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Editorial

Vaccine Development against Dengue, a Viral Disease of Increasing Significance to Global Public Health

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Abstract

Dengue is a mosquito-borne viral disease of humans that is a major public health concern in tropical and sub-tropical regions of the world. The increasing mortality and morbidity rates caused by infection in recent years are attributable partly to a lack of availability of effective antiviral therapies and vaccines. In a concerted attempt to arrest this global expansion, several dengue vaccine candidates have progressed from pre-clinical testing into clinical trials. However, the advancement of vaccine development has manifest challenges to be overcome. A principal tenet underpinning dengue vaccine design is balanced immunity to all five recognised virus serotypes, but difficulties in achieving optimal attenuation of each virus and interference between individual attenuated viruses are significant hurdles to successful implementation. Currently, the most advanced vaccine candidate, the recombinant, live-attenuated ChimeriVax-DEN1-4 preparation developed by Sanofi Pasteur, is likely to achieve licensure at the completion of phase III trials and undergo population safety surveillance. Subunit and DNA vaccines are also in various stages of clinical evaluation. The intrinsic rationale is to produce a safe, efficacious and cost-effective vaccine. While this remains an achievable goal, progress is limited by an incomplete understanding of dengue viral pathogenesis, together with a lack of suitable animal models for fundamental pre-clinical development. This editorial highlights current approaches and future directions for vaccine strategies to combat the global expansion of this infectious disease.

Keywords
dengue; virus; immunity; vaccine; serotype; antibody.

Introduction

Dengue is a vector-borne disease of humans that is transmitted by mosquitoes of the species *Aedes aegypti* and, less frequently, *Aedes albopictus* [1]. The causative agent of infection is Dengue Virus (DENV), which belongs to genus *Flavivirus* of the family *Flaviviridae* [2]. Other members of this family are responsible for a number of notable infectious diseases in humans, such as yellow fever, West Nile encephalitis, Japanese encephalitis and hepatitis C virus infection. DENV is an enveloped, single-strand, positive sense RNA virus. The 10.7 kilobase genome encodes a precursor polypeptide in which post-translational cleavage by a host cell and virus-encoded protease results in formation of three structural proteins – capsid (C), membrane (M), and envelop (E), and seven non-structural proteins – NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [2,3]. These structural and non-structural proteins play several functions in the life cycle and pathogenesis of DENV. They are therefore potential targets for host immune responses and hence also for vaccine design. In particular, E protein, as its name suggests, is the principal surface protein of the virion, and is involved in host cell attachment, typically to keratinocytes, the predominant cell type in human skin. E protein presents a major target for humoral immunity and mutation affects DENV virulence [3]. Of the non-structural proteins, NS1 is a highly conserved glycoprotein that, while detailed biological functions are yet to be ascribed, appears essential to pathogenesis. It is present in elevated concentrations in sera of dengue-infected patients during the early clinical phase of infection [4].

There are five antigenically distinct serotypes of DENV, the last one of which was discovered only very recently [5]. Named DENV-1 to -5, they are distinguished by a plaque reduction neutralization test. Separate serotypes frequently co-circulate in the same locality. Infection will engender lifelong immunity to the exposed serotype but provide only transient protection against others [3,6]. However, in cases of re-infection by a heterologous dengue serotype with the presence noted of cross-reactive antibodies [7] and/or cross-reactive T cells [8,9], the potential risk of antibody-dependent enhancement of disease is increased. Indeed, the greatest risk factor for dengue haemorrhagic fever is secondary infection.

Over several decades the rising public health burden of dengue has provoked widespread international concern. The causes for an increase in cases are multifactorial but include urbanization, climate change, globalization and human population growth, which have facilitated both clustering and dispersal of hosts, vectors and viruses [10]. At present, more than 2.5 billion people are at risk of infection in over 125 countries of

the tropics and subtropics [1]. Recent estimates suggest that there are 390 million cases of dengue annually, of which 96 million present with clinicalor subclinical severity [11]. Of these, around 500,000 require hospitalization with life-threatening complications, resulting in up to 20,000 deaths [2,12]. Dengue infection presents clinically as a spectrum ranging from mild, self-limiting febrile illness to severe forms like haemorrhagic fever with warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, increased haematocrit with decreasing platelets), and life-threatening shock syndrome (with profound plasma leakage, severe bleeding, or organ failure) [13].

There is no specific therapy for dengue, only supportive treatments for symptoms, including oral rehydration, administration of intravenous fluids and/or blood transfusion. Hence, there is an imperative need for intervention approaches based on either vector control or human vaccination if the current upward epidemiological trend is to be reversed [14,15]. Although clinical testing of attenuated vaccine candidates began in the 1980s [16], a licenced vaccine is not yet available. The challenge to vaccinologists is to achieve pan-serotype immunity without triggering associated pathology [7]. An overriding principle driving vaccine design is balanced immunity to all five now identified viruses. An intensive, cross-disciplinary research effort aims at producing an effective multivalent vaccine for prophylactic immunization [17]. To date, a variety of approaches has been undertaken to produce a prototype vaccine.

Live-attenuated tetravalent dengue vaccine

Live-attenuated vaccines (LAV) aim to mimic natural infection by inducing humoral and cellular responses to both structural and non-structural proteins of the virion using a harmless or less virulent form of the live virus. The first dengue tetravalent LAV evaluated in clinical trials was initiated by the University of Hawaii, USA, then developed by Mahidol University, Bangkok, Thailand, and licensed to Aventis Pasteur (now Sanofi Pasteur, Lyon, France) [18]. Attenuation of DENV-1 to -4 viral strains for this vaccine was achieved by serial passage through *in vitro* epithelial cell cultures of primary dog kidney (PDK) or primary African green monkey kidney lines [19]. Another classic LAV was developed by the Walter Reed Army Institute of Research, Silver Spring, USA, and licensed to GlaxoSmithKline, Brentford, UK. This candidate has used serially diluted DENV attained by passaging in PDK cells and foetal rhesus lung cells [20]**.** Reports indicated promising seroconversion rates for each monovalent vaccine but unsatisfactory antibody responses to the tetravalent form [21]. Serial passage of virus in cell culture often produces erratic molecular changes through spontaneous genetic mutation and hence the degree of attenuation may vary. Interference between four individual attenuated viruses is potentially a significant obstacle to progress [22]. Attenuation has also been attempted by introducing a specific genetic mutation into the virus genome, which has the effect of interfering with the virion's ability to replicate. Initial difficulty lay in achieving optimal attenuation of each of the four tested DENV serotypes, all of which are needed to provide a required minimal level of reactogenicity and optimum immunogenicity [23,24]. Subsequent attempts to address this problem used reverse genetics to attenuate the virus with mutations in the 3'UTR region in order to generate genetically stable attenuated strains [25]. In addition to viral interference, symptoms of dengue-like fever were observed when the LAV preparation was administered to Thai and Australian healthy volunteers [26,27]; this led to the discontinuation of the Sanofi Pasteur vaccine in phase II clinical trials. Mahidol University,in collaborationwith the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) and the Pune-based Serum Institute of India, has since resumed further evaluation of this preparation in pursuit of a second generation LAV that shows suitable multivalent immunogenicity but with much reduced side effects [28,29].

Recombinant tetravalent dengue vaccine

A chimeric vaccine strategy uses genetic engineering technology to produce a recombinant construct using the‘backbone’of the related yellow fever virus fromwhich the pre-membrane (prM) and E gene products are replaced with the corresponding proteins from DENV. Hence, the backbone still contains the capsid, non-structural proteins and 5'- and 3'-untranslated regions. The principle of this formulation is to retain the attenuation properties from the parent viral vaccine while simultaneously incorporating dengue antigenicity [29]. Various chimeric vaccines are in different phases of clinical trial. Sanofi Pasteur’s ChimeriVax tetravalent DENV vaccine (TDV), which contains prM/E genes derived from each of the first four characterized serotypes of DENV and 17 D yellow fever virus as backbone, has been tested already in adult human volunteers in the United States, Philippines and Mexico [25]. Pre-clinical phase I and II trials demonstrated mild to moderate adverse effects [30]. It is encouraging to note that seroconversion rates after completion of a three dose immunization of ChimeriVax-DEN1-4 vaccine ranged from 77-100% and were considered satisfactory [31]. Furthermore, a subsequent study showed that this vaccine was well-tolerated and elicited neutralizing antibody responses against all four DENV serotypes in both flavivirus seropositive and seronegative participants [32]. A higher immune response to vaccination was observed in seropositive individuals, suggesting that pre-existing seropositivity may increase the vaccine-induced antibody response to immunization[29].If ongoing phase IIb/III trials are successful, Sanofi Pasteur will prepare an application for licensure from federal health authorities in the near future [15,33].

DNA-based dengue vaccines

An alternative method described to attenuate DENV is the use of reverse genetics to mutate the 3'-untranslated region of cDNA clones [34]. The method was developed by the National Institute of Allergy and Infectious Diseases, Bethesda, USA, and is based on deletion of a sequence of 30 nucleotides. A successful balance between immunogenicity and attenuation was reported for DENV-1 and -4 when immunized in rhesus monkeys and human volunteers. However, optimism was tempered by the observation in macaques of only partial attenuation of DENV-2 and no attenuation of DENV-3 [35]. A possible resolution of this limitation is to make a chimeric vaccine using the backbone of the DEN4Δ30 candidate to create similar preparations to target DENV-1, -2 and -3. The DEN4Δ30-based tetravalent vaccine has shown to be attenuated and immunogenic in rhesus monkeys, warranting further phase I/II testing [36]. In spite of excellent results for DENV-1 and -2, over-attenuation was detected for DENV-3 (rDV3/4 Δ30), such that its immunogenicity was insufficiently low. In order to enhance this candidate, an additional 31-nucleotide deletion was performed, with improved performance [37].

The Centers for Disease Control and Prevention (Atlanta, USA) and Takeda Pharmaceuticals (Osaka Japan) have also produced a chimeric vaccine candidate, DENVax, using the infectious cDNA clone derived from a DENV-2-PDK-53 strain developed at Mahidol University as backbone [38]. Intertypic recombination of DENV prM/E to replace the corresponding DENV-2 parental gene produced attenuated chimeric strains, each of which was transfected into Vero immortalized monkey kidney cells. Three sequential plaque purifications of each virus were performed to reduce genetic variation and to thereby ensure safety of the master seed virus. The resultant candidate vaccine was safe and immunogenic when tested in cynomolgus monkeys [39]. It has now progressed to phase II clinical trials in Puerto Rico, Colombia, Singapore and Thailand [29].

Subunit tetravalent dengue vaccine

A subunit formulation aims to deliver a TDV through overcoming issues of viral interference by adjusting the dose for each of the four recombinant proteins. Furthermore, it avoids the unpredictable interactions between viruses as experienced for a LAV. Bacteria (*Escherichia coli*), yeast (*Pichia pastoris*), virus (*baculovirus*) and plant cells have each been utilized successfully to express recombinant dengue E protein [16]. Correct folding is required to preserve the integrity of its neutralizing epitopes which requires co-expression of the prM protein that acts as a chaperone for E by facilitating folding, then trafficking through the secretory pathway[25]. Co-expression of E and prM can induce the formation of highly antigenic virus-like particles. Low yield and improper folding of E protein were overcome by C-terminally truncated E proteins produced in an insect cell expression system. Hawaii Biotech (now part of Merck, Kenilworth, USA) has developed the vaccine DEN-80Es (full length prM and 80% E derived from four serotypes) produced in *Drosophila* S2 cells [40].

Tetravalent DNA vaccine with adjuvant

The US Naval Medical Research Center (Bethesda, USA) and Vical Inc. (San Diego, USA) developed a DNA-based TDV consisting of DENV epitopes expressed in a plasmid. The fundamental principle behind this design is that when injected subcutaneously or into skin or muscle, antigen-presenting cells phagocytose the plasmid whereupon the encoded genes are expressed intracellularly to generate target antigens. When presented in association with major histocompatibility complex class II molecules to the immune system this will stimulate both humoral and cellular responses. The antibody response to dengue in humans is directed at a variety of DENV proteins, including C, prM, E, NS1, NS3, NS4B and NS5. The majority of anti-dengue neutralizing antibody epitopes have been mapped to the E protein. The lipid-based adjuvant Vaxfectin, a tetravalent formulation, increased immunogenicity in non-human primates [41], and has recently entered a phase I clinical trial [37].

Current challenges and future directions

Recent research efforts have been directed to produce a highly effective dengue vaccine against homotypic and heterotypic infection. A priority is that this should be safe to administer and economic to produce. However, several obstacles for the development of dengue vaccines are making this a very challenging goal. These include an insufficiently defined pathogenesis and a lack of suitable experimental models [42]. The most widely used animal model is the laboratory mouse, in which DENV replicates poorly. Vero and LLC-MK2 monkey kidney cell lines are standardly used for performing the neutralization test. However, as the widely accepted hypothesis of dengue pathogenesis involves binding of a virion to Fcγ receptor-bearing phagocytic cells, more appropriate cell lines for experimental studies include U937, THP-1, K562 and BHK. The efficacy of vaccines currently on a path to licensing should be assessed in different settings and clinical conditions [15]. Using immunocompromised animal models would be an effective method to identify vaccine outcomes in immunosuppressed hosts. Furthermore, detailed examination of dose and delivery of each TDV may overcome the potential hurdles of reducing viral interference and achieving balanced immunity. Inactivated whole virus is an alternative option to live vaccines and offers the perceived advantages over TDV approaches of no possibility of reversion to virulence and relative ease of induction of a balanced immune response. However, a number of challenges remain, such as

lack of immunity to NS proteins and a requirement to employ adjuvants to enhance immunogenicity.

Conclusions

Although there are quite a few TDV candidates undergoing pre-clinical testing or in clinical trials, an effective DENV vaccine has yet to become commercially available. Several of the TDV candidates have not fulfilled their early promise in animal models by eliciting immunity against each of the four dengue serotypes tested in human participants. Hence, 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.

Competing Interests

The authors have declared no competing interests.

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