Which Dengue Vaccine Approach Is the Most Promising, and Should We Be Concerned about Enhanced Disease after Vaccination?

The Challenges of a Dengue Vaccine

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A dengue vaccine has been pursued for more than 50 years and, unlike other flaviviral vaccines such as that against yellow fever, progress has been slow. In this review, we describe progress toward the first licensed dengue vaccine Dengvaxia, which does not give complete protection against disease. The antibody response to the dengue virion is reviewed, highlighting immunodominant yet poorly neutralizing responses in the context of a highly dynamic structurally flexible dengue virus particle. Finally, we review recent evidence for cross-reactivity between antibody responses to Zika and dengue viruses, which may further complicate the development of broadly protective dengue virus vaccines.

GREAT DEBATES

What are the most interesting topics likely to come up over dinner or drinks with your colleagues? Or, more importantly, what are the topics that *don't* come up because they are a little too controversial? In ***Immune Memory and Vaccines: Great Debates***, Editors Rafi Ahmed and Shane Crotty have put together a collection of articles on such questions, written by thought leaders in these fields, with the freedom to talk about the issues as they see fit. This short, innovative format aims to bring a fresh perspective by encouraging authors to be opinionated, focus on what is most interesting and current, and avoid restating introductory material covered in many other reviews.

The Editors posed 13 interesting questions critical for our understanding of vaccines and immune memory to a broad group of experts in the field. In each case, several different perspectives are provided. Note that while each author knew that there were additional scientists addressing the same question, they did not know who these authors were, which ensured the independence of the opinions and perspectives expressed in each article. Our hope is that readers enjoy these articles and that they trigger many more conversations on these important topics.

Editors: Shane Crotty and Rafi Ahmed

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Dengue virus (DENV) is a flavivirus transmitted to man by *Aedes* mosquitoes, principally *Aedes aegypti*. There are estimated to be ~400 million infections annually of which around a quarter are symptomatic and 1%–5% of these can present with severe disease characterized by vascular leak and hemorrhage, termed dengue hemorrhagic fever. There are four distinct serotypes of dengue, which differ by 30%–35% amino acid identity; infection with one serotype leads to lifelong immunity to that serotype but not to infection with the other serotypes (Sabin 1952; Guzman et al. 2007). In many countries in the tropics and subtropics, all four serotypes frequently cocirculate or cyclically replace each other, meaning that multiple sequential infections are common or indeed the norm.

ENHANCED DISEASE ON SECONDARY INFECTION

One of the interesting immunological features of dengue is that the most severe symptoms occur more frequently following a secondary or sequential infection than occur following a primary infection, implying that some form of acquired immune response to the primary infection is driving more severe outcomes on subsequent reinfection.

One theory for more severe disease occurring on secondary infection is antibody-dependent enhancement (ADE), which was put forward by Halstead some 40 years ago (Halstead and O'Rourke 1977a,b). The ADE hypothesis proposes that during a secondary infection, antibodies formed to the primary infecting DENV, which differs substantially in sequence from the secondary infecting serotype, will not be of sufficient avidity or concentration to fully neutralize the secondary infecting dengue serotype (Screaton et al. 2015). Instead, they may partially coat and opsonize the virus for Fc-receptor-mediated uptake into myeloid cells, such as monocytes/macrophages, which are believed to be the principal site for virus replication, thus driving higher virus loads.

DENGUE VACCINES

The first successful flavivirus vaccine against yellow fever virus (YFV) was developed by Theiler in the 1930s and the same attenuated 17D strain is still in use today. Successful vaccines have also been produced against Japanese encephalitis virus (live attenuated or inactivated virus) and tick-borne encephalitis virus (inactivated virus).

There have been many different approaches to developing a vaccine against DENV over the last 50 years, and these vary from live attenuated and inactivated whole-virus vaccines to subunit, vaccine-like particles (VLPs), and DNA-based approaches. A central tenet to vaccine design to date is that individual DENV serotypes lead to type-specific protective immunity, meaning that vaccines are constructed as a tetravalent formulation, containing components from each of the four DENV serotypes.

The most advanced DENV vaccines are live attenuated tetravalent formulations of which there are three leading candidates produced by Sanofi Pasteur, Takeda, and National Institutes of Health (NIH)/Butantan; the former has been licensed and the other two are entering phase III trials. All three vaccines contain precursor membrane (prM) protein and E-sequences from the four DENV serotypes grafted onto an attenuated backbone containing the non-structural components. The Takeda and NIH vaccines use DENV as the backbone, whereas the Sanofi vaccine uses the YFV 17D as backbone (Fig. 1) (Screaton et al. 2015).

The Sanofi vaccine has undergone extensive phase IIb and III trials, which have shown an overall efficacy of 66%, with efficacy against DENV2 consistently lower than the other serotypes (Sabchareon et al. 2012; Capeding et al. 2014; Villar et al. 2015). The efficacy was somewhat below expectations based on early phase work in which the vaccine produced respectable *in vitro* neutralizing antibody titers and has prompted both a search for an explanation for this discrepancy and also better correlates of protective immunity. One suggestion for the less-than-expected performance of this vaccine was that it may not have produced a full T-cell

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A Denvaxia (CYD-TDV, Sanofi Pasteur) **B** NIH/Butantan (NIH) **C** DENVax (Takeda)

Figure 1. Construction of three leading live attenuated dengue virus (DENV) vaccines. (A) The Sanofi Pasteur vaccine Denvaxia contains four chimeric live flaviviruses, each derived from the genome of the yellow fever virus 17D vaccine strain (shown in yellow) with the precursor membrane (prM) and envelope (E) gene segments replaced by the corresponding gene segments of each of the four DENV serotypes (DENV1 to DENV4). (B) The National Institutes of Health (NIH)/Butantan vaccine contains a mixture of four recombinant DENV genomes color coded to represent the origins of the component parts. The vaccine strains were attenuated by deleting 30 nucleotides ($\Delta 30$) from the 3' untranslated region of the dengue viral genome. (C) The DENVax vaccine from Takeda contains a mixture of four recombinant DENV2 genomes. (From Screaton et al. 2015; reprinted, with permission.)

response as only the structural antigens were from dengue, while the nonstructural antigens came from YFV. Further analysis of the trials also suggested that the vaccine-protected individuals who were previously dengue exposed but was less efficacious in vaccinees who were

dengue-naïve at enrollment (Capeding et al. 2014; Villar et al. 2015).

Follow-up of the Sanofi vaccine trials substantiated the vaccine efficacy, but there was also a safety signal in the younger age groups included in the trial (Hadinegoro et al. 2015).

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Specifically, 3 years postvaccination, there were more hospitalizations in those aged <9 years in the vaccine group than in the control group. One explanation for this is that, as the <9-year age group is likely to be enriched for dengue-naïve subjects, the vaccine may be priming but not protecting these naïve individuals and leading to ADE on natural dengue infection.

The Sanofi vaccine, Dengvaxia, has been licensed for use in a number of countries and the World Health Organization (WHO) Strategic Advisory Group of Experts on immunization has recommended its use in those >9 years in areas of high dengue transmission with >70% dengue seropositivity and estimated that deployment of the vaccine in such areas will reduce hospitalization by 10%–30% over a 30-year time frame (see who.int/immunization/sage/meetings/2016/april/SAGE_April_2016_Meeting_Web_summary.pdf) (Screaton et al. 2015).

WHY HAVE DENGUE VACCINES PROVED PROBLEMATIC TO PRODUCE?

The challenge for dengue vaccines is formidable and part of this challenge relates directly to the fact that there are four related serotypes:

1. The vaccine will need to be effective against four similar but distinct serotypes.
2. The risk of ADE mandates that a successful vaccine will produce protection against all four serotypes, otherwise the vaccine may prime individuals for more severe disease on secondary infection.
3. Tetravalent formulations need to produce balanced immunity between the four serotypes when coadministered.
4. Original antigenic sin may complicate vaccine strategies relying on multiple boosts and, in endemic countries, a vaccine will ideally be given to both dengue-naïve and previously dengue-exposed vaccinees.

In addition to these challenges, there may be additional features unique to DENV, which are described below.

INCOMPLETE prM CLEAVAGE

Two transmembrane-anchored structural proteins are found in the glycoprotein shell of dengue virions, prM, and envelope protein (E) (Mukhopadhyay et al. 2005; Li et al. 2008; Yu et al. 2008; Screaton et al. 2015). Virions bud into the endoplasmic reticulum (ER) initially as immature forms in which 180 copies of E and 180 copies of prM are arranged into trimers (3prM:3E), giving the virion a spiky appearance. In the acidic environment of the Golgi, the virion undergoes a conformational change whereby it reassorts from prM/E trimers to prM/E dimers. Also in the Golgi, prM is cleaved by furin protease and, following cleavage, the pr peptide remains associated with the virion and disassociates on release of the virus particle from the infected cell. In dengue, prM cleavage is frequently incomplete, leading to the production of a spectrum of viral particles containing differing prM:E ratios, varying from the spiky, fully immature particle in which prM remains associated with E, to the mature virus particle, in which prM is fully cleaved and 180 copies of the E protein are arranged into 90 head-to-tail dimers to form smooth virus particles (see Fig. 3a in Mackenzie et al. 2004 and Fig. 3c in Kuhn et al. 2002). Between the fully immature and fully mature particles, a spectrum of partially mature forms exist, which have been shown by cryoelectron microscopy (cryo-EM) to contain varying amounts of smooth (E-dimer) and spiky (prM/E trimer) surfaces (Junjhon et al. 2008).

To add to this complexity, there are cell-type-specific differences in the degree of prM cleavage; insect cells produce high-prM virus particles, whereas primary human dendritic cells (DCs) produce relatively low prM virus particles (Dejnirattisai et al. 2010). Analysis of the memory B-cell response following DENV infection shows a high proportion of cells that produce antibodies reacting to prM (Dejnirattisai et al. 2010). These antibodies are potent inducers of ADE but show poor neutralization, which plateaus at ~50%. The reason for this plateau is that fully mature DENV particles contain no prM and are therefore not a target for anti-prM antibodies. Low prM particles do not

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contain enough prM antigen to allow neutralization, but can still be opsonized and promote ADE; therefore, only a fraction of prM-containing particles can be neutralized. We believe that, ideally, prM responses should be minimized or avoided in DENV vaccines; however, prM is an obligatory component of all live attenuated or inactivated virus vaccines currently under investigation.

STRUCTURAL FLEXIBILITY OF THE DENGUE VIRION

Further complexity is added by the structural flexibility of DENV. The dengue virion can adopt a variety of different conformations with differing thermodynamic stabilities in a process termed breathing. This can affect the accessibility of some antigenic sites that may be occluded in some conformations but exposed in others and explains why binding of some antibodies may be enhanced by prolonged incubation or by increased temperature (Dowd et al. 2014). A “bumpy” conformation of DENV2 has been described in which the virus particle is expanded and the interaction of the 90 E-dimers is changed relative to the standard mature virus particle, which may disrupt some quaternary epitopes formed between opposing dimers (Fibriansah et al. 2013; Zhang et al. 2013). It is interesting in this regard that the Zika virus (ZIKV) particles seem to be relatively more rigid than DENV particles (Goo et al. 2016; Kostyuchenko et al. 2016).

THE IMMUNODOMINANT FUSION LOOP EPITOPE

Many antibodies have been made to dengue starting with mouse monoclonals and, more recently, several hundred human monoclonal antibodies have been produced by different investigators. Antibodies binding the fusion loop epitope (FLE) are a major immunodominant component of the response to DENV (Smith et al. 2013; Dejnirattisai et al. 2015). During virus infection, acidification in an endosomal compartment triggers conformational reorganization of the E-protein from dimers to trimers

exposing the fusion peptide, which drives apposition of viral and host-cell membranes, allowing membrane fusion and escape of the viral RNA into the host-cell cytoplasm (Screaton et al. 2015).

In DENVs, access to the FLE is not restricted to virions at endosomal pH but also found at neutral pH. This is likely attributed to two of the processes described above. The presence of prM in partially mature DENV particles increases accessibility of the FLE (Cherrier et al. 2009; Dejnirattisai et al. 2015). Anti-FLE monoclonal antibodies (mAbs) can fully neutralize high-prM DENV produced in insect cells. However, low-prM DENV produced in primary human DCs, which represents virus produced in the infected host following the initial mosquito inoculation, cannot be fully neutralized by anti-FLE mAbs, typically plateauing at ~80%, yet anti-FLE mAbs can potently induce ADE (Dejnirattisai et al. 2015).

In addition to the presence of prM, structural flexibility or breathing of the DENV E-dimer may also allow access to the FLE. Interestingly, the FLE is highly conserved between DENVs and ZIKV and anti-FLE produced from DENV-infected patients can bind with high affinity to monomeric ZIKV E-protein (Dejnirattisai et al. 2016; Stettler et al. 2016). However, these anti-FLE mAbs fail to neutralize ZIKV infection but promote ADE, which is consistent with the concept that ZIKV is more rigid than the DENVs, thereby limiting access to the FLE (Barba-Spaeth et al. 2016; Dejnirattisai et al. 2016; Kostyuchenko et al. 2016).

We believe that the immunodominance of the FLE in DENV infection may be related to the incomplete cleavage of prM and to structural flexibility of the virus. Because anti-FLE antibodies poorly neutralize low-prM-containing viruses. The ideal DENV vaccine would thus aim to minimize responses to the FLE.

CONFORMATIONAL QUATERNARY EPITOPES

Analysis of panels of human anti-DENV mAbs has identified a number that are potently neutralizing with 50% neutralization titer (NT50)

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values into the low picomolar range (Screaton et al. 2015). The most potent antibodies react to conformational epitopes on the E-protein that are only found when E is displayed on virus particles, but not on recombinant monomeric-E (Screaton et al. 2015). A number of such epitopes have now been structurally characterized, most of which are serotype-specific; mAb-1F4 (DENV1) binds E-monomers only when in the intact virion, mAb-HM14c10 (DENV1) binds to two opposing E-dimers, mAb-5J7 (DENV3) binds three adjacent monomers, and mAb-2D22 (DENV2) binds two monomers in the E-dimer (Teoh et al. 2012; Fibriansah et al. 2014, 2015a,b; Screaton et al. 2015).

We have recently reported a new epitope for conformational quaternary antibodies, the E-dimer epitope (EDE), of which two categories EDE1 and EDE2 are distinguished by the lack of sensitivity or sensitivity to removal of glycan at position N153 in E, respectively (Dejnirattisai et al. 2015). A number of such antibodies were isolated from dengue-infected patients and their epitopes mapped by X-ray crystallography and cryo-EM (Dejnirattisai et al. 2015; Rouvinski et al. 2015). The antibodies bind across the interface of two head-to-tail E-monomers making up the E-dimer (Fig. 2). They occupy a site where prM binds to E as it passes through the Golgi, which is highly conserved between all DENV serotypes; hence, many of the EDE mAbs are broadly neutralizing of all four serotypes. The EDE-mAbs are potently neutralizing in the low picomolar range and, unlike the anti-FLE antibodies described above, they potently neutralize high- and low-prM-content viruses produced in insect and DC, respectively.

In summary, antibodies to conformational epitopes seem to be the most potent neutralizers of DENV. The mAbs themselves are potential prophylactics or therapeutics and elicitation of broadly neutralizing antibodies to the EDE should be prioritized in future vaccine approaches.

DENGUE AND ZIKA INTERACTIONS

ZIKV was first isolated in 1947 and, until recently, has been relatively understudied because

infection was frequently asymptomatic, caused relatively mild disease, and cases were largely sporadic with no epidemic activity (Musso and Gubler 2016). This has dramatically changed with large-scale outbreaks spreading eastward across the Pacific reaching Brazil in 2014, leading to an explosive epidemic spreading across South America associated with Guillain-Barré syndrome (estimated risk 0.24%) and a large increase in cases of microcephaly in children born to mothers infected during pregnancy, particularly the first trimester (estimated risk, 1%–22%) (Brasil et al. 2016; Cauchemez et al. 2016). The WHO declared Zika a global health emergency in February 2016 and there is now a concerted effort to develop a ZIKV vaccine.

ZIKV is a flavivirus most closely related to the DENV serocomplex (41%–46% amino acid difference in the envelope protein) and like DENV is also transmitted by the *A. aegypti* mosquito (Musso and Gubler 2016). In South America, there has been geographical spread of DENV, meaning that in recent ZIKV-affected areas DENV seropositivity is frequently 80% or more (Brathwaite Dick et al. 2012; Castanha et al. 2013). The difficulty in distinguishing previous DENV or ZIKV infection serologically suggests that there is substantial cross reactivity in the antibody responses to the two viruses (Lanciotti et al. 2008).

This cross-reaction leads to the possibility that anti-DENV responses may be either protective against ZIKV infection or, by promoting ADE, may actually increase ZIKV replication. Several reports have now explored this possibility (Castanha et al. 2016; Dejnirattisai et al. 2016; Priyamvada et al. 2016; Stettler et al. 2016), showing that serum from dengue-immune donors strongly binds to ZIKV by enzyme-linked immunosorbent assay (ELISA). Although most of these sera are non-neutralizing of ZIKV, some anti-dengue serum samples showed quite respectable neutralization of ZIKV (50% activity in focus reduction neutralization test [FRNT50] <1 in 200) (Dejnirattisai et al. 2016; Priyamvada et al. 2016). However, although most anti-DENV serum samples fail to neutralize ZIKV, they potently promote ADE of ZIKV in vitro

Figure 2. Anti-E-dimer epitope (EDE) monoclonal antibodies (mAbs) cross-react between dengue virus (DENV) and Zika virus (ZIKV). (A,B) Binding of EDE mAb-A11 to the DENV E-dimer. (From Rouvinski et al. 2015; reprinted, with permission, from Nature Publishing Group.) (C) Conservation of amino acid sequence and footprint of the EDE between DENV and ZIKV. (From Barba-Spaeth et al. 2016; reprinted, with permission, from Nature Publishing Group © 2016.)

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and ADE induced by an anti-dengue serum has also been demonstrated in a murine model (Bardina et al. 2017).

Analysis of panels of monoclonal antibodies made from DENV- or ZIKV-infected donors have now been reported (Dejnirattisai et al. 2010, 2015; Dai et al. 2016; Priyamvada et al. 2016; Sapparapu et al. 2016; Stettler et al. 2016; Swanstrom et al. 2016; Wang et al. 2016; Robbiani et al. 2017). These mAbs show substantial cross-reaction between DENV and ZIKV, suggesting that ZIKV could be considered as a fifth member of the DENV serocomplex. Interestingly, antibodies generated from DENV-infected donors, which are directed to the FLE and which can neutralize DENV bind strongly to recombinant ZIKV envelope protein but show poor neutralization of ZIKV, yet still potently promote ADE of ZIKV infection (Barba-Spaeth et al. 2016; Dejnirattisai et al. 2016; Paul et al. 2016). This may be the result of differences in breathing of the DENV and ZIKV virions as described above (Kostyuchenko et al. 2016).

Although most anti-dengue mAbs show poor neutralization of ZIKV anti-EDE mAbs, particularly the EDE1 subclass show potent neutralization of ZIKV in the low picomolar range (Dejnirattisai et al. 2016). Crystal structures of EDE1 antibodies binding to the ZIKV envelope dimer reveal the strong conservation of this epitope between ZIKV and DENV (Barba-Spaeth et al. 2016).

The cross-reaction between serological responses to ZIKV and DENV may have implications for the pathogenesis of ZIKV infection by driving increased virus replication by ADE of ZIKV in previously DENV-exposed individuals, and there is also the possibility the ADE may directly drive transplacental spread of ZIKV leading to fetal brain infection and microcephaly. The close serological cross-reactivity between DENV and ZIKV needs to be borne in mind in future vaccine development. It is possible that dengue vaccination may prime individuals for ADE of future ZIKV infection and conversely that ZIKV vaccination may lead to ADE of future DENV infection. In addition, it is likely that, in the future, DENV and ZIKV vaccines will need to be deployed in populations

that have already been naturally exposed to one or other viruses, and original antigenic sin may well shape the subsequent response to the vaccine in a way that could differ substantially from vaccination of DENV/ZIKV-naïve individuals.

SUMMARY

Following >50 years of work, the first dengue vaccine has been licensed for use primarily in previously DENV-exposed individuals in areas of high endemicity. Two further tetravalent live attenuated vaccines from Takeda and NIH/Bu-tantan, which differ from Dengvaxia in terms of the vector backbones, are reaching phase III and the results of these trials are awaited. If these two products fail to advance greatly on Dengvaxia, new approaches will be required to control the spread of DENV infection. We believe that anti-prM and anti-FLE responses to DENV are not desirable and that their immunodominance may indeed be an immune-evasion strategy used by DENV. One possible new avenue is to produce a subunit immunogen consisting of stabilized E-dimers; this would both remove the need for prM and also restrict breathing of the E-dimer, reducing accessibility and immunogenicity of the FLE. Furthermore, because the EDE response is broadly neutralizing between the DENVs and extends to ZIKV, a universal pan-DENV or DENV/ZIKV immunogen may be possible.

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