Strategic Approaches to Multivalent Vaccine Development against Dengue Virus Infection

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Abstract

The mosquito-transmitted viral infectious disease dengue is a major public health problem in tropical and sub-tropical countries, where it causes dengue fever, dengue haemorrhagic fever and dengue shock syndrome. Almost 400 million people are infected by dengue each year, causing around 20,000 deaths. There are no vaccines or therapeutic regimens currently available for the prevention and treatment of disease. A number of vaccines are under development and several have entered clinical trials while others are still in the pre-clinical phase. Most dengue vaccines in animal models have been found to provide a degree of protective immunity by eliciting neutralizing antibodies against all tested serotypes of the dengue virus, and this forms a foundation for future vaccine design. It is probable that a refined, second generation multivalent vaccine will be required to target each of the five now recognized dengue serotypes.

INTRODUCTION

Dengue is an acute febrile illness caused by dengue virus (DENV), a small, enveloped, positive sense, single-stranded RNA virus belonging to the family Flaviviridae and genus Flavivirus [1]. Based on neutralization assays, there are five distinct serotypes, DEN-1 to DEN-5, the last of which was discovered only very recently [2]. DENV is transmitted from human to human by mosquitoes of the species Aedes aegypti and, less frequently, Aedes albopictus [1]. During the 19th century dengue was considered a sporadic disease, causing epidemics at long intervals. However, dramatic changes in this pattern have occurred and currently dengue ranks as the most important arthropod-borne viral disease worldwide. The nature of infection varies from a mild self-limiting febrile illness to severe forms like haemorrhagic fever with warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, increasing haematocrit (with decreasing platelets), and life-threatening shock syndrome (with profound plasma leakage, severe bleeding, or organ failure) [3]. In recent decades, the global prevalence of dengue has increased markedly, partly due to differences in serotype genetic diversity, geographical origin and distribution. The disease is now considered endemic in more than 100 countries in Africa, the Americas, the eastern Mediterranean, South East Asia and the Western Pacific, such that more than 2.5 billion people are under threat of infection. Worldwide, recent estimates suggest that 390 million people are infected by dengue annually, of which 96 million present with clinical or subclinical severity [4]. Of those persons, 500,000 require hospitalization with life-threatening complications, resulting in up to 20,000 deaths [1].

Typically, serotype-specific protection occurs in which infection with one dengue serotype confers long-term protection against re-infection by the same serotype. However, in cases of re-infection by a heterologous dengue serotype with the presence noted of cross-reactive antibodies [5] and/or cross-reactive T cells [6,7], the potential risk of antibody-dependent enhancement of disease is increased. The greatest risk factor for dengue haemorrhagic fever is secondary infection. Therefore, the challenge of vaccine development against DENV is to achieve pan-serotype immunity without triggering associated pathology. This has focused on tetravalent formulations that can simultaneously provide protection to all four well-characterized DENV serotypes. At present, there are several candidate tetravalent DENV vaccines at various stages of preclinical and clinical testing [8]. Evaluation of DEN-5 is in its infancy but this serotype will no doubt be incorporated into future vaccine formulations.

CURRENT DENGUE VACCINATION STRATEGIES

The first dengue candidate vaccine was evaluated in 1929 [9,10]. Since then, dengue vaccines have been continually under development. In an attempt to produce an effective vaccine,
several technologies are now being implemented using live-attenuated virus, purified inactivated virus, recombinant subunits, virus-like particles and plasmid or viral vectors. All of these approaches are at different stages of development and each has its advantages and disadvantages [11]. Currently, CYD tetravalent dengue vaccine (TDV) developed by Sanofi Pasteur and a live-attenuated TDV developed by the Walter Reed Army Institute of Research (WRAIR) in collaboration with GlaxoSmithKline Vaccines (GSK) have been found in clinical trial studies to be acceptable for human use [12,13].

There are several types of TDV which have undergone recent pre-clinical or clinical evaluation:

**Live-attenuated tetravalent dengue vaccine**

Development of a first live-attenuated dengue vaccine by serial passage of virus in a non-human host was started at the University of Hawaii and then transferred to Mahidol University in Bangkok, Thailand, for further evaluation and testing [14,15]. This candidate vaccine was used for phase I and II clinical trials in Thai adults and children but neutralizing antibody (NAb) was not found in those volunteers who did not seroconvert to all four then known dengue serotypes. Furthermore, unacceptable reactogenicity was experienced by some volunteers and consequently further clinical testing ceased [16-18]. WRAIR and GSK developed a TDV candidate representing each of the four DENV serotypes attenuated by serial passage in primary dog kidney cells [19,20]. This vaccine was identified as safe, well-tolerated and immunogenic in a phase II trial conducted in adult subjects in the US [21]. The re-derived form of this vaccine was evaluated in a phase II clinical trial and approved as clinically acceptable in healthy adults [12].

In a similar tetravalent live-attenuated DENV vaccine strategy, a TDV consists of a molecularly characterized attenuated DEN-2 strain (TDV-2) and three chimeric viruses containing the pre-membrane (prM) and envelope (E) genes of DEN-1, -3 and -4 expressed in the context of the TDV-2 genome. In a pre-clinical trial, efficient NAb responses to all four DENV serotypes were primed and induced after administration subcutaneously or intradermally of two full doses of this vaccine. Cynomolgus macaques challenged with DEN-2 were protected, showed no detectable viraemia and exhibited sterilizing immunity (no increase of neutralizing titre post challenge) [22].

Robust, long-lasting, broad humoral and cellular immune responses were elicited by a live-attenuated vaccine but are associated with a higher rate of adverse events that cannot be used in some at-risk groups, such as immune compromised individuals. Although inactivated vaccines have reduced potential for reactogenicity they tend to develop a diminished immune response. Conventional tetravalent DENV vaccines based on live-attenuated viruses have shown low efficacy during clinical trials, to which viral interference and incomplete monotypic, heterotypic and multitypic immune protection have been suggested as possible contributory factors [24,25,38].

**Recombinant tetravalent dengue vaccine**

A recombinant, live-attenuated TDV (CYD-TDV; Sanofi-Pasteur, Lyon, France) is in the late stages of clinical development. It has been evaluated in clinical trials in Thailand and Philippines and showed promising results [24,26]. It contains four recombinant viruses (CYD-1 to -4), each of which expresses the dengue prM and E proteins of one or four dengue serotypes together with the non-structural and capsid proteins of the attenuated yellow fever (YF) vaccine virus YF-17D [27,28]. A phase Iib study conducted in the Ratchaburi province of Thailand investigated the efficacy of the vaccine against virologically confirmed symptomatic dengue [24], while another clinical trial was conducted in Latin America to determine the immunogenicity and safety of this vaccine in children and adolescents aged 9-16 years in preparation for a large phase III study [29]. Findings from previous phase II studies were confirmed in this trial. Vaccination with a three-dose CYD-TDV regimen showed that this was well-tolerated and elicited NAb responses against all four dengue virus serotypes in both flavivirus (FV) seropositive and FV seronegative participants. A higher immune response to vaccination was observed in FV seropositive individuals in comparison to their FV seronegative counterparts. This finding suggests that pre-existing FV seropositivity may increase the vaccine-induced antibody response to CYD-TDV [29].

Most recently, the promise of this vaccine was substantiated by publication of a phase III study undertaken in five countries in the Asia-Pacific region [13]. The primary objective was to estimate protective efficacy against symptomatic, virologically confirmed dengue after the completion of three doses of CYD-TDV given six months apart from birth. The incidence of dengue during the 25-month active surveillance period was 1.8% among vaccinated children and 4.1% in those in the control group, translating into an overall, serotype-specific protective efficacy of 56.5%. Moreover, vaccine efficacy against dengue haemorrhagic fever was impressive; 80% after one or two injections and 88.5% after a third [13]. Hence, the principal benefit of this vaccine would be seen in protecting against severe disease, thereby reducing hospital admissions and hence health-care costs, and potentially preventing deaths.

**DNA-based dengue vaccines**

The concept of using DNA to immunize people was first advanced in the early 1990s when it gained immediate widespread recognition due to its apparent simplicity and elegance [30-32]. As five antigenically related serotypes of DENV commonly co-circulate, an effective vaccine must cover all serotypes. Safety, balance between immunogenicity and attenuation, and ‘interference’ among DENV serotypes represent hurdles to be overcome for live-attenuated dengue vaccine candidates [25,33-38]. A DNA-based vaccine is an alternative strategy that both circumvents the concern of these issues and provides many direct benefits [40]. Unlike a live-attenuated dengue vaccine which requires at least six months’ gap for an effective boosting dose from the last immunization [41,42], an advantage of a DNA vaccine is its ability to boost much sooner. There are several DNA based dengue vaccines:

**Chimeric tetravalent dengue DNA vaccine**: Chimeric vaccines are created by cloning segments of DNA from one virus into another virus to generate a ‘chimera’. The advantage of chimeric tetravalent dengue DNA vaccine strategy is that the DNA
shuffling and screening technology produces single DNA vaccine candidates that express antigens containing immunogenic epitopes from four (and potentially five) dengue serotypes. The DNA chimeric vaccine constructs encoding antigens comprised of prM and E epitopes of each of the four tested DENV serotypes in a single construct was developed by utilizing this DNA shuffling technique [43,44]. A regimen of three doses of this vaccine was administered to rhesus macaques on days 0, 28 and 84. Variable NAb responses were detected four weeks after the third dose with individual shuffled DNA clones, only one clone inducing NAb to multiple DENV serotypes [44]. Higher NAb titres were induced to all four tested DENV serotypes by a formulation containing all three shuffled DNA clones. A disappointing observation of only partial protection against DEN-1 and no protection against DEN-2 was made upon challenge at week 32 with wild-type DEN-1 or DEN-2. The immune response induced by these constructs may be improved by incorporating immune stimulatory sequences or adjuvants [44]. DNA remains the only platform that does not induce anti-vector immunity, thereby making it suitable for vaccine regimens that include both priming and boost immunizations. Furthermore, since manufacturing of plasmid DNA relies primarily on bacterial hosts for production, it is considerably faster and easier than for most other vaccine platforms. Moreover, DNA is relatively stable at room temperature, making the requirement to maintain the vaccine cold-chain less critical compared to other vaccine platforms. In addition, the manufacture of DNA is extremely safe especially compared to killed pathogenic vaccine platforms.

**Syncon tetravalent dengue DNA vaccine:** DENV contains a 10.7 Kb RNA genome that encodes three structural proteins (coat, C; prM and E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). E protein, which induces NAbs, is divided into three domains (EDI, EDII and EDIII) based on structural characterization by X-ray crystallography [45,46].

In Syncon tetravalent dengue DNA vaccine strategy, tetravalent immunity was elicited by a synthetic consensus (SynCon) human codon-optimized DNA vaccine. The consensus DIII domains of E protein from all four tested serotypes were cloned and expressed as a single open reading frame in a mammalian expression vector, pDV-U-DIII (dengue-vaccine universal). A significant level of anti-DIII antibody that neutralizes each dengue serotype was elicited in BALB/c mice by this construct, thereby preventing cell death induced by dengue infection [47].

**Tetravalent DNA vaccine with adjuvant:** In this strategy, prM and E proteins of DEN-1, -2 and -3 were each expressed in plasmids and minor changes were introduced in the sequences just upstream of the initiating methionine codon in order to remove certain redundant sequences [48,49]. A DEN-4 vaccine construct was similarly prepared and the tetravalent dengue DNA vaccine (TVDV) was produced by mixing the four plasmids in equal ratio. Vaxfectin, a cationic lipid-based adjuvant, was combined with TVDV to produce an adjuvanted vaccine containing both DNA and lipid at a final concentration of 1 mg/mL (TVDVwax). Intramuscular injection of this vaccine in New Zealand white rabbits elicited a strong NAb response to all serotypes. Subsequently, a phase 1 clinical trial of this vaccine was initiated in early 2012, the results of which are awaited [50].

**Electroporation-enhanced tetravalent DNA vaccine:** The method of introducing macromolecules such as nucleic acids into cells, either in vivo or in vitro, by application of brief electric pulses to increase the transient and reversible permeability of the cell membrane is known as electroporation. Utilization of this technique has escalated from an experimental protocol to its current use in several clinical trials to deliver nucleic acids as well as drugs to a variety of target tissues [51]. When injected by intramuscular electroporation as a tetravalent cocktail into ICR mice, the recombinant dengue prM and E DNA vaccine candidates generated respectable NAb titres against all serotypes of DENV [52]. The dengue-specific NAb induced by this TVDV candidate was detectable a minimum of three months following the last immunization. A prior study demonstrated that NAb induced by DNA vaccine encoding prM and E can endure until 30 weeks [53]. This persistence of immunological memory may be explained by observations that DNA vaccines are capable of inducing both memory Band helper T cells [54,55]. Further research is warranted in non-human primates and thereafter in clinical trials in order to determine whether this vaccine is effective in a sequential boosting regimen. The rationale is that administration before a rainy season would enhance recall memory cells and NAb responses in a manner similar to the strategy deployed for seasonal influenza vaccines [56-59].

**Sub-unit tetravalent dengue vaccine**

The most potent NAbs against DENV bind to EDIII and have been shown in some cases to be effective as passive prophylaxis in rodents [60-62]. Hence, rather than full-length E protein, EDIII can be used as a promising region for a subunit vaccine candidate. Several reports have indicated that EDIII can be produced in various expression systems, including *Escherichia coli*, *Pichia pastoris* yeast and insect cells, and induces specific immune responses against DENV in mice or non-human primates [63-67].

In order to develop a sub-unit TDV, the tandem EDIII of two serotypes of DENV was first constructed. These were connected by a (Gly4Ser)3 flexible linker to create bivalent antigens. Tandem EDIIIIs of two serotypes (type 1-2 and type 3-4) of DENV connected by a Gly-Ser linker were expressed in *E. coli* to produce bivalent recombinants. The TDV candidate MixBiEDIII was then formed by mixing these two bivalent recombinant EDIIIIs. Specific immunoglobulin (Ig)G and NAbs against all four tested serotypes of DENV were induced successfully by immunization of BALB/c mice with MixBiEDIII in Freund’scomplete adjuvant. Furthermore, significant protection against tetravalent DENV challenge was achieved recently in similar suckling mice immunized with MixBiEDIII [68].

**Nanoparticle-adsorbed tetravalent dengue vaccine**

Generation of polymeric nanoparticles with adsorbed or entrapped antigens represents a novel method for controlling the release of immunogens and to optimise the immune response via selective targeting of antigen-presenting cells (APC) [69]. Biodegradable nanoparticles made by coating bovine serum albumin (BSA) with proteins represent a promising method for *in vivo* delivery of protein-based vaccines directly to the immune system. Biodegradable nanoparticles are currently used as drug carriers or as adjuvants for vaccines [70]. For adsorption
of inactivated viral particles to the surface of nanoparticles, a suspension of tetravalent DENV antigens (equivalent of \(1.2 \times 10^4\) plaque forming units per serotype) was incubated with nanoparticles. Six-week-old Swiss Webster mice were immunized subcutaneously on days 0, 7 and 14. Anti-dengue IgG to all serotypes was elicited but no neutralizing activity was found [71].

Since nanoparticles are phagocytosed by dendritic cells (DC) and other APC, the adjuvant effect of BSA nanoparticles may be explained by their rapid internalization by skin-resident APC, such as DC. A similar mechanism was described for whole inactivated influenza virus, for which it was demonstrated that the viral RNA present in inactivated virus particles could trigger Toll-like receptor 7 to augment the adaptive humoral response [72].

Vector-based vaccines

Recombinant poxviruses and adenoviruses expressing foreign proteins have been demonstrated to induce strong humoral and cellular responses in humans against various pathogens. Several live virus vectors such as adenovirus, alphavirus and vaccinia virus are designed for direct administration to the host and have been engineered to express DENV E protein for evaluation as dengue vaccine candidates [73,74].

It has been demonstrated recently that a non-propagating Venezuelan equine encephalitis virus replicon expression vector (VRP) expressing the ectodomain of DENV E protein (E85) overcomes maternal interference in a BALB/c mouse model [75]. NAb and T cell responses to each serotype may be induced by a single immunization with a tetravalent VRP vaccine at a level equivalent to the monovalent vaccine component, suggesting that this vaccine modality can overcome serotype interference. Although the neonatal immune response was found to be lower in magnitude than responses in adult mice, neonatal immunization was durable and could be boosted later in life to increase further NAb and Tcell responses. Hence, since VRP vaccines expressing DENV antigens are immunogenic and protective in neonates, they are promising candidates for safe and effective vaccination in early life [75].

Another encouraging vaccine platform is the use of replication-defective recombinant adenovirus (Ad) vectors. In a wide variety of cell types, these vectors have the ability to express antigens at high levels making them ideal for inducing potent immune responses. Ad vectors have been studied as a delivery vehicle for a wide variety of infectious agents, including dengue virus [76], tick-borne encephalitis virus [77], human immunodeficiency virus [78], Ebola virus [79-81] and Marburg virus [82,83]. However, the major limitation to the traditional Ad vector vaccine approaches is the volume of antigenic information that can be carried by a single vector. Specifically, in the Ad-based dengue vaccine, only a fraction of the E gene sequence of a single DENV serotype was expressed [76]. ATDV based on a novel complex adenovirus platform that is capable of expressing multiple antigens de novo has also been developed [84]. This dengue vaccine was constructed as a pair of vectors that each expresses prM and E genes of two different DENV serotypes. Upon vaccination of C57BL/6 mice, natural infection was mimicked and both NAb and cellular immune responses against multiple serotypes of DENV were induced [84]. As several epitopes of the E protein are known to play a significant role in the induction of both NAb and T cell-mediated immunity, both prM and E were selected as antigens [85,86], while expression of the flavivirus prM protein is necessary for correct processing and expression of E [87-89]. The vast majority of the global human population becomes rapidly immune to adenovirus, particularly subtype-5, that most common used for vector-based gene therapy and vaccine design [90]. Thus, a common criticism of Ad vector use in humans is the likelihood of pre-existing immunity. The presence of circulating Ad NAb has the potential to limit the efficacy of an Ad-based vector vaccine. However, a recent clinical trial found no correlation between pre-existing Ad-NAb titres and potency of an Ad-based influenza vaccine in humans [91].

Challenges in the development of tetravalent dengue vaccines

Despite numerous studies to date on DENV, no effective vaccine is currently available [92,93]. In order to provide long-term broad protection against all serotypes, dengue vaccine design has targeted a multivalent strategy (Table 1). However, a number of major scientific challenges have slowed down development. The lack of an entirely appropriate animal model, coupled with insufficient understanding of correlates of successful human immunity and immune pathology following infection, are significant obstacles to the development of an effective vaccine [94,95]. In addition, within each of the DENV serotypes there are multiple genotypes, reflecting a high degree of mutation due to evolutionary selective pressure. Specific genotypes may exhibit differences in viral fitness and be associated with more severe clinical phenotypes. It remains unclear how important identifying genotypic variation is to dengue vaccine development [24]. Furthermore, infection by one of the DENV serotypes has been shown to confer lasting protection against homotypic reinfection but only transient protection against a secondary heterotypic infection. Secondary heterotypic infection is associated with an increased risk of severe disease which is referred to as immune enhancement of disease [12]. A paucity of knowledge of the contribution of NAb to protection versus other immune mechanisms such as cytotoxic T cell responses, a lack of appreciation of the most suitable target epitopes for vaccines, an unrecognized role of non-structural proteins in dengue immunity, viral interference of multivalent combinations, and concern about reactogenicity in flavivirus-exposed individuals are examples of further challenges associated with the development of safe and effective dengue vaccines [95].

**FUTURE PERSPECTIVES**

Most of the TDVs developed so far have shown qualified success, generating NAb responses but not necessarily reducing viraemia. Importantly, although research is ongoing to date these candidates have not provided sufficient indication of delivering broad protection against all tested serotypes (Table 1) [16]. Many TDVs are based on live virus vectors that can potentially elicit an adverse effect in immune suppressed patients, while the newly discovered dengue serotype, DEN-5, is also found to be associated with infection [2]. In this context, there is a pressing need to identify novel pentavalent vaccine candidates which
Table 1: Characteristics of various tetravalent dengue vaccines under trial.

<table>
<thead>
<tr>
<th>Vaccine type</th>
<th>Animal model/human</th>
<th>Route of vaccination</th>
<th>Important observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live-attenuated</td>
<td>Cynomolgus macaques</td>
<td>Subcutaneous/intradermal</td>
<td>Neutralizing antibody response to DEN-2 showed no significant increase in titre in all vaccinated groups</td>
<td>Ambuel et al. 2014 [22]</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Human (children &amp; adolescents)</td>
<td>Subcutaneous</td>
<td>Vaccination elicited neutralizing antibody response against all serotypes and was well tolerated in children/adolescents</td>
<td>Dayan et al. 2013 [29]</td>
</tr>
<tr>
<td>Chimeric DNA</td>
<td>Rhesus macaques</td>
<td>Intramuscular</td>
<td>Reduction in viraemia for DEN-1 but not DEN-2, i.e. partial protection against DEN-1 but no protection against DEN-2</td>
<td>Raviprakash et al. 2006 [44]</td>
</tr>
<tr>
<td>DNA Syncon</td>
<td>BALB/c mice</td>
<td>Intramuscular electroporation</td>
<td>Significant level of neutralizing antibodies to all serotypes</td>
<td>Ramanathan et al. 2009 [47]</td>
</tr>
<tr>
<td>DNA with adjuvant</td>
<td>New Zealand white rabbits</td>
<td>Intramuscular</td>
<td>All animals elicited strong neutralizing antibody response to all serotypes</td>
<td>Raviprakash et al. 2012 [50]</td>
</tr>
<tr>
<td>Electroporation-enhanced DNA</td>
<td>ICR mice</td>
<td>Intramuscular electroporation</td>
<td>A good neutralizing antibody response was induced in all animals</td>
<td>Prontpetchara et al. 2014 [52]</td>
</tr>
<tr>
<td>Sub-unit</td>
<td>BALB/c mice</td>
<td>Subcutaneous</td>
<td>Neutralizing antibodies were induced against all serotypes</td>
<td>Zhao et al. 2014 [68]</td>
</tr>
<tr>
<td>Nanoparticle-adsorbed DNA</td>
<td>Swiss Webster mice</td>
<td>Subcutaneous</td>
<td>Anti-dengue IgG to all serotypes was elicited but no neutralizing activity</td>
<td>Silva et al. 2012 [71]</td>
</tr>
<tr>
<td>Alphavirus-based</td>
<td>BALB/c mice</td>
<td>Intraperitoneal</td>
<td>Elicited protective immunity by neutralizing antibodies against all serotypes in neonatal mice</td>
<td>Khalil et al. 2014 [75]</td>
</tr>
<tr>
<td>Adenovirus-based</td>
<td>C57Bl/6 mice</td>
<td>Intraperitoneal</td>
<td>Neutralizing antibody against all serotypes was elicited</td>
<td>Holman et al. 2007 [84]</td>
</tr>
</tbody>
</table>

may engender effective protection against all acknowledged serotypes.

CONCLUSION

Although several TDV candidates are undergoing pre-clinical testing and a number of others are currently in clinical trials, an effective DENV vaccine has yet to become commercially available. Several of the tetravalent vaccine candidates have not fulfilled their early promise in animal models by eliciting immunity against each of the four dengue serotypes measured in human participants. Therefore, development of a second generation of new or refined vaccine candidates is most likely required to provide protection against all serotypes, including the recently recognized fifth serotype.

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REFERENCES


