism of increased disease severity from DF to DHF/DSS has remained the central focus of researchers since this will eventually pave a way to better identify, treat and prevent severe dengue. Despite many decades of intensive investigation, there continues to be a wide gap in our knowledge on dengue pathogenesis. Several hypotheses have been proposed, including mechanisms involving both host and viral factors.

Viral strains

There are four dengue viral serotypes, and many genotypes within each serotype have been documented as well. Epidemiological data indicate that depending upon the geographic location, a particular serotype appears to be more virulent than others.[54] In addition, results also suggest that certain genotypes within a serotype may have a determinant (sequence) that is correlated with virulence, attenuation and tissue tropism.[55–57] There have been some indication that these sequences play a role in disease severity but without a suitable animal model, these virulence markers cannot be fully validated.[58]

Immune-mediated enhancement

Literature reports, mainly from epidemiological data, suggest that individuals who succumb to severe dengue were likely to have pre-existing dengue antibody circulating in their vasculature.[59] Results from additional studies suggest that previous infection with a given serotype predisposes select individuals to a more severe form of dengue virus infection upon subsequent exposure to a different serotype.[10,60] In addition to the concerns about pre-existing antibody, the adaptive T-cell response resulting from re-infection has been suggested to play a contributory role in the pathogenesis as well.[61,62] However, several lines of recent results contradict these long held notions. Thus, no difference was observed in the severity of disease between primary and secondary dengue virus infection in non-endemic regions.[63] In an elegant prospective study conducted by Libraty *et al.*[64] found no detectable association between the presence of maternal antibodies and the development of severe dengue in infants. Also the incidence of severe dengue disease in naïve travelers was similar between those with naïve primary dengue and secondary dengue.[65] Importantly, the frequency of immunologic non-responders in natural dengue virus infection in dengue endemic zones is unknown. These immunologic non-responders could be repeatedly re-exposed to the virus, and develop a primary infection profile. Since currently there is no biomarker available to differentiate this group from naïve individuals, hence, in order to further advance the understanding of the causes of DHF/DSS, disease should be divided into three major categories (naïve primary infection, definition primary infection in endemic zones, and secondary infection) and considered separately. In addition, these findings suggest a note of caution in the notion of the antibody enhancement theory and also suggests that an alternative pathogenic factor may account for severe dengue, especially in infants and others experiencing naïve primary infection.

Biological enhancement

Biological components circulating in the patient's blood stream may have an important role in controlling

infection or in enhancing the progression of the disease. Importantly, DSS often occurs at the time in which virus has been cleared off from the system, thus is independent of viral load. Consequently, severe dengue has been viewed to be an immune-mediated disease. An increase in vascular permeability is the salient feature of severe dengue, which may result in the translocation of microbial products from the intestinal lumen into the systemic circulation in the absence of overt bacteremia.[66] These products may have a role in driving persistent immune activation, resulting abnormal physiology responses including aberrant T- and B-cell response and producing extraordinary levels of pro-inflammatory mediators.[67,68] Gut injury has been proposed to be one of the mechanisms leading to multiorgan failure in hemorrhagic shock.[69] Interestingly, it has been reported that endotoxin was detected in about 50% of serum samples collected from DHF/DSS patients.[70] Furthermore, gut mucosal injury in patients suffering dengue virus infection has been reported and the degree of injury appears to correlate with the severity of the illness.[71] Very recently, reports show that more than 35% of DF patients have evidence of bleeding in the gut,[72] and that the levels of lipopolysaccharide in serum samples of dengue patients correlate with disease severity.[73] In light of these concepts, it is important to note that phagocytic cells such as monocytes and macrophage, in general, are very difficult to be infected by dengue virus *in vitro*.[74] However, if these cells are pretreated with macrophage activators, then the infectivity rate increases significantly,[75] likely as a result of enhancing phagocytic activity of these cells.[76] The cumulative evidence therefore implicates that alternative parameters may contribute to the severity of dengue and that rethinking or refinement of our current hypotheses on dengue pathogenesis is urgently needed.

Alternative factors

Obviously, many factors may contribute to why only a small percentage of affected subjects progress to severe dengue. These include but are not limited to an individual's genetic background, sex, age, nutrition and underlying diseases.[1,77] The harmonizing and balancing of these factors may be critical in restricting disease progression.

Dengue vaccines

The need for effective vaccines to control dengue is urgent and compelling in light of the increasing burden of the public health system globally. Despite efforts that have been ongoing for several decades, a licensed dengue vaccine is not yet available. However, several vaccine candidates are currently being evaluated in clinical trials and are likely available within the next 5–10 years. Key aspects relevant to the dengue vaccine developments are discussed below.

Immunity to dengue virus

Although Ashburn[78] demonstrated the etiology of dengue by showing that it is caused by a filterable agent, the true identity of this pathogen was not verified until 1940, during World War II, by Dr S Hotta in Japan[7] and Dr A B Sabin in the USA.[6] The isolation of the virus provided a platform for the development of biological and functional assays for the study of the immune responses to dengue virus.[9,79] Immunity to dengue virus was first observed in mice using formalin-inactivated virus and in human volunteers with attenuated dengue vaccines derived from suckling mice.[80,81] These volunteers remained asymptomatic upon re-exposure to viruses of the same dengue serotype for at least 18 months.[9]

Early attempts

Cleland *et al.*[82] observed a modulation of virulence of dengue virus after successive passages of infected human serum through human volunteers. This is the first observation on the loss of putative virulent determinants of the virus and is the basis for the concept of attenuated dengue viral vaccine development. Subsequently, numerous attempts with a variety of other techniques were made for the development of attenuated and inactivated dengue vaccines.[15] Experimental evaluation of these dengue vaccines were performed in animal models and human volunteers by Sabin and Hotta in the early 1950s.[6,7,9,79,83] However the results were mixed and viral interference was suggested to be one of the contributing factors responsible for the unsatisfactory outcome.[9]

With the availability of neutralization assays, multiple dengue serotypes were identified.[9,84] Considering the potential risk to vaccine recipients potentiated by an imbalance of the antibody response to each serotype, the concept of needing a tetravalent vaccine that simultaneously protects against all four dengue serotypes was established.[11] However, evaluations of clinical trials have thus far revealed that an imbalance in the levels and/or quality of immune responses against the four dengue strains and the potential of interference in the immune responses between the four strains in the formulation is difficult to overcome, particularly in human populations.[16,85] Thus, any candidate vaccine has the potential to selectively show enhanced T-cell responses to select determinants of one of the four viruses based on the MHC type of the individual, which as the theory goes, may predispose the individual to a severe form of disease when exposed to an immunogenic subdominant dengue serotype. Consequently, obtaining the right tetravalent vaccine formulation has been a major challenging task plaguing the advancement of dengue vaccine development for many years.

Current dengue vaccines

There are several dengue vaccines either under development or in the pipeline for clinical trials.[15] Despite the various strategies employed, these vaccines were designed with the aim of neutralizing all four serotypes simultaneously and equally. Although the majority of these tetravalent vaccines exhibited good immunogenicity and safety profiles in tested animals, many issues have surfaced in clinical trials in humans. One of the most obvious problems

is an imbalanced immune response.[13] This issue was also highlighted in the recently released news on the tetravalent vaccine trials in Thailand.[86] In spite of this setback, the results from their preclinical trials in rhesus monkeys demonstrated that a proficient trivalent antibody response induced by the tetravalent dengue virus inoculation was protective upon challenge with the fourth virus.[87] Thus the current clinical phase IIB trials were considered to be a success since the vaccine could effectively elicit an antibody response in three out of the four serotypes. Accordingly, the first dengue human vaccine may be available within five years. Nevertheless, an uneven immune response is always a potential issue of concern, especially on why the vaccine does not work for certain viral serotypes but seems to modestly work for other serotypes. Consequently, designing a formulation of tetravalent vaccines that can generate equivalent antibody production and elicit equivalent cellular immune responses to all four serotypes in recipients is an ongoing challenge.

Challenging issues on dengue tetravalent vaccines

Effectiveness and efficacy are the two critical parameters used to evaluate the overall performance of a candidate vaccine. An effectiveness trial, conducted as post-licensure, can capture direct and indirect effects, such as magnitude and duration of vaccine-induced herd immunity, and can address outcomes of public-health concerns such as serious but rare vaccine-associated adverse effects. An efficacy trial, designed to support vaccine licensure, mainly measures the vaccine protection in a restricted populations using idealized schedules and does not take into account the pattern and the extent of vaccine coverage of a population. Thus, the protection demonstrated in efficacy trial may not accurately predict the level of protection that can be achieved in real world. The center of discussion point for the current dengue vaccine is overwhelming on the vaccine efficacy rather than vaccine effectiveness. The basic features needed in a dengue vaccine are its ability to induce effective cellular responses that are associated with protection which is central to the topic of current discussion. For instance, (i) what is (are) the protective element(s) in asymptomatic individuals? (ii) How are dengue viruses neutralized? (iii) What are the roles of the adaptive immunity in clearance of dengue virus?

Protective parameters

The major obstacle in developing an effective dengue vaccine is the lack of known correlates of protection. What are the protective factors present in asymptomatic persons but absent in dengue patients? Why is it that such a small percentage of infected subjects develop severe clinical symptoms? Consequently, dengue vaccine developers measure the traditional protective parameters that are easily measured. Generally, it is assumed that neutralizing antibody is critical in controlling and clearance of circulating virus in the bloodstream, because this has been a good correlate of protection for many of the existing effective vaccines.[88] But in the case of dengue, the antibody titer has not translated into protection from dengue virus *in vivo*, since high dengue specific serum antibody and neutralizing titers can be demonstrated in dengue patients, and yet, as a function of time, high viremia may be observed in the same subject.[89–91] The results taken from dengue confirmed cases from a walk-in clinic support this scenario as well (Figure 1). In addition, although adaptive T-cell immunity has been demonstrated to play a critical role in viral clearance,[40] exactly how this process is engaged is unclear. Innate immunity and other associated soluble factors may be important as well, but their roles in dengue virus infection remain largely unknown due to the inability to test them in a suitable animal model recapitulating the cardinal features of human dengue disease. In order to see progress made in determining correlates of protection, a parameter should be identified that can be linked to protection in humans, then a controlled experiment in an animal model, such as the HIV-negative rhesus macaques, can be implemented and verify the observation. Implementing this new strategy to fine tune our knowledge of dengue pathology will aid in resolving the dilemma faced in the current dengue vaccine clinical trials.

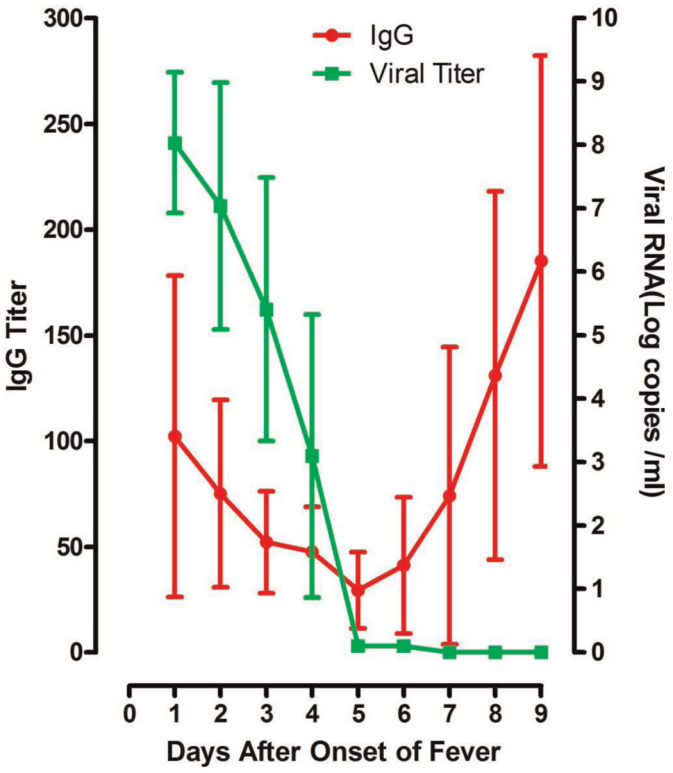

Figure 1 High viral load in the presence of specific immunoglobulin (IgG) to dengue virus. Results were from cumulative dengue confirmed cases (57 cases). Serum samples were collected from walk-in febrile cases that were clinically diagnosed as dengue fever according to the World Health Organization guidelines. Serological and viral polymerase chain reaction (PCR) assays were used to confirm the etiological agent. The levels of IgG antibody specific to dengue viral antigen were determined as previously reported[108] and the viral loads were measured by realtime reverse transcriptase-PCR as previously described[108]

Multiple serotypes

There are four genetically related but serological distinct dengue viruses, DENV1, DENV2, DENV3 and DENV4. These four serotypes constantly co-circulate in endemic regions. Populations inhabiting these areas normally have been exposed to multiple serotypes by school age. It is likely that subjects in endemic regions have been exposed to one, two, three or even all four serotypes throughout the course of their lifespan. Such exposure leads to the development of DENV specific antibody that may be detectable for decades. A large fraction of this antibody is specific for epitopes present on all four serotypes some of which are also expressed by other flaviviruses and thus serum samples from endemic population cross-reacts with all four viral serotypes and to some extent even other flaviviruses. Theoretically, specific antibody to DENV should be sufficient to contain the incoming virus since several of the mechanisms (complement, ADCC, neutralization, etc.) are functional and should contribute to virus containment. High levels of neutralizing antibody titers have been demonstrated in these subjects, but currently there is no assay that can differentiate the sequence of serotype infections in an individual.[3] Yet, still some succumb to debilitating illness in spite of detectable neutralizing antibody. This should raise a red flag on questions such as (i) Why does neutralizing antibody not function *in vivo* accordingly? (ii) How many times does a subject get infected prior to illness? Two aspects have been considered important for the performance and interpretation of neutralizing antibody assay results: the source of the virus and the levels and quality of neutralizing antibody from patient serum. This kind of antibody is noted since multiple isotypes, primarily IgM or IgG, have the capacity to neutralize virus of any serotype. Importantly, it is believed that antibody to one serotype can cross-react with other serotypes within a certain time frame. To test the concept, a pilot investigation was launched, in which DENV2 purified from infected Vero and C6/36 cells was used to evaluate the presence of neutralizing antibody from dengue patients who were acutely infected; the infecting viral serotype was confirmed with these patients. As expected, serum from a DENV2-infected subject could neutralize DENV2 efficiently regardless of the source of the tested virus (Figure 2a). With serum from a DENV3-infected subject, the source of the tested virus had a significant impact on the antibody titer, providing better protection from mamalian-derived rather than insect-derived virus (Figure 2b). In contrast, serum samples from DENV1 and DENV4-infected patients were unable to neutralize DENV2, regardless of what cell lineage the virus was propagated in (Figures 2c and d). Mouse monoclonal antibody (clone 3H5) has been shown to be a potent neutralizing antibody against DENV2. Experimentally, this proved to be the case (Figure 3). These data suggest that (i) the cellular source of the stock virus can be critical in evaluating the neutralizing antibody titer, (ii) the human antibodies that are capable of neutralizing homotypic DENV represent a small fraction of the total DENV-specific antibody response and (iii) human and mouse antibodies likely recognize distinct epitopes on the dengue virion. Vaccine-related viremia has always been a concern with live-attenuated dengue vaccines. Low-level viremia has been documented in clinical phase I/II trials, even though neutralizing antibody was detectable prior to subsequent vaccination.[92–94] In addition, although the live, attenuated vaccine is safe and immunogenic, questions remain regarding the frequency of reversion resulting from intraserotypic recombination of the vaccine viruses to a virulent form of the dengue virus.[95] The likelihood of this scenario may or may not be visible in phase III clinical tetravalent vaccine trials and remains a subject of future study.

Formulation and interference

Dengue viral interference was first observed by Sabin,[9] and since then the phenomenon has been described *in vitro* and in clinical trials of tetravalent dengue vaccines.[96–100] The consequence of viral interference potentially results from the suppression or delay of other viral serotypes from entering cells and/or replicating and then triggering the subsequent corresponding type specific immune response.[100] When infecting with multiple related viruses, which likely compete with each other for the same cellular hosts, there is always a tendency for one of them to serve as a predominant virus. This results in an uneven immune response, eliciting better antibody titers to a few serotypes rather than all of them. Thus development of a vaccine with the right combination is critical in achieving a balanced immune response that does not contribute to immune-mediated severe dengue in vaccinees.[101] In addition, multiple dosing of the right combination and proper interval administration have been suggested to have the capacity to circumvent this viral interference.[102,103] Interestingly, the interference likely occurs in protein synthesis in a host cell since puromycin treatment can overcome the effects.[96] The observation also implies that inclusion of a cellular protein synthesis modulator such as puromycin in the tetravalent vaccine formula may reduce the level of viral interference and levels out the immune response. However, a successful vaccine may be also unlikely because there is no known correlate of protection; the levels of neutralizing antibody have not been proven so far to predict disease severity.[12,91]

Duration of immune components

How long do the protective parameters against dengue last, if there is any? The measurable neutralizing antibodies may be cross-reactive but they are not protective for a sufficient period of time. Protection against disease by heterotypic dengue virus was seen in human volunteers given heterotypic virus two months after primary infection, but after three months, such protection waned.[9] This is the major reason why it is simply not adequate to evaluate the protective potential of a candidate vaccine by demonstrating neutralization or resistance to challenge early after immunization, when the immune system's capacity is always at its highest potency. These strategies

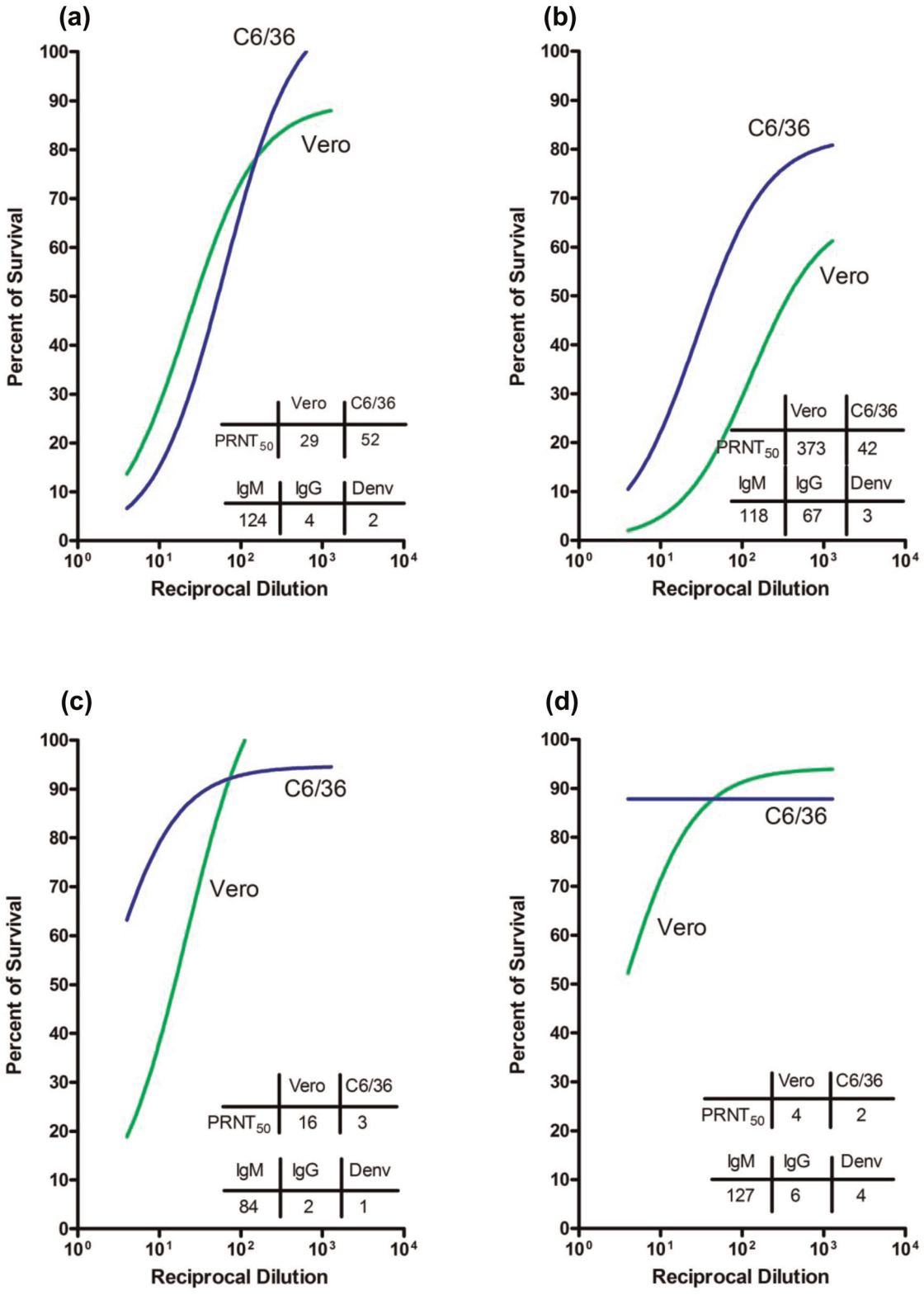

Figure 2 Source of virions dramatically impacts neutralizing antibody titers. Serum samples from serotype confirmed dengue patients were utilized to evaluate the variations of neutralizing antibody capacity to serotype 2 virions purified from supernatants of infected Vero and C6/36 cells. Dengue neutralizing assays were performed according to the published methods.[109] (a) Serum from DENV2-infected patients. A good neutralization curve can be observed against homologous DENV2 virus with DENV2-specific patient serum, independent of the source of the virions. (b) Serum from DENV3-infected patients. A good neutralizing curve could be seen as well. But, virions prepared from the supernatant of DENV2-infected Vero were easier to get neutralized than those from C6/36 cells. (c,d) Serum from DENV1- and DENV4-infected patients, respectively. Both serum samples appeared not to have the capacity to neutralize DENV2 virions efficiently, regardless of the source of dengue virions

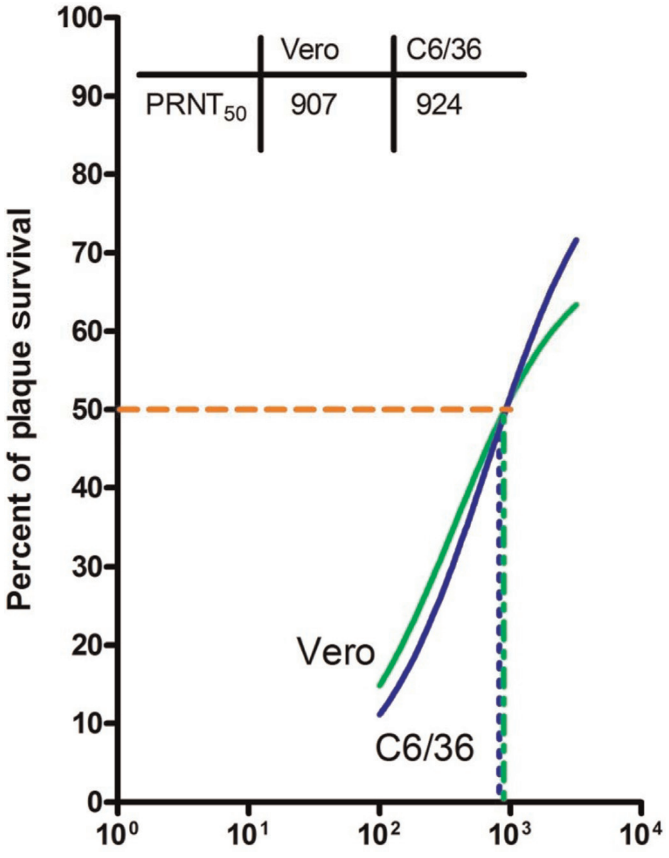

Figure 3 Mouse monoclonal antibody neutralized cell culture derived virions efficiently. Virions used for the evaluation were identical to Figure 2. Mouse monoclonal antibody (clone 3H5) was purchased from ATCC (Manassas, VA, USA). This well-established mouse monoclonal antibody is very effective at neutralizing DENV2 virions, regardless of the source of virus

do not in fact test for the presence of immunological memory.

The time interval between the immunizing doses can dictate the strength of the antibody response. However, determining the best vaccine schedule can be complicated in endemic areas. In these regions the population is constantly challenged by wild-type virus, and the measurable immunity is likely the result of a combination of pre-existing and vaccine-induced immunity along with re-exposures to virus present at this stage in the environment; however, the importance of these variables remains unknown. Perhaps the vaccine formulation and schedule may be best determined with naïve travelers. In short, further investigation is required for us to gain a better understanding of how the four serotypes participate in altering the reactogenicity and immunogenicity to the vaccines and to obtain a better vaccine-induced memory response.

Animal models

Several key questions concerning dengue tetravalent vaccines remain to be tested via animal models. Unfortunately, a suitable animal model that can recapitulate the cardinal features of severe human dengue virus infection is currently unavailable and urgently needed. Though some aspects of human dengue disease can be investigated and evaluated in certain animal models,[5] these aspects are only the tip of the iceberg, considering the plethora of presentations seen in human dengue. While these animal models may be very useful for antiviral propagation evaluation,[21] they lack the ability to assess the quality of the immune response and the potential for a reduction in disease pathogenesis. In order to get a better glimpse of how tetravalent dengue vaccines might behave, immunocompetent animal models that display the key clinical features of human dengue would be required. Otherwise, the vaccines would have to be directly evaluated with human volunteers, which would not only be time consuming but also not without considerable cost.

Perspective for dengue vaccines

Ideally, dengue attenuated vaccines should be capable of reducing the deleterious effects to a minimum, stifling the opportunities at immune evasion and amplifying the immune responses that potentiate protective immunity. Without an animal model capable of evaluating immunogenicity or reactogenicity, vaccines demonstrating only a few good characteristics often get considered ideal candidates. A way to more critically analyze vaccine candidates before beginning human trials is needed.

Broadly neutralizing antibodies against all four serotypes

Despite the presence of multiple dengue viral serotypes, the fundamental life cycle, in regards to host–pathogen interactions, is virtually identical. For instance, each serotype is capable of inducing DF and DHF in infected patients and antibodies to one can also transiently cross-react and protect against the others. This suggests that a highly conserved site, likely associated with an essential function, can be targeted by broadly neutralizing antibodies. This antibody can be either targeted against linear or complex conformational epitopes. As aforementioned, antibodies generated from natural human dengue virus infection are different from those produced in mice immunized with virions prepared utilizing tissue culture. Considering this dichotomy, a more suitable and reliable way to isolate a broad spectrum antibody that can neutralize dengue virus efficiently may be to use virions isolated from high viremia patients during the screening process. The respective target epitope could subsequently be identified and used as an immunogen for vaccine potential. Alternatively, broadly neutralizing antibodies may be generated from a primitive system, such as the lamprey[104,105] or with the artificial nano-antigen technique.[106] The artificial nano-antigen can be generated by imaging the *in vivo* morphology of viral particles derived from dengue patients with Cryo-electron microscopy. A nano-antigen can be modeled according to the dimension observed from the Cryo image.

Long-lasting immunity

The generation of a long-lasting immunity to all four dengue viral serotypes remains a major challenging task. This may require the identification of an effective immune parameter that can serve as an indicator of the protective index. As aforementioned, the measurement of the elicited neutralizing antibody response may not be a valuable parameter for assessing the success of a dengue vaccine. For instance, it has been noted that the Th1 T-cell response to all four serotypes can be effectively induced with tetravalent vaccines, even in the absence of neutralizing antibody.[107] However it is unclear whether individuals who develop a strong cell-mediated immunity without robust neutralizing antibody to all four serotypes after vaccination are adequately protected against disease. It is therefore anticipated that the tetravalent seroconversion along with adequate T-cell immunity responses, especially CD8+ T memory, should be included as part of the efficacy index in order to preclude enhanced disease many years following vaccination.

Additionally, to obtain a vaccine with long-lasting protection, we need a better understanding of the factors involved in waning immunity, especially why protection from some serotypes diminishes faster than others. This aspect can be achieved by studies in dengue endemic regions where multiple dengue serotypes co-circulate constantly.

Protective assays

Currently, neutralization assays are performed with virus derived from *in vitro* propagated cell cultures. The resulting data from these experiments are very difficult to translate into *in vivo* protection. The evidence suggests that patient antibodies recognize other viral parameters besides envelope domain III and that other dengue proteins may participate in neutralization.[36] A mosaic morphology for dengue virus has been proposed.[28] Thus, the assessment of the safety and efficacy of dengue vaccines cannot be limited to the conventional criteria, for instance neutralizing antibody titer against insect cell-derived virus. Determining the possibility of immune evasion and potentiation of disease *in vivo* must be included. One of the approaches for better deciphering the quality of the immune response elicited by a candidate vaccine will be to perform the standard assays but switching the source of virions. Instead of cell culture propoagated virus, primary dengue virus isolates from acutely infected dengue patients (or from vaccine trial participates) could be used to evaluate the protective neutralizing antibody or T-cell response. Alternatively, virus derived from more relevant cell lines (if identified) could be used in these assays.

Conclusion

A successful dengue vaccine product must achieve several criteria in order to be capable of ameliorating dengue disease as a special concern to public health. These necessary requirements include long-lasting immunity, a balanced immune response to all four dengue serotypes, avoidance of reversion to pathogenic strains, and low or no viremia to prevent uptake by mosquitoes in endemic areas. In addition, suitable animal models that capture the cardinal features of human dengue is urgently needed; these tools could facilitate the development of the right vaccine formulation with the proper viral composition that can provide the best balance between reactogenicity and immunity. Although the development of the dengue vaccine has been a big challenge, significant progress has been made in recent years and the progress towards clinical efficacy trials has accelerated substantially. If studies go forward as planned, a dengue vaccine for humans may be available within five years. Until a vaccine is licensed, disease surveillance and vector population control remain the mainstay of dengue prevention.

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