Dengue vaccine design: issues and challenges

M J Cardosa

Institute of Health and Community Medicine, Universiti Malaysia Sarawak, Malaysia

Dengue virus infection is now a global problem affecting tens of millions of people. The spread of the four dengue virus serotypes had led to increased incidence of dengue haemorrhagic fever (DHF) reported and with 2.5 billion people at risk, efforts towards the development of safe and effective vaccines against dengue must be accelerated.

This chapter reviews some of the important lessons of pathogenesis which may be learnt from classical studies in the field and place these in the context of current knowledge about the molecular biology of the virus. The issues which have to be addressed in designing a safe vaccine against dengue are raised and the problems of designing subunit as well as whole virus vaccines are pointed out, particularly with regard to the phenomenon of antibody dependent enhancement and, more generally, the problem of immune potentiation of disease. More efforts must be made to understand the basis of pathogenesis in DHF and in finding out what nature has to teach about protection against and recovery from dengue virus infection.

The dramatic emergence of dengue fever (DF) and dengue haemorrhagic fever (DHF) as a global public health burden in the last decade has emphasized the urgency of addressing the problem of vaccination against dengue viruses. Although the first reported outbreaks of dengue fever occurred in the late 18th century in Asia, Africa and North America, in modern times dengue was really only considered a problem post World War II, and then only as a problem in southeast Asia, particularly because of the increasing incidence of the disease entities referred to as dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). The subsequent spread of DF and DHF through Asia, the Pacific, Africa and the Americas is well documented in a number of reviews of the global emergence of dengue, for example the documents available at the website *http://emergency.com/dnguefv.htrr*.

Correspondence to Dr M J Cardosa, Institute of Health and Community Medicine, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

With 2.5 billion people at risk and tens of millions estimated to be infected by dengue virus annually, the reported incidence of DHF has risen drastically over the last decade. Although a successful vaccine against the prototype flavivirus yellow fever virus (YFV) has been in use since the 1930s and vaccines to two other flaviviruses, Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBE) are also

available today, there is as yet no dengue vaccine approved for use. This gap has less to do with any lack of interest in the subject than in the complicated nature of the pathogenesis of DHF/DSS.

This chapter will address the question of dengue vaccination and issues in the design of vaccines against DF and DHF. Some important concepts relevant to the design of such vaccines will be discussed in the context of current research into the development of dengue vaccines.

The viruses and the disease syndromes

Innis[1] has written an excellent detailed review of the epidemiology, biology, pathology and clinical features of dengue fever and dengue haemorrhagic fever, and the reader is referred to this for details. Briefly, dengue viruses belong to the family *Flaviviridae* and the most important of the vectors transmitting the dengue viruses to humans is *Aedes aegypti*. The family *Flaviviridae* comprises nearly 70 viruses, almost half of which can cause disease in humans. There are three major clinical syndromes associated with the pathogenic flaviviruses: (i) fever with rash, often associated with myalgia or arthralgia; (ii) encephalitis or meningoencephalitis; and (iii) haemorrhagic fever. Dengue, however, is associated more commonly with two of these syndromes, although there have been some reports recently of dengue encephalitis, such as that by Lum and coworkers[2].

Typically a dengue infection begins with an abrupt onset of high fever accompanied by facial flush and headache, sometimes also with vomiting and abdominal pain. DF is often unremarkable, the patient presenting with a maculopapular rash and/or petechiae, the platelet count may be variable and a bleeding tendency may or may not be present. DHF, however, is characterised by a rising haematocrit and thrombocytopenia with the nadir platelet counts dropping to below 100 000 per mm^3, the critical stage being after the viraemic febrile period. It is important to realise that it is the leakage of plasma rather than the haemorrhagic manifestations *per se* which distinguishes DHF from DF, although invariably the platelets counts are lower in DHF than in DF. Dengue shock syndrome (DSS), as its name suggests, is DHF with circulatory collapse.

There are undoubtedly many instances of DF cases with severe bleeding tendency but without the attendant plasma leakage, and by World Health Organization definitions, such cases should be classified as DF rather than DHF[3]. It is important to maintain a standardised approach to classifying the various dengue syndromes particularly in studies of pathogenesis and host responses in human disease, in order that results may be more accurately interpreted by others in the field.

The 10.5 kb dengue viral genome is a single stranded positive sense RNA organised into a single long open reading frame with the genes encoding the structural proteins C, prM, and E followed by the genes encoding the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. There are 4 serotypes of dengue viruses named DEN 1 to 4. These are all able to cause disease and are distinguished classically by neutralization tests but may also be distinguished today by monoclonal antibodies[4] and by the polymerase chain reaction[5,6].

The genome is encapsidated by the single 12–14 kDa core protein C with an envelope consisting of the major structural 55–60 kDa protein E and the 8 kDa membrane protein M which is cleaved late in maturation from the 19–23 kDa prM. The major nonstructural proteins are NS1 (44–49 kDa), NS3 (67-76 kDa) and NS5 (91–98 kDa) and these are expressed in the infected cell[7]. However, by far the most immunogenic of these is NS1, although the function of this protein has not yet been determined. NS3 is known to be a protease, and studies with recombinant vaccinia viruses expressing polyproteins of DEN4 or YFV have shown that NS3 as well as NS2B are required for cleavages of NS2A–NS2B, NS2B–NS3, NS3–NS4A and NS4B–NS5[8,9]. NS5 is the most conserved of all the nonstructural proteins and is believed to be an RNA-dependent RNA polymerase[7].

Pathogenesis of dengue haemorrhagic fever

The major risk factor associated with the development of DHF rather than DF is a secondary immune response. The spread of all four serotypes globally, therefore, increases the risk of sequential infection with different serotypes, and thus also may explain the rise in reported outbreaks of DHF in recent times. One dominant hypothesis on the immunopathogenesis of DHF derived from *in vivo* monkey studies as well as *in vitro* models has been referred to as the immune enhancement hypothesis and is based on an antibody dependent enhancement (ADE) of viral infectivity. This is reviewed by Porterfield[10] and Morens[11] who both register concern about the need to understand more about the mechanisms and determinants involved in ADE, particularly with respect to vaccine development.

Antibodies to dengue virus E may be detected in a variety of assays including the haemagglutination inhibition (HI) assay, enzyme linked immunosorbent assays (ELISA) and the neutralization test (NT). Some assays merely detect binding to E (HI and ELISA) while the NT is a functional assay and is a measure of the potency of an antibody preparation in the inactivation of dengue virus *in vitro*. Most antibodies directed against E also have the ability to effect ADE, which is a means by which antibody bound to the virion provides an efficient ligand for

virus binding and entry into Fc receptor bearing cells. Even neutralizing antibodies may effect ADE when diluted to beyond neutralizing levels, and are thus effective mediators of ADE at very low concentrations. This phenomenon is postulated to be one of the reasons why patients with a secondary immune response to dengue virus are more likely to develop DHF than are patients with a primary immune response.

The outcome of ADE is increased infectivity of virus, but there has as yet been no direct evidence that a greater virus load will lead to more severe disease *in vivo*, nor is there any knowledge of the mechanism by which high virus loads may predispose to DHF. Alternative hypotheses about the pathogenesis of DHF include arguments that DHF is a consequence of complement activation on the surface of infected cells expressing dengue virus antigens against which anti-dengue antibodies have bound[12], or that secondary infection with heterotypic dengue results in increased numbers of infected monocytes resulting in a marked increase in the activation of cross-reactive $CD4^+$ and $CD8^+$ memory CTL thus mediating a deluge of cytokines and other mediators[13]. Both these arguments actually implicitly accept that a secondary immune response is a risk factor for more severe disease, and that the presence of heterotypic antibody will lead to increased infection. While ADE as a mechanism for pathogenesis of DHF might be an oversimplification in the current understanding of the role of cytokines and cell mediated immunity, it is nevertheless quite clear that **immune potentiation** plays a role as a risk factor for DHF.

Quite apart from immune potentiation, there is also evidence that dengue viruses do have determinants for virulence and attenuation[14,15] as well as different tissue tropisms. An example of this is the demonstration by Chen and coworkers[16] that a single amino acid substitution $Glu_{406} \rightarrow Lys$ in a chimeric DEN3–DEN4 expressing the DEN3 structural proteins in a background of DEN4 nonstructural genes renders the mutant virus neurovirulent, while the parent or wildtype DEN3 is not.

The development of live whole virus vaccines for dengue clearly has had to focus on these characteristics of attenuation and neurovirulence and the successful development of such attenuated vaccine candidates by Bhamarapravati and coworkers[17] as well as Marchette and coworkers[18] is testimony to the existence of such markers. However, the evaluation of the safety and efficacy of such vaccines cannot be limited to conventional criteria and must include an evaluation of the possibility of immune potentiation of infection and disease *in vivo*. The difficulty in carrying out such determinations satisfactorily lies in the fact that so little is really known about the mechanisms taking place aside from the contribution of Fc receptors and antibodies to E. Much more thought has to be given to the problem of an effective evaluation of the possibility of immune potentiation in dengue infection in any vaccine development study.

The virion surface

Of the structural proteins, E and prM have been implicated in the development of antibodies able to neutralize dengue viruses. A major development in the effort to understand the structure of E has been the beautiful crystal structure of the dimerized ectodomain of E of the flavivirus TBE elucidated by Rey and coworkers[19]. The structure of E is unusual in that the protein does not extend outwards from the virion surface but, instead, lies flat in a direction that can only be described as lying along or parallel to the membrane. E appears to comprise three domains which have been named domains I, II and III in which domain I is a central domain, domain II is involved in dimerization of E by contacting adjacent molecules of E along the surface of the virion, and domain III is an immunoglobulin-like domain which is analogous to the domain B described previously (see for example, Heinz *et al*[20]). Domains I and II are roughly analogous to domains C and A, respectively.

At physiological pH, E forms dimers on the surface of the virion, but, on exposure to a pH below 6.5, a conformational shift occurs which leads to a re-arrangement of E to form trimers, an event which also allows fusion between the virion and the host cell membrane[21]. This phenomenon is analogous to the fusion event described for influenza virus haemagglutinin[22,23] which allows virus in the endosomal compartment to release its nucleocapsid into the cytosol. Indeed this phenomenon of low pH driven conformational change of virus envelope proteins which mediates fusion of enveloped viruses with host cell endosomal membranes[24] is distinct from the initial event of virus binding to the host cell. In the case of flaviviruses, low pH driven fusion from the endosomal compartment has been shown to be a necessary event for infection whether the virus enters through its 'normal' receptor or whether it enters the cell through an Fc receptor route[25].

The recent report[26] that highly sulphated heparan sulphates act as target cell receptors for DEN2 virus and that the polysulphonate drug Suramin can block dengue infection of target cells is an important contribution to the understanding of one pathway of dengue virus infection. However, this finding has to be placed in the context of other known pathways of infection[10] and the design of antivirals or vaccines against dengue must be able to account for all of these.

The protective immune response

Central to the discussion of protection is the question: how are dengue viruses neutralized? Polyclonal antibodies generated against dengue virus are very potent neutralizing antibodies. Mouse monoclonal

antibodies have also been described to be able to neutralize dengue and other flaviviruses and by far the most common means by which neutralizing antibodies effect this function is by blocking attachment of virus to target cells.

He and coworkers[27] have shown that this is also true for neutralizing antibodies generated in the normal human immune response against dengue virus infection. This is the reason why almost invariably neutralizing antibodies will cause ADE in Fc receptor bearing cells when diluted to subneutralizing levels. This is also the 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 level of neutralizing antibodies will be at the highest potency.

Most neutralizing antibodies have specificity for E and most are in fact directed against conformational epitopes. Rey *et al*[19] have summarised much information on neutralizing epitopes of flaviviruses and have located these on their crystal structure of TBE E protein. Some of these epitopes are abolished by the conformational transitions which take place in low pH and are postulated to be important in the fusion event necessary for infection to be established. Thus it is evident that the blocking of virus attachment to the target cell is not the only mechanism of neutralization of the flaviviruses. Prevention of pH dependent fusion as an alternative mechanism of neutralization for flaviviruses was predicted by Gollins and Porterfield[28] in 1986 in their studies of neutralization of another flavivirus West Nile virus (WNV). Two types of antibody mediated interactions can be envisaged to achieve this. In the first and most direct, antibody can be directed against the actual determinant which effects fusion between the virus and the endosomal membrane. This requires some engineering as the fusion epitope is not normally expected to be exposed and is effectively a neo-epitope in the mature virion. Another approach is to generate antibodies directed against epitopes formed by the dimerized E in such a way as to lock E into the dimer form, thus making the virus incapable of exiting the endosomal compartment, since it is thought that the trimer formation is critical for exposure of the fusion moiety[21]. This has been shown in nature to be an effective way to reduce virus infectivity. When virions are released from an infected cell, prM on the virion surface is cleaved to M. If this cleavage is prevented, the immature virions containing prM and E have been shown to have markedly reduced infectivity probably because the whole prM molecule serves to stabilize the E homodimer[29,30], thus preventing premature exposure of the fusion moiety on the virus envelope.

In our laboratory, we have generated mouse monoclonal antibodies against DEN2 virus which were selected by their ability to bind to E in

a sandwich ELISA. Only 2 of 8 such antibodies were able to neutralize DEN2. All the non-neutralizing antibodies and one of the two neutralizing antibodies were able to enhance dengue infectivity in P388D1 cells. The other neutralizing monoclonal antibody, which we have named MAS1, does not exhibit ADE even at subneutralizing dilutions (unpublished data). It recognises a discontinuous epitope and represents an example of an antibody which exhibits neutralization of dengue in the manner predicted by Gollins and Porterfield[28]. Further elucidation of the determinant recognised by MAS1 and the mechanism by which it effects neutralization will go a long way towards learning how to make antibodies which neutralize dengue virus without the danger of ADE. One approach we are using to recreate this conformational epitope is to use random peptide display to fish out peptides which bind to MAS1 and thus mimic the neutralizing epitope of interest.

The nonstructural proteins

When Schlesinger and coworkers first showed that immunization by NS1 could protect monkeys against YF infection[31] and mice against DEN2 infection[32] this major flavivirus antigen was considered to be an alternative immunogen in dengue vaccines, since antibodies directed to NS1 will not effect ADE. The mechanism by which protection occurs is thought to be by destruction of infected cells by antibody mediated cellular cytotoxicity or by complement activation. NS1 is highly immunogenic and exists naturally as a dimer[33]. Western blotting studies in our laboratory on antibodies generated in patients with a natural dengue infection show that the convalescent sera of most patients have very high titre antibodies to the dimeric form of NS1 compared to the monomeric form and we postulate that the integrity of the molecule in its oligomeric state is important for the success of vaccine candidates utilising NS1.

The other major nonstructural proteins NS3 and NS5 do elicit a humoral response in dengue patients, but the magnitude of this response is marginal compared to that against NS1. NS3 has been shown to have a major role as a source of flavivirus T cell determinants generating both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes[34–36]. NS1-NS2A[37] also contains T cell determinants as do the structural proteins.

The way forward

In the past decade, dengue and other flavivirus proteins have been intensively studied as recombinant fusion proteins in *Escherichia coli*[38,39]

or as products of recombinant baculoviruses[40–42] or vaccinia viruses[43,44]. The review by Venugopal and Gould[45] gives a very comprehensive overview of the achievements of recombinant DNA technology in the progress towards new vaccines for flaviviruses. Here, it remains necessary only to summarise some of the important lessons learned about what kind of expression products are likely to be useful in eliciting a strong immune response against dengue virus.

There is no doubt that E is important. Since it is widely accepted that the most important neutralizing epitopes are discontinuous, correct folding and post translational modification of the polypeptide is critical. This means that probably the best choices would be a recombinant virus such as vaccinia or adenovirus. Good immunogenicity is achieved when structural proteins are expressed as subviral particles and this can be achieved in a baculovirus system as well. For flaviviruses, it has been shown that co-expression of the genes coding for prM and E can generate subviral particles[21,41] and these studies offer insight into how the structural proteins are organised on the virion surface.

The choices abound. Yet, in any approach which involves E, it is necessary to consider how to avoid the caveat of ADE. One approach is to isolate or separate determinants. This is difficult because of the marked conformation dependence of most of the important epitopes. Furthermore, what epitopes on E would one want to use? Nearly all 'protective' epitopes elicit neutralizing antibodies which also cause ADE, making a protective response potentially a 'potentiating response' at a different point in time. There is no doubt that one of the pressing research questions in the quest for a dengue vaccine has to be the mechanisms of dengue virus neutralization. A better understanding of the basis of virus inactivation by antibody will lead to the engineering of minigenes which can be used to elicit protective responses without risk.

Protective responses associated with a humoral response to the nonstructural protein NS1 need also to be better understood. NS1 still remains a good candidate for a subunit approach to a vaccine. However, because of the very narrow specificities of antibodies directed against NS1 dimer, it is necessary to take a tetravalent approach and design formulations which include NS1 dimer from all four serotypes to ensure that antibodies are elicited against all serotypes. It is appropriate also to remember in this regard that, in the broader understanding of the immunopathogenesis of DHF, immune potentiation may not be limited to immune responses directed against E.

The avoidance of ADE is also one reason why, in the development of whole virus vaccines, it is necessary to produce a tetravalent formulation. It is thought that immunization with any one dengue serotype will lead to an ADE risk free protection against challenge with the homologous virus. The use of tetravalent formulations of attenuated viruses may run

into problems of the emergence of revertants to virulent forms or to domination of one or another serotype due to the well known phenomenon of viral interference. Some of these issues are being addressed very innovatively by the creation of infectious cDNA clones[46,47] with the intention of designing chimeric constructs containing the genes of one dengue virus in a background of another. Another approach has been to renew attempts to develop inactivated whole virus vaccines as exemplified by the work of Putnak and coworkers[48,49].

The detailed studies of the cell mediated immune response and the role of cytokines in DHF which are being published by Ichiro Kurane and Francis Ennis will also teach us many lessons about how to harness the T cell response to advantage in the development of dengue vaccines which may combine both B cell and T cell responses. At the very least, their studies on T cell and cytokine dependent immunopathogenesis will guide efforts to produce safe and effective vaccines.

An exciting development in the design of dengue vaccines has been the demonstration that a DNA vaccine for dengue is possible. Kochel *et al* inoculated plasmid constructs containing the gene for DEN2 E into mice and were able to report that neutralizing antibodies were elicited[50]. It seems appropriate to conclude here that the field of dengue vaccines offers both challenge and promise and that in order to make a leap forward, much more effort has to be invested in understanding the processes of pathogenesis, of protection and of recovery from infection.

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