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The Development of Recombinant Subunit Envelope-Based Vaccines to Protect Against Dengue Virus Induced Disease

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Abstract

Challenges associated with the interference observed between the dengue virus components within early tetravalent live-attenuated vaccines led many groups to explore the development of recombinant subunit based vaccines. Initial efforts in the field were hampered by low yields and/or improper folding, but the use of the *Drosophila* S2 cell expression system provided a mechanism to overcome these limitations. The truncated dengue envelope proteins (DEN-80E) for all four dengue virus types are expressed in the S2 system at high levels and have been shown to maintain native-like conformation. The DEN-80E proteins are potent immunogens when formulated with a variety of adjuvants, inducing high titer virus neutralizing antibody responses and demonstrating protection in both mouse and non-human primate models. Tetravalent vaccine formulations have shown no evidence of immune interference between the four DEN-80E antigens in preclinical models. Based on the promising preclinical data, the recombinant DEN-80E proteins have now advanced into clinical studies. An overview of the relevant preclinical data for these recombinant proteins is presented in this review.

Introduction

Dengue is the most important vector-borne viral disease in terms of morbidity and mortality with an estimated 2.5 billion people throughout the tropics and subtropics at risk of infection. An estimated 50 million infections with dengue occur worldwide annually, with approximately 2.1 million severe cases, 500,000 cases of dengue hemorrhagic fever (DHF), and 20,000 deaths [1–4]. Disease caused by dengue virus infection ranges from asymptomatic to severe life-threatening disease generally referred to as DHF and dengue shock syndrome (DSS).

Dengue is caused by any one of the four dengue viruses (Family *Flaviviridae*). While infection with one type of dengue virus is widely believed to provide life-long immunity against that virus type, it does not provide long-term protection against infection from the other dengue virus types. Furthermore, while the more severe forms of the disease occur relatively infrequently (< 5%), the vast majority of DHF cases occur following secondary infection [1, 4, 5]. Although there are several hypotheses to explain the association between DHF and secondary infection, nearly all postulate that the severe disease manifestation includes an immunopathogenic component [6–10]. These hypotheses have led to the

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common theme underlying all dengue vaccine development: vaccines must be tetravalent, and must induce potent balanced immunity to all four viruses. This approach to vaccine development is widely believed to be the best way to limit both dengue virus-induced disease and to mitigate the risk of exacerbated disease.

Up until the late 1980s work on dengue vaccines focused exclusively on live attenuated viruses produced using classic methods (e.g. passage through foreign substrates such as primary dog kidney cells) [11,12]. The traditional approach of a whole killed vaccine was not pursued until recently, mainly because of the generally low productivity of dengue virus in cell culture. As live attenuated viruses advanced from monovalent to tetravalent trials, the interaction/interference between the viruses emerged as an issue [13]. The concept of interference has continued to plague the development of live attenuated dengue vaccines [13–15]. More recently, the use of molecular biology methods has allowed for the development of attenuated viruses based on defined mutations conferring attenuation (e.g. chimerization with other flaviviruses, deletion of portions of the genomes, etc.). The use of these molecularly defined viral vaccine strains and use of extended dosing schedules has helped reduce the impact of vaccine virus interference (see other articles in this Special Issue).

The application of molecular biological tools to dengue vaccine development also led to the pre-clinical development of a number of alternative vaccine approaches including recombinant subunit vaccines, viral vectored vaccines (adenovirus, pox, alphavirus or measles) and DNA delivery [16–22]. All of the vaccine approaches have their own associated advantages and disadvantages in terms of safety, immunogenicity, and general feasibility in terms of the ability to develop into a commercial product. Viral vectored vaccines and DNA vaccines will be the topics of other articles in this Special Issue and will not be considered here. Recombinant subunit vaccine approaches offer one of the safest alternatives, a means to avoid the issue of viral interference, and the ability to administer a tetravalent formulation on an accelerated schedule. One advantage of an accelerated schedule is that full protective immunity would be induced more quickly, thus avoiding the potential of exacerbated disease due to partial immunity during an extended immunization course. Other advantages of an accelerated schedule include better general compliance, more suitability for travelers and military personnel, easier integration into existing immunization schedules, and the potential for use in an outbreak setting. A balanced tetravalent immune response may also be more easily achieved through simple adjustments of dose for each of the four recombinant proteins, compared to live virus vaccines where the interactions between viruses can be complex and unpredictable. For these reasons several groups launched efforts to develop recombinant subunit based dengue vaccines as described below.

The dengue envelope (E) glycoprotein has been the focus of subunit-vaccine development as it is the primary target of neutralizing antibodies [23–26]. In an effort to express recombinant E proteins several different expression systems have been utilized, including *Escherichia coli*, baculovirus vectors, yeast, mammalian cells, insect cells and more recently plant based expression systems (recently reviewed in ref. 27 and 28 and summarized in Table 1). Proper folding of the envelope protein is required to preserve the integrity of its neutralizing epitopes and this generally requires the coexpression of the premembrane (prM) protein along with E [29]. The prM protein acts as a chaperone for E facilitating proper folding and trafficking through the secretory pathway [18, 30]. Successful expression also requires that the expression system is capable of the proper processing of the prM and E proteins including disulfide bond formation, proteolytic cleavage and glycosylation.

Due to the inability of bacteria to efficiently process membrane-associated glycoproteins, much of the work in *E.coli* has focused on the expression of subregions of E typically fused

to other proteins. Epitope mapping of E and NS1 was done using *E. coli* expressed TrpE-Dengue fusion proteins [31, 32]. The *E. coli* system has also been used to produce vaccine candidates consisting of sub domains of DENV2 E fused with the meningococcal P64K protein, the Staphylococcal A protein, or the *E. coli* Maltose Binding Protein (MBP) [33–35]. These subdomain based vaccines are the subject of another article in this Special Issue.

The baculovirus expression system has been most widely utilized for the expression of E alone or co-expressed with prM [30, 36–40]. The construction of C-terminal truncations of E, which removes the membrane anchor sequence, was demonstrated to improve its secretion, facilitate purification and improve its immunogenicity [37, 38, 41].

Coexpression of prM and E can induce the formation of virus-like particles (VLP's). VLP's are expected to be more antigenically similar to dengue virions since they contain glycosylated prM and E in association with a lipid membrane. Dengue VLPs have been expressed from baculovirus, yeast, mammalian cells and insect cells [39 42–46]. Although VLP's are recognized by monoclonal antibodies specific for different domains of E and can induce neutralizing antibodies in mice and non human primates [44], the responses are only weak to moderate. Low production yields have also hindered their commercial application.

While production challenges have slowed progress in the field, it is our view that recombinant subunits provide a promising approach to the development of a tetravalent dengue vaccine. In order for this approach to be successful, it is imperative that an expression system is used that can produce recombinant E proteins that maintain relevant native-like characteristics with yields high enough to support commercial production. The focus of this review is on the C-terminally truncated E proteins produced utilizing the *Drosophila* S2 cell expression system. The S2 system has been demonstrated to produce high levels of high quality dengue E antigens that are suitable as vaccine candidates.

Cloning and Expression of Recombinant Envelope Proteins

Initial efforts by Hawaii Biotech Inc. (HBI) scientists to express the recombinant dengue E proteins focused on yeast expression (*Saccharomyces cerevisiae* and *Pichia pastoris*) [47]. Using the C-terminal truncations first identified by Men et al., [41] soluble forms of the E ectodomain were expressed in the yeast expression systems. While high levels of E domain III (EDIII, amino acids 296–395) were expressed in yeast systems and showed modest immunogenicity in mice, the levels of expression of larger E subunits including various C-terminal truncations (e.g. 60% truncated at amino acid 295, 80% truncated at amino acid 395 (80E), and 90% truncated at amino acid 445) were much lower (10–100 fold). Furthermore efforts to produce full-length E co-translationally with prM in an effort to generate VLP's were even less successful.

Based on the low expression levels and modest immunogenicity of the yeast-expressed E proteins, alternative expression systems were explored including evaluation of mammalian expression systems (e.g. Chinese hamster ovary cells with dihydrofolate reductase (DHFR) amplification) and insect cell expression systems. The breakthrough in expression levels came with the evaluation of the *Drosophila melanogaster* S2 cell expression system originally developed by SmithKline Beecham [48–50] and licensed to Invitrogen (Carlsbad, CA). The system utilizes Schneider 2 (S2) cells that are derived from *Drosophila melanogaster* embryos [51]. This expression system is based on the generation of stably transformed cell lines that express the protein of interest. Targeting the proteins for secretion helps to assure relevant post-translational modifications are efficiently incorporated and facilitates purification. The use of this system has been shown to express heterologous proteins that maintain native-like biological structure and function [52, 53].

Using the *Drosophila* S2 cell system, HBI was able to overcome the limitations in expression levels that had challenged the field for years. Focusing on expression of a C-terminally truncated version of E (80E), the cloning of the relevant dengue genes from all four dengue virus types into the S2 cell system led to unprecedented levels of expression and protein quality. Details on the expression constructs have been described [47, 54, 55]. Briefly, dengue sequences encoding the full-length prM protein and 80% of the E protein (80E, truncation at amino acid 395 for DEN1, DEN2 and DEN4, 393 for DEN3) were inserted into the pMttΔXho vector (derived from pMttPA [49]). The 80E truncation removes carboxy-terminal stem region and trans-membrane domain. The dengue gene sequences were derived from the following stains: DENV1 strain 258848, DENV2 strain PR159 S1, DENV3 strain CH53489, and DENV4 strain H241. The expression of the prM-80E sequences in this manner produces a prM-80E polypeptide that is processed by the cells and results in the 80E being secreted into the culture medium. Transformation of *Drosophila* S2 cells line results in the integration of the expression cassette into the genome to generate stable cell lines. The recombinant proteins are easily detected in the unconcentrated media by Coomassie blue stained SDS-PAGE gels as shown in Figure 1. While the expression levels vary between the dengue types, all DEN-80E proteins are expressed at levels exceeding 10 mg/L and ranging up to approximately 40 mg/L. The high expression levels are illustrated by the prominent band visible in unconcentrated culture supernatant stained with Coomassie blue (Figure 1). It is important that expression levels in this range, or greater, are achieved in order to be able to support cGMP manufacturing. The secreted recombinant DEN-80E subunits are purified from clarified culture medium by immunoaffinity chromatography using conformationally sensitive monoclonal antibodies [54, 55]. The transformed S2 cells are easily adapted to serum free suspension growth which is critical for vaccine manufacture.

The native-like conformation of DEN2-80E and DEN3-80E were subsequently established based on reactivity with conformationally sensitive monoclonal antibodies and the crystal structure determination of these recombinant proteins [56–58]. The DEN-80E structure is very similar to that previously determined for the virally-derived tick-borne encephalitis E ectodomain [59]. The recombinant DEN-80E proteins are glycosylated at both of the N-linked sites encoded by the E protein.

Formulation Evaluations

Soluble recombinant proteins are typically not very immunogenic without an adjuvant [60], so a significant effort was invested to evaluate various candidate vaccine formulations for their ability to induce robust immune responses while still maintaining a safety profile that could be acceptable for humans. Until recently, aluminum salt based adjuvants were the only adjuvants approved for human use. However, over the last 5–10 years a number of vaccine formulations containing novel adjuvants have been approved for human use in various countries around the world. These include adjuvants based on oil-in-water emulsions (e.g. MF59 and AS03 from Novartis and GlaxoSmithKline [GSK] respectively) and adjuvants based on toll-like receptor agonists (e.g. Monophosphoryl lipid A – MPL from GSK) [61]. A number of additional products containing other novel adjuvants are currently undergoing early and late stage clinical development (e.g. ISCOMATRIX™ adjuvant from CSL and AS01 and AS02 from GSK) and may be licensed for human use in the coming years [62, 63].

The combination of the recombinant DEN-80E antigens with these modern adjuvants may offer advantages in terms of the magnitude, breadth, and durability of the immune response induced compared to classic aluminum salt-based formulations. To that end several potent modern adjuvants, in addition to aluminum salt-based adjuvants, were evaluated in

combination with the recombinant DEN-80E antigens in mice and non-human primate studies. Through these studies several formulations were identified which showed potent immunogenicity and efficacy and which can be considered for clinical development. A summary of some of the key studies and their findings is presented below.

Immunogenicity of the Recombinant DEN-80E Subunits in Mice

Immunogenicity of the recombinant DEN-80E antigens was evaluated in inbred and outbred mice with a variety of adjuvant formulations. In an early study utilizing DEN2-80E, the variability of the antibody responses induced by various formulations was noted, Table 2 [55]. In these studies the highest virus neutralizing titers were obtained with formulations containing ISCOMATRIX™ adjuvant or MF75 with threonyl-MDP. A formulation containing Ribi700 adjuvant induced an intermediate virus neutralizing response, while formulations containing aluminum hydroxide, MF59 or Freund's adjuvant induced lower virus neutralizing titers. In comparing the relative ability of the adjuvants to induce virus neutralizing titers when formulated with DEN2-80E, it should be noted that responses following two doses of ISCOMATRIX™ adjuvant administered with 10 µg of antigen were compared with the titers induced by three doses of the other adjuvants formulated with 12.5 or 25 µg of antigen. Despite the lower doses of antigen and fewer immunizations, ISCOMATRIX™ adjuvant formulated with DEN2-80E produced the most potent virus neutralizing antibodies, with a PRNT₈₀ titer of 4000.

Evaluation of each of the four recombinant DEN-80E proteins was conducted following formulation with ISCOMATRIX™ adjuvant. Various doses of each DEN-80E were administered to determine the effective immunizing dose for each recombinant protein. All four of the DEN-80E proteins induced a potent virus neutralizing antibody response which reached maximum neutralizing antibody titers (reciprocal PRNT₅₀ titers ranging from 526 to 10,556) at protein doses ranging from 3 to 10 µg of each DEN-80E, Table 3 [55]. While the virus neutralizing titers are not identical for each DEN-80E, it is clear that there is a dose response and that adjustment of the protein dose will impact the virus neutralizing antibody titers induced.

The durability of the immune response in mice was examined with DEN2-80E formulated with selected adjuvants and the virus neutralizing antibody responses were assessed at monthly intervals for 6 months as shown in Table 4 [55]. As with the study comparing various formulations described above, mice were immunized with only 2 doses of vaccine formulated with ISCOMATRIX™ adjuvant at a 4 week interval compared to 3 doses of vaccine containing the other adjuvants (MF75 or Ribi) administered at a 3 week interval. High neutralizing titers of several thousand were elicited following immunization with the all of the formulations tested and the virus neutralizing antibody titers remained at levels of greater than 1000 through 6 months for all but one of the formulations (906 for Ribi at 6 months). At 6 months the mice were given a booster dose to test for a memory immune response for two of the formulations. Virus neutralizing antibody titers were increased for both formulations when tested one month following the 6 month booster immunization [55]. These results demonstrate that DEN2-80E formulated with various adjuvants generated high virus neutralizing antibody responses that persisted for at least 6 months in mice and that a subsequent booster resulted in an anamnestic response indicating that good memory component has been induced.

In order to assess potential immune interference between the four recombinant proteins, the DEN-80E proteins were mixed together in a tetravalent formulation. A mixture consisting of 10 µg of each of the four DEN-80E subunits formulated with ISCOMATRIX™ adjuvant was used to immunize mice. The tetravalent results were compared against results generated

from immunization with each individual DEN-80E protein. As shown in Table 5, mice immunized with the tetravalent formulation developed virus neutralizing antibody titers against each dengue virus type similar to the titer obtained in mice immunized with a single DEN-80E type suggesting that immune interference would not be a major issue with this tetravalent recombinant subunit dengue vaccine [55].

Protection of Mice from Viral Challenge

The protective efficacy of the DEN2-80E formulated with ISCOMATRIX™ adjuvant was assessed in a mouse challenge model. Groups of weanling mice were immunized with formulations consisting of 1 and 5 µg doses of DEN2-80E and 2 µg of ISCOMATRIX™ adjuvant and then challenged intracranially with 100 LD₅₀ of mouse-adapted DENV-2 (Figure 2). Complete protection from morbidity and mortality was observed with both doses of recombinant antigen, while 7 of the 10 unimmunized control mice succumbed to viral challenge. These results demonstrate the protective potential of the recombinant DEN-80E antigens [55].

Immunogenicity and Protective Efficacy of the Recombinant DEN-80E Proteins in Non-Human Primates

In a series of immunogenicity and efficacy studies conducted in non-human primates, the ability of monovalent and tetravalent formulations to induce virus neutralizing antibody and protect from viral challenge was evaluated. The rhesus monkey model is not a disease model since the monkeys do not develop dengue-like illness. However, the monkeys do develop a measurable viremia and prevention of viremia is generally taken as an indicator of protective efficacy. Since viremia levels in humans generally correlate with disease severity [5, 64], and the level of viremia developed in monkeys is generally lower than that typically seen in humans who develop disease (e.g. Putnak et al 2005, [54]), it is reasonable to assume that the ability to prevent significant viremia in monkeys will indicate an ability to prevent viremia and therefore disease in humans.

The studies conducted included both monovalent and tetravalent formulations and included the evaluation of several different adjuvants. In one study [54], DEN2-80E was evaluated in formulations containing adjuvants developed by GSK and referred to as the AS series of adjuvants. The adjuvants evaluated in this study included aluminum hydroxide alone (Alum), AS04 (MPL + either of two different aluminum salts – AS04-OH aluminum hydroxide and AS04-PO aluminum phosphate), AS05 (MPL + aluminum hydroxide + the saponin QS21), and AS08 (aluminum hydroxide + QS21). In two other studies formulations with ISCOMATRIX™ adjuvant were evaluated [55]. One focused on DEN2-80E alone and the other focused on tetravalent formulations containing all four DEN-80E proteins plus non-structural protein 1 (NS1) from dengue virus type 2 (DEN2-NS1). The results of each study are summarized in the following paragraphs and demonstrate the potent immunogenicity and protective efficacy of the recombinant DEN-80E proteins formulated with various adjuvants when tested in non-human primates.

Immunogenicity and Efficacy of DEN2-80E formulated with AS adjuvants in rhesus macaques

In this study the efficacy of purified DEN2-80E with several of the GSK (AS) adjuvants were evaluated. Groups of three animals were immunized with two doses of each vaccine formulation or saline placebo on day 0 and day 90. The primary immunization was administered by the subcutaneous route and dose 2 was administered by the intramuscular route. Measureable virus neutralizing antibodies were detected post dose 2 in all animals receiving the DEN2-80E regardless of vaccine formulation or antigen dose, Table 6. The

highest geometric mean titer (GMT) of neutralizing antibody after the second dose was observed in the group that received 5 µg of DEN2-80E in AS08 (GMT=7,723), followed by groups that received 5 µg of DEN2-80E in AS05 (GMT=4,660), 1 µg of DEN2-80E in AS05 (GMT=3,222), and 20 µg of DEN2-80E in AS04-OH (GMT=2,671). For recipients of the DEN2-80E formulations, the GMT of neutralizing antibody peaked following dose 2 and then declined at various rates, but all animals still had measurable antibody at 2 months following dose 2 and prior to the virus challenge, Table 6.

The animals were challenged subcutaneously with approximately 10^4 plaque forming units (pfu) of the near wild-type strain of DENV2 S16803 at two months post dose 2. The challenge strain is heterologous relative to the vaccine formulation (PR159 S1 strain) and therefore represents a stringent challenge. The level of viremia was determined for 12 consecutive days after challenge. Viremia was measured using virus isolation on C6/36 mosquito cells (to screen for positive samples) followed by direct plaque assay on Vero cells to determine actual virus titer in the serum (Table 6). All three saline control animals developed viremia starting 4 to 5 days after challenge and lasted through day 8. In contrast, several formulations including the 20 µg dose of DEN2-80E + AS04, and both the 1 and 5 µg dose of DEN2-80E + AS05 had no viremia detectable by plaque assay. In all cases there was an anamnestic antibody response to challenge consistent with limited viral replication and strong memory. High virus neutralizing antibody titers (e.g. >200) were generally associated with protection from viremia as measured by plaque assay, but interestingly a few animals developed detectable viremia despite the presence of high titer virus neutralizing antibody (e.g animal GTF). This highlights the complexity of assessing protection for dengue and a limitation of the model with no disease endpoint. Non-replicating vaccine formulations, and in particular formulations containing aluminum salts, are generally administered by the intramuscular route so the potential impact of administration of the first vaccine dose in this study by the subcutaneous route is not known. Therefore, it is possible that responses were less than ideal under these conditions and the potential for even higher antibody titers and efficacy with these aluminum salt-based formulations may have been seen with intramuscular vaccination for both doses.

Immunogenicity and Efficacy of DEN2-80E formulated with ISCOMATRIX™ adjuvant in rhesus macaques

In this study the ability of the DEN2-80E formulated with ISCOMATRIX™ adjuvant to protect monkeys from virus challenge was tested. Animals were immunized with different doses of purified DEN2-80E antigen formulated with ISCOMATRIX™ adjuvant or aluminum hydroxide adjuvant. The animals were immunized by the subcutaneous route at days 0, 34 and 97 and then challenged subcutaneously with a near wild-type DENV-2 (strain S16803) approximately one month after the last dose (challenge strain heterologous to vaccine strain). Virus neutralizing antibodies measured over the course of the immunization schedule and following virus challenge are presented in Table 7. All DEN2-80E formulations elicited virus neutralizing antibodies by two weeks post dose 2. Peak titers post dose 3 were in the range of 660 to >1280 and do not appear to be dose dependent.

The protective efficacy results of the vaccine formulations as determined by the level of viremia are shown in Table 8. The unimmunized controls developed detectable viremia for an average of 9 days. In contrast, all the animals vaccinated with the DEN2-80E formulations exhibited some degree of reduction in viremia levels which in all cases was statistically significant compared to the control group ($p < 0.0001$, 0.0219, and 0.0496, for the low, mid, and high dose groups respectively). Interestingly, there was no detectable viremia in animals that received the lowest dose (5 µg) of DEN2-80E, while animals immunized with higher doses of vaccine of 25 µg or 100 µg showed some breakthrough viremia. Since the numbers of animals are small it is not clear if this represents a

reproducible result or not. However, follow on experiments conducted in mice suggested that lower antigen doses administered in a formulation containing ISCOMATRIX™ adjuvant tended to have a more Th1-type profile compared to higher antigen doses (Coller et al., unpublished data) which could be consistent with better protective efficacy. As with the previous study, vaccinated animals exhibited strong anamnestic responses following challenge. In this study the subcutaneous route was used for all immunizations. Based on published data [65] it appears that the intramuscular route is the preferred route for clinical testing of vaccine formulations containing ISCOMATRIX™ adjuvant, so like the previous study where aluminum-salt based formulations were administered partly by the subcutaneous route, this study may represent a less than ideal test of the capabilities of the formulation.

Immunogenicity and Protective Efficacy of Tetravalent Formulations in Monkeys

In a small pilot study the safety and immunogenicity of a tetravalent DEN-80E vaccine formulation was assessed. In this study two groups with two monkeys each were immunized with different doses (1 µg or 5 µg each) of tetravalent DEN-80E formulated with ISCOMATRIX™ adjuvant. Both tetravalent formulations included DEN2-NS1. Animals were vaccinated by the intramuscular route at 0, 1, 2, and 3 months. Virus neutralizing antibodies measured over the course of the immunization schedule are presented in Table 9. All vaccinated animals developed virus neutralizing antibodies to each of the four dengue types, although the antibody titers varied among animals.

To assess the protective efficacy of the tetravalent DEN-80E vaccine formulation the vaccinated animals and unvaccinated controls were challenged with DENV2 or DENV-4 (one animal each from each group) five months after the last dose. The challenge results are presented in Table 10. The DENV2 control animal developed viremia which lasted for four days and the DENV4 control animal developed viremia lasting for five days. In contrast, 3 out of 4 immunized animals did not have any detectable viremia as measured by cell culture. One of the animals (T206) that received the 5 µg dose of the tetravalent formulation did exhibit some breakthrough viremia. This animal had an anti-DENV-4 virus neutralizing titer of 14 one month before and 17 at the time of DENV-4 challenge consistent with the trend noted in a previous study that higher virus neutralizing antibody titers are generally associated with protection [54]. These data demonstrated that properly formulated tetravalent vaccines based on recombinant DEN-80E subunit proteins can induce virus neutralizing antibodies and protective immunity against more than one dengue virus type.

Summary and Conclusions

Challenges associated with interference between component dengue viruses within early tetravalent live-attenuated vaccines led researchers at Hawaii Biotech to target development of a recombinant subunit envelope-based vaccine. Though initial efforts in the field at expressing dengue envelope subunits were stymied by low yields and/or improper folding, the use of the *Drosophila* S2 cell expression system provided the means to overcome these limitations. The DEN-80E recombinant subunit proteins for all four dengue virus types are expressed at high levels and have been shown to maintain native-like conformation. When formulated with a variety of adjuvants the antigens are potent immunogens and induce high titer virus neutralizing antibody responses. Furthermore, the antigens have been shown to protect against viral challenge in both mouse and non-human primate models. Tetravalent vaccine formulations have also been evaluated in preclinical models with no evidence of immune interference or competition between the four DEN-80E antigens being observed. These proof of concept preclinical studies led to the advancement of a monovalent DEN1-80E vaccine candidate into clinical testing. The phase 1 trial was a double-blind, placebo-controlled safety study conducted in 16 healthy adult, flavivirus-naïve individuals.

Two dose levels of DEN1-80E (10 and 50 µg) both adjuvanted with aluminum hydroxide were evaluated. Three intramuscular injections of each vaccine dose level were administered to subjects at 4 week intervals. The study is now complete and data unblinding and publication is expected in the near future. Preparations for clinical testing of a tetravalent formulation are now ongoing following the transfer of the HBI program to Merck.

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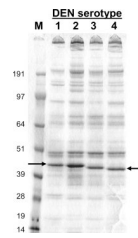


Figure 1. SDS-PAGE Analysis of Secreted Recombinant DEN-80E Products. Ten μ l of unconcentrated culture medium from induced S2 cell cultures was separated on a 4–12% SDS-PAGE gel under non-reducing conditions and the proteins stained with Coomassie blue. Size of molecular weight markers in kilodaltons is indicated on the left. Position of DEN-80E bands are indicated by arrows.

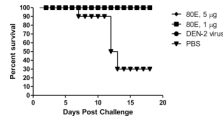


Figure 2. Survival of Mice Immunized with two different doses of DEN2-80E formulated with ISCOMATRIX™ adjuvant and Challenged with Live DEN2 Virus.

Table 1

Expression platforms used for recombinant dengue protein expression

	Strengths	Weaknesses	References
<i>Escherichia coli</i>	<ul style="list-style-type: none"> - Easy to produce - High level expression* of E domain III - Low cost of goods 	<ul style="list-style-type: none"> - Lack of post-translational modifications – not able to support proper folding of E - Low level expression# of full-length or truncated E 	31–35
<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> - Easy to produce - High level expression of E domain III - Low cost of goods 	<ul style="list-style-type: none"> - Hyperglycosylation - Lack of native conformation for E → low immunogenicity - Low level expression of full-length or truncated E 	47
<i>Pichia pastoris</i>	<ul style="list-style-type: none"> - Easy to produce - High level expression of E domain III - Low cost of goods 	<ul style="list-style-type: none"> - Lack of native conformation for E → low immunogenicity - Low level expression of full-length or truncated E 	42, 43, Clements et al., unpublished data
Chinese Hamster Ovary DHFR system	<ul style="list-style-type: none"> - Capable of producing proteins with native conformation 	<ul style="list-style-type: none"> - Expression levels very low - Moderate cost of goods 	Coller et al. unpublished data
Vaccinia expression in mammalian cells	<ul style="list-style-type: none"> - Capable of producing proteins with native conformation - Immunogenicity demonstrated in mice 	<ul style="list-style-type: none"> - Expression levels low - Not a commercially viable production system (safety and expression) 	41
Baculovirus in Sf9 or High Five cells	<ul style="list-style-type: none"> - Capable of producing proteins with native conformation - Immunogenicity demonstrated in mice 	<ul style="list-style-type: none"> - Expression levels moderate@ - Moderate cost of goods 	30, 36–40
Stably transformed <i>Drosophila</i> S2 cells	<ul style="list-style-type: none"> - Produces proteins with native conformation - High expression levels for truncated E (> 10 mg/L) - Immunogenicity and efficacy in primates 	<ul style="list-style-type: none"> - Expression platform not yet registered for a commercial product - Moderate cost of goods 	54, 55

* High expression level > 10 mg/L

@ Moderate expression level > 1 mg/L < 10 mg/L

Low expression level < 1 mg/L

Table 2

Virus Neutralizing Responses in Mice Immunized with DEN2-80E Formulated with Various Adjuvants

Antigen	Adjuvant	Geometric Mean PRNT ₈₀ Titer
DEN2-80E	MF59	26
DEN2-80E	MF75+ThrMDP	2297
DEN2-80E	Ribi	333
DEN2-80E	Alum	24
DEN2-80E	Freund's	<10
DEN2-80E	ISCOMATRIX™ Adjuvant	4000
DEN2-80E	no Adjuvant	10
PBS	no Adjuvant	<10

Table 3

Virus Neutralizing Antibody Titers in Mice Immunized with Various Doses of Recombinant DEN-80E Proteins Formulated with ISCOMATRIX™ Adjuvant

DEN-80E Antigen Dose	Geometric Mean PRNT ₅₀ Titer			
	DENV1	DENV2	DENV3	DENV4
30 µg	NT*	NT	1741	728
10 µg	1450	10,556	1550	526
3 µg	1053	8574	1908	278
1 µg	504	4595	1414	144
0.3 µg	35	2096	400	28
0.1 µg	<20	590	NT	NT
PBS	<10	<10	<10	<10

NT - Not tested

Table 4
Duration of Immunity in Mice Immunized with DEN2-80E Formulated with Various Adjuvants

Vaccine Formulation	Geometric Mean PRNT ₈₀ Titer							Post-Boost
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6		
10 µg DEN2-80E ISCOMATRIX™ adjuvant *	6500	6062	5278	3703	4924	3732	12,996	
2.5 µg DEN2-80E Rib ⁱ #	3177	4816	3249	2276	1784	906	6858	
2.5 µg DEN2-80E MF75#	6063	6063	4756	2000	1207	2000	NT	
2.5 µg DEN2-80E MF75+ThrMDP#	8000	5278	3031	3031	1320	3482	NT	

NT – not tested, animals did not receive booster dose of vaccine.

* Animals were immunized with 2 doses of vaccine administered at a 4 week interval.

Animals were immunized with 3 doses of vaccine at a 3 week interval. Dose 1 included 25 µg of DEN2-80E while the booster doses included 12.5 µg of DEN2-80E.

Table 5

Homologous Virus Neutralizing Antibody Titers in Mice Immunized with Monovalent or Tetravalent DEN-80E Proteins Formulated with ISCOMATRIX™ Adjuvant

Immunogen	Geometric Mean PRNT ₅₀ Titer			
	DENV1	DENV2	DENV3	DENV4
Monovalent*	2759	3031	381	174
Tetravalent ⁺	1589	2639	564	159
PBS	<20	<20	<20	<20

* 4 groups of mice were immunized with 10 µg of each of the 4 monovalent 80E subunits. Each group was tested for homologous serotype virus neutralizing antibody titers.

⁺ 1 group of mice was immunized with a tetravalent mixture containing 10 µg of each of the four 80E subunits. The group was tested individually for virus neutralizing antibody titers for each of the dengue serotypes.

Table 6

Immunogenicity and Efficacy of DEN2-80E Formulated with Several AS Adjuvants in Rhesus Macaques

Vaccine Formulation *	Animal ID	PRNT ₅₀ Titer Post Dose 2	PRNT ₅₀ Titer Day of Challenge	Viremia by Cell Culture (pfu/mL serum)
5 µg DEN2-80E + aluminum hydroxide	GTF	660	1,300	3.6
	GPE	680	110	8.2
	FVF	345	60	10.5
5 µg DEN2-80E + AS04-OH	GTP	1100	270	1.7
	GCE	790	100	9.2
	JEJ	570	200	0
20 µg DEN2-80E + AS04- OH	KBV	4200	450	0
	GKF	1900	400	0
	FXX	2400	880	0
5 µg DEN2-80E + AS04-PO	GPH	360	100	1.7
	EKB	250	60	13.4
	GVV	490	250	0
1 µg DEN2-80E + AS05	DBP	1400	1,200	0
	GEV	4500	730	0
	FKT	5300	700	0
5 µg DEN2-80E + AS05	GPT	4700	650	0
	GBA	5700	2,500	0
	EEE	3200	750	0
5 µg DEN2-80E + AS08	JFE	8400	740	1.7
	FGC	9600	5,300	0
	HPH	5800	1,200	0
Saline	JEH	<10	<10	7.6
	GKB	<10	<10	8.7
	GWP	<10	<10	10.10

* Animals were immunized with 2 doses of vaccine administered on day 0 and day 90 by the subcutaneous and intramuscular routes respectively.

Table 7
 Virus Neutralizing Antibody in Sera from Rhesus Monkeys Immunized with Recombinant DEN2-80E ISCOMATRIX™ Adjuvant Formulations

Vaccine Formulation#	Animal ID	Virus Neutralizing Antibody Titers (PRNT ₅₀)											
		Day 0	Day 34	Day 49	Day 69	Day 97	Day 113	Day 132	Day 159	Day 180	Day 230	Day 250	Day 270
100 µg DEN2-80E + ISCOMATRIX™ adjuvant	G617	<10	10	180	460	230	920	400	>12,800				
	B7487	<10	110	480	480	600	>640	>640	>12,800				
25 µg 80E + ISCOMATRIX™ adjuvant	F477	<10	<10	300	230	450	660	470	>12,800				
	I613	<10	90	3000	1000	1100	>1280	>1280	>12,800				
5 µg 80E + ISCOMATRIX™ adjuvant	I619	<10	50	1600	590	1900	>1280	1200	>12,800				
	H7J	<10	10	620	760	530	>1280	>640	>12,800				
100 µg DEN2-80E + Aluminum hydroxide	F485	<10	60	650	1200	250	770	600	>12,800				
	N637	NT*	NT	NT	NT	NT	NT	NT	<10	860			
Placebo	N670	NT	NT	NT	NT	NT	NT	NT	<10	1600			
	N816	NT	NT	NT	NT	NT	NT	NT	<10	990			

* NT - not tested

Animals were immunized with 3 doses of vaccine administered by the subcutaneous route on days 0, 34, and 97.

Table 8
Viremia in Vaccinated Rhesus Monkeys After Challenge with Live DEN2 Virus

ID	Vaccine	Viremia on Day:												
		1	2	3	4	5	6	7	8	9	10	11	12	
G617	100 µg DEN2-80E ISCOMATRIX™ adjuvant	0 ¹	0	0	0	0	0	0	0	0	0	0	0	0
B7487	100 µg DEN2-80E ISCOMATRIX™ adjuvant	0	0	0	0	0	+	+	+	0	0	0	0	0
F477	25 µg DEN2-80E ISCOMATRIX™ adjuvant	0	0	0	+	+	+	+	+	0	0	0	0	0
I613	25 µg DEN2-80E ISCOMATRIX™ adjuvant	0	0	0	0	0	+	0	0	0	0	0	0	0
I619	5 µg DEN2-80E ISCOMATRIX™ adjuvant	0	0	0	0	0	0	0	0	0	0	0	0	0
H71	5 µg DEN2-80E ISCOMATRIX™ adjuvant	0	0	0	0	0	0	0	0	0	0	0	0	0
F485	100 µg DEN2- 80E/Alum	0	0	0	0	0	0	+	+	+	+	0	0	0
517Z	PIV//Alum	0	0	0	0	0	0	0	0	0	0	0	0	0
N637	none	0	+	+	+	+	+	+	+	+	+	0	0	+
N670	none	0	+	+	F ³	+	+	+	+	+	+	+	+	0
N816	none	0	+	+	+	F	+	+	0	+	+	+	0	0

¹ 0 - no virus plaques

² + - > 5 virus plaques

³ F - 1-5 virus plaques

Table 9

Virus Neutralizing Antibody Responses Induced in Rhesus Macaques Following Vaccination with a Tetravalent Recombinant Subunit Vaccine Formulation

Group/ Vaccine*	Animal ID	Dengue Serotype for PRNT Testing	Day 0 (Dose 1)	Day 28 (Dose 2)	Day 67 (Dose 3)	Day 102 (Dose 4)	Day 275 (Challenge)	Day 293	
1 µg each tetravalent DEN-80E + 0.1 µg NS1 + ISCOMATRIX™ Adjuvant	AA37	DENV-1	<10	<10	68	290	510	72	161
		DENV-2	<10	<10	112	194	604	232	135
		DENV-3	<10	<10	29	136	127	33	61
		DENV-4	<10	<10	47	83	300	89	107
	FTH	DENV-1	<10	<10	232	1230	1361	231	711
		DENV-2	<10	<10	105	887	1969	293	548
		DENV-3	<10	<10	<20	231	305	90	747
		DENV-4	<10	<10	39	313	894	105	812
	T206	DENV-1	<10	<10	34	88	193	20	1300
		DENV-2	<10	<10	53	363	602	NA	630
		DENV-3	<10	<10	<10	75	147	136	6845
		DENV-4	<10	<10	<10	32	59	17	5958
AJ14	DENV-1	<10	<10	82	420	326	117	700	
	DENV-2	<10	<10	340	1048	2536	200	9500	
	DENV-3	<10	<10	157	206	201	355	4020	
	DENV-4	<10	<10	92	170	282	237	1889	

NA – no valid result available

* Animals were immunized with 4 doses of vaccine administered at monthly intervals by the intramuscular route.

Table 10

Live Virus Challenge of Monkeys after Tetravalent Immunization.

Group	Animal ID	PRNT ₅₀ for each serotype one month before challenge	Challenge serotype assigned randomly	Viremia (positive days)
1 µg each tetravalent DEN-80E + 0.1 µg NS1 + ISCOMATRIX™ Adjuvant	AA37	DEN1: 63 DEN2: 139 DEN3: 59 DEN4: 61	DEN4	None
	FTH	DEN1: 242 DEN2: 309 DEN3: 64 DEN4: 181	DEN2	None
5 µg each tetravalent DEN-80E + 0.5 µg NS1 + ISCOMATRIX™ Adjuvant	T206	DEN1: 49 DEN2: 39 DEN3: 192 DEN4: 14	DEN4	Days 4, 5, 6, 7
	AJ14	DEN1: 51 DEN2: 530 DEN3: 270 DEN4: 148	DEN2	None
Naive controls	B34Z	<10 for each serotype	DEN4	Days 6, 7, 8, 9, 10
	B08Z	<10 for each serotype	DEN2	Days 7, 8, 9, 10