Evaluation of Dengue Envelope Glycoprotein Domain III as a Subunit Vaccine
Candidate for Prophylaxis of Dengue Hemorrhagic Fever/Shock Syndrome

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Submitted in Partial Fulfillment of the
Requirements for the Degree
Doctor of Philosophy

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University of Rochester
Rochester, NY
2010
Dedication

I dedicate this thesis to my parents, Walter and Rosario Tono, my husband Noah, and my daughter Mila Isabella, for their conditional and unconditional love, support, guidance, and patience.
Curriculum Vitae

Olivia Block was born in Silver Spring, Maryland on July 8, 1981. She attended Saint John Fisher College from 1999 to 2003, and graduated with a Bachelor of Arts degree in Biology. She came to the University of Rochester in June of 2003 as a PREP trainee in the lab of Dr. Barbara Iglewski. She began graduate studies in the Department of Microbiology and Immunology in June of 2004 and joined the laboratory of Dr. Robert C. Rose in the spring of 2005. She received the Master of Science degree from the University of Rochester in 2007.
List of Publications

**Block OK**, Rodrigo WW, Jin X, Rose RC, Schlesinger JJ. A tetravalent recombinant dengue domain III protein vaccine stimulates neutralizing and enhancing antibodies in mice. (Manuscript submitted to Vaccine July 21, 2010).


Acknowledgments

I would like to acknowledge and thank my advisor, Dr. Robert Rose for his guidance throughout my graduate studies. I would also like to thank my committee members, Drs. Frelinger, Zeng, and Kim, the Dengue research group at the University of Rochester, especially Drs. Schlesinger and Jin for their encouragement, suggestions, and discussions. I would also like to thank Dr. Rodrigo and Matt Quinn for their assistance and guidance in virology techniques, and especially Christopher Lane for his patience, encouragement, and helpful discussions. This work was partially funded by the predoctoral training program in Viral Diseases, Vaccines and Biodefense (T32 AI 007169) and by grants from the Pediatric Dengue Vaccine Initiative of the International Vaccine Institute, Award TR 03/04 (J.J.S).
Abstract

Antibody dependent enhancement (ADE) of dengue viral (DENV) infectivity poses a significant challenge to the development of broadly effective vaccines for the prevention of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In this phenomenon, antibodies generated during an initial (primary) infection with one of four circulating DENV serotypes cross-react with, but fail to neutralize, a subsequently infecting serotype. The formation of such immune complexes is thought to enhance DENV infectivity by facilitating entry and subsequent replication of the virus in cells of the immune system that bear antibody Fcγ receptors. To address the need to stimulate a balanced neutralizing antibody response against all four DENV serotypes, most current vaccine strategies involve the administration of tetravalent live, attenuated or recombinant viral formulations that typically elicit both serotype-specific neutralizing, and antigenically cross-reactive but non-neutralizing, antibody responses. Thus, in the event that a balanced response is not achieved, all such formulations have the potential to facilitate ADE. In this thesis, I describe a DENV subunit vaccine strategy that involves administration of only one of three domains of the DENV envelope (E) glycoprotein derived from all four DENV serotypes. Domain III (dIII) is attractive for this purpose because it appears to contain epitopes capable of eliciting primarily serotype-specific virus neutralizing antibody responses. Here I examine the potential utility of an E glycoprotein dIII-based strategy to mediate broadly protective (i.e., tetravalent) DENV neutralizing antibody responses with minimal associated ADE activity.
In addition, I investigate the mechanism by which DENV-immune complexes enhance entry and viral infectivity in cells expressing Fcγ receptors utilizing a panel of epitope-matched immunoglobulin switch variants and cell lines engineered to express either FcγRI or FcγRII. Results demonstrate that DENV neutralization is modulated by the antibody Fc region in a manner that is dependent upon IgG subclass, likely through effects on virion and FcγR binding. Thus, the IgG antibody subclass profile generated by DENV infection and/or vaccination appears to be a critically important parameter in the development of safe and effective vaccines for the prevention of DF, DHF and DSS.
List of Abbreviations

6HIS  Hexa-Histidine
Ab   Antibody
ADE  Antibody Dependent Enhancement
C    Capsid
CFA  Complete Freund’s Adjuvant
dI   Domain I
dII  Domain II
dIII Domain III
DEN  Dengue
DENV Dengue Virus
DENV1 Dengue Virus 1
DENV2 Dengue Virus 2
DENV3 Dengue Virus 3
DENV4 Dengue Virus 4
DF   Dengue Fever
DHF  Dengue Hemorrhagic Fever
DNA Deoxyribonucleic Acid
DSS  Dengue Shock Syndrome
E    Envelope
ER   Endoplasmic Reticulum
ELISA Enzyme-Linked Immunosorbent Assay
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>FcγR</td>
<td>Fragment Crystalline Gamma Receptor</td>
</tr>
<tr>
<td>GMT</td>
<td>Geometric Mean Titer</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s Adjuvant</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>JE</td>
<td>Japanese Encephalitis</td>
</tr>
<tr>
<td>LAV</td>
<td>Live Attenuated Vaccine</td>
</tr>
<tr>
<td>M</td>
<td>Membrane</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-Structural Protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Non-Structural Protein 2</td>
</tr>
<tr>
<td>NS3</td>
<td>Non-Structural Protein 3</td>
</tr>
<tr>
<td>NS4</td>
<td>Non-Structural Protein 4</td>
</tr>
<tr>
<td>NS5</td>
<td>Non-Structural Protein 5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>prM</td>
<td>Pre-Membrane</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em></td>
</tr>
<tr>
<td>TBE</td>
<td>Tick Borne Encephalitis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile Virus</td>
</tr>
<tr>
<td>YF</td>
<td>Yellow Fever</td>
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Foreword

The following chapters of this dissertation were jointly produced. My participation and contribution to the research is as follows:

Chapter 1 of my dissertation was solely authored by me.

I am the primary author of Chapter 2. I collaborated with Drs. Jacob Schlesinger, Robert Rose, Xia Jin, and Shanaka Rodrigo. My contribution to this chapter is the production, characterization, and analysis of recombinant DENV immunogens as antigens in mice as well as the ability of mouse immune sera to mediate in vitro neutralization and enhancement. This work was submitted to Vaccine on July 21, 2010.

Chapter 3 of my dissertation was co-authored with Drs. Shanaka Rodrigo, Jacob Schlesinger, Xia Jin, Robert Rose, Ching-Juh Lai, Michael Diamond, Syd Johnson, Ana Goncalvez, and Soliva Sukupolvi-Petty in addition to Christopher Lane. My contribution to this chapter includes evaluation of the antibody binding affinity of epitope-matched IgG subclass variant antibodies as well as data analysis. This chapter has been published in Virology, 2009, pages 175-182.
Introduction

I. Dengue Virus

Flaviviruses are small spherical enveloped RNA viruses belonging to the family *Flaviridae*. The genus flavivirus consists of over 70 members including important human pathogens such as dengue (DEN), Japanese encephalitis, West Nile, and yellow fever virus (14). Phylogenetic analysis suggests that all flaviviruses share a common non-vectored ancestor and are divided into three distinct clades based on mode of transmission: mosquito-vectored, tick-vectored, and non-vectored viruses (37, 61). Dengue virus, transmitted to humans through the bite of an infected *Aedes aegypti* mosquito, accounts for the majority of arboviral diseases worldwide (40). Notably, dengue viruses alone accounts for an estimated 50-100 million infections each year, with approximately 500,000 of these cases resulting in severe debilitating illness, hemorrhagic fever, shock, and in some cases death (125).

Dengue exists as four serologically distinct co-circulating serotypes, DENV1-4. The positive sense RNA genome encodes a large viral polyprotein approximately 3,500 amino acids long that is translated on the surface of the endoplasmic reticulum (14). Proteolytic processing of the polyprotein results in three structural proteins, the capsid, precursor membrane, and envelope glycoprotein, as well as seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1). Host-encoded proteases cleave the anchC/prM, prM/E, E/NS1, and NS4A/NS4B junctions (16). Cleavage at NS1/NS2A, NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4b/NS5 junctions is mediated by the viral NS2B-NS3 protease (8, 16).
**Figure 1**: Schematic diagram of the dengue polyprotein. Translation of the viral genome results in production of a single polyprotein on the surface of the endoplasmic reticulum. Proteolytic cleavage by host and viral proteases yields three structural, and seven nonstructural proteins. Arrows denote cleavage sites of the viral NS2B-NS3 protease as well as host cell factors including furin and signal peptidases. Adapted from (5, 8).
`A. Viral Gene Products

1) Structural Proteins

Processing of the dengue virus polyprotein results in the generation of three structural proteins: capsid, pre-membrane, and envelope. Cryo-electron microscopy has been used to elucidate the structure of intact immature and mature dengue virions (60, 130). At the center of each virus is the nucleocapsid core surrounded by a host-derived lipid bilayer containing copies of the prM and E glycoproteins (Figure 2).

Capsid (C)

Each mature dengue virion contains a single positive sense RNA genome complexed with several copies of the viral capsid protein (C). The C protein is a helical, basic protein, approximately 12kDa in size (73). It is translated on the surface of the ER as the first component of the viral polyprotein. The carboxyl terminal end of C contains a hydrophobic signal sequence that serves as a membrane anchor and is responsible for the translocation of pre-M into the luminal side of the ER during translation. Cleavage by the viral NS2A/NS3 protease on the cytoplasmic side of the ER lumen frees C from pre-M (103).

Capsid protein readily forms dimers in solution. A large central hydrophobic patch present in the C protein dimer interacts with ER-derived lipid droplets and is essential for infectious virus particle formation (33, 52, 100). In addition to being found in the cytosol of infected cells, C protein dimers have also been identified in the nucleus and nucleolus, and are required for apoptosis of infected cells (120).
Figure 2: **Structure and cross section of dengue virions.** (A) Central cross section viewed along an icosahedral three-fold axis of symmetry. Darkness of shading is proportional to electron density. (B) Central cross section of cryo-EM density showing the prM-E glycoprotein shell (blue), membrane bilayer (light blue), and nucleocapsid core (red). Also shown is the averaged (red) and maximum (blue) density as a function of radius. Adapted from: (130)
Precursor M (prM)

The viral precursor M (prM) protein is a 166 amino acid (18 kDa) glycoprotein present only in immature virus particles as hetero-dimers with the envelope glycoprotein E (14) in the form of 60 trimeric spikes (Figure 3) stabilized primarily by the pr peptide regions of each dimer (66, 131). The amino terminus of the prM protein (residues 1-91) is glycosylated at an Asp residue and composed of the mostly hydrophilic “pr” region; whereas the carboxyl terminus is designated the M protein, and includes an ectodomain (residues 92-130) and transmembrane anchor region (residues 131-166) (66, 83).

The pr peptide functions as a chaperone, preventing premature fusion with host cell membranes during virus maturation (66). Cleavage of prM by host-cell furin protease results in the release of the pr peptide, destabilization of the trimeric spikes, and rearrangement of the structural proteins into 90 antiparallel E homodimers. This step is required for the production of mature infectious virus (Figure 3B) (60, 129, 133, 134). Incomplete cleavage of prM however occurs in several cell lines used to propagate virus, including C6/36 mosquito cells and African Green Monkey Vero and LLC-MK2 cells, resulting in the secretion of both non-infectious subviral particles and immature prM-containing virus in addition to mature infectious virions (19, 53).

Antibodies against prM are detected following both primary and secondary DENV infection (24, 63). Importantly, anti-prM antibodies against dengue, Japanese encephalitis, and West Nile are complex specific and can be used to distinguish between previous flavivirus infections (17).
Figure 3

**Figure 3:** The flavivirus maturation pathway. (A) Immature particles bud into the ER and are transported through the Golgi into the TGN. Acidification induces conformational changes of the virus. Furin cleavage of prM occurs in the TGN and the pr peptide remains associated until the virus is released to the extracellular milieu. (B) The structure of the E protein in the secretory pathway is largely unchanged, except for movements at the hinge between domains I and II. In contrast, the oligomerization states of the glycoproteins are critically dependent on pH. The fusion loops are indicated by red stars. Adapted from: (130).
**Envelope (E) Glycoprotein**

The dengue Envelope glycoprotein, approximately 50 kDa in length, is the principal antigen inducing virus-neutralizing antibodies. There is an approximately 40% homology in E protein among all Flaviviruses (14). Glycosylation sites are not conserved but generally occur on two separate asparagine residues (Asn-67 and Asn-153) in all four serotypes of DENV (92). E protein is attached to the viral lipid bilayer through a C-terminal stem-anchor (residues 396-495) (Figure 4B) (132). The soluble ectodomain (residues 1-395) can be divided into three structurally and functionally distinct regions: domains I, II, and III (Figure 4).

Domain I is the central structural domain of E, forming an 8-stranded beta-barrel which functions as a hinge during fusion of the virus with cellular membranes (81). Domain I is made up of three discontinuous segments (amino acids 1-52, 132-193, and 280-296 in Figure 4A), contains two disulfide bridges (81), and is flanked by either Domain II or Domain III.

Domain II serves as the envelope protein dimerization domain and is an elongated finger-like structure (81, 93) made up of the two antiparallel strands joining domain I regions to each other (residues 53-131 and 194-279 in Figure 4A) (1). The base of domain II is made up of two alpha helices and a short beta sheet. Three disulfide bridges serve to stabilize the structure (93). The distal end of domain II, amino acids 98-110, contains a highly conserved flavivirus 12-amino acid sequence corresponding to the viral fusion peptide (1, 93, 99). At neutral pH envelope protein heterodimers on mature virions are tightly packed; the viral fusion peptides remain
inaccessible, buried within a crevice formed by domains I and III of the opposing glycoprotein (Figure 4C) (93, 123). A decrease in pH induces conformational changes in mature virions (2) resulting in a reorganization of E protein dimers into fusion-competent trimeric spikes with the fusion peptide loops present at each tip (80, 81). This rearrangement is achieved primarily through a hinge motion in domain II of the envelope glycoprotein (Figure 5) (80). Exposure in the viral fusion loop allows fusion with host cell membranes and release of the viral nucleocapsid into the cytosol of infected cells. The majority of the immune response against E is cross-reactive and directed against the conserved fusion peptide sequence (63).

Domain III of the flavivirus E glycoprotein is approximately 10 kDa in size (residues 296-394 in Figure 4A). It has a characteristic immunoglobulin-like fold stabilized by a single disulfide bridge (93). Each domain III subunit is in close contact with the domain I and the fusion peptide loop of its corresponding heterodimer partner (93). Antibodies directed against domain III are potent neutralizers of infection (71, 104) and are able to block entry of DENV into mammalian and insect host cells (23, 48, 71). On the mature virus, the domain III subunit projects furthest out of the surface. For these reasons, domain III is thought to be the putative receptor binding motif (93).
Figure 4

(A) The three domains of soluble E protein are colored in red, yellow, and blue respectively. A 53-amino acid stem region at the carboxyl-terminus links the ectodomain with the transmembrane anchor region of E (blue hatched bars). 

(B) Diagram of DENV E protein ectodomain and transmembrane domain, side and top views. 

(C) Ribbon diagram of Envelope dimers with the fusion peptide indicated in green. 

Figure 4: Structure of E proteins in mature flavivirus virions. (A) The three domains of soluble E protein are colored in red, yellow, and blue respectively. A 53-amino acid stem region at the carboxyl-terminus links the ectodomain with the transmembrane anchor region of E (blue hatched bars). (B) Diagram of DENV E protein ectodomain and transmembrane domain, side and top views. (C) Ribbon diagram of Envelope dimers with the fusion peptide indicated in green. Adapted from: (81, 82, 123).
Figure 5: Structural rearrangement of envelope glycoproteins during flavivirus fusion with host cell membranes. (A) Envelope protein dimers shown at neutral pH lying flat on the surface of the virus particle (left panel). Domains I (red), II (yellow), and III (blue) are indicated. Reduced pH in the endosome causes domain II to hinge outward from the virion surface, exposing the fusion loop (orange), and allowing E monomers to rearrange laterally in the plane of the viral membrane (right panel). (B) The fusion loop at the tips of domain II (orange) inserts into the hydrophobic layer of the host-cell membrane, promoting fusion. Adapted from: (13, 81)
2) Non-Structural (NS) Proteins

NS1

Glycoprotein NS1 is approximately 48 kDa in size and contains 2 conserved N-glycosylation sites as well as 12 cysteine residues. Following synthesis of NS1 in the endoplasmic reticulum, monomeric subunits interact via sequences in their carboxyl termini to form stable homodimers (14). In its dimeric form, NS1 is found associated with components of the viral replication machinery. Intracellular NS1 also interacts with viral protein NS4A, a membrane bound protein associated with replication (70). Although found in association with cell plasma membranes, NS1 does not contain a membrane anchor domain. It is thought to bind infected cells and participate in cell signaling through its interaction with a glycosyl-phosphatidylinositol (GPI) anchor in response to binding of NS1-specific antibody (50). NS1 is also found attached to uninfected cells through interactions with heparin and chondroitin sulfate, preferentially binding subsets of microvascular endothelial cells (6). Hexameric NS1, secreted by mammalian cells, is formed by oligomerization of three dimer subunits, and accumulates in the blood of infected patients (35). Additionally anti-NS1 antibodies found in patient sera recognize common epitopes on integrin/adhesion proteins, human endothelial cells, and platelets (14, 31) suggesting a role in the characteristic vascular leakage that occurs during severe infection. NS1 antibodies are able to both prevent platelet aggregation and activate complement mediated lysis of platelet(67) and endothelial cells(68).
. **NS2A and NS2B**

Little is known about NS2A and NS2B, which are small hydrophobic proteins. NS2A is approximately 22 kDa in size and speculated to participate in recruitment of RNA templates to the membrane-bound replicase complex (14). Dengue NS2B, a small membrane-associated protein, contains a 40 amino acid hydrophilic domain essential for the stabilization of the protease activity of the viral NS2B-NS3 complex (32).

**NS3**

Cytoplasmic protein NS3 is a multifunctional protein central to both replication of the virus genome and processing of the viral polyprotein. It contains two globular folds connected by a short flexible linker (5). The amino terminal 180 amino acids contain a serine protease that functions, in complex with NS2B, both in *cis* and in *trans*, at several junctions ([**Figure 1**](#)) including NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 (8). The carboxyl terminal two-thirds of NS3 mediates ATP-dependent RNA strand separation through its helicase and nucleoside triphosphatase functions (65). The NS3 helicase belongs to the SF2-superfamily, containing Walker A and B motifs responsible for RNA and Mg$^{2+}$ binding (5). It is thought to facilitate the release of newly synthesized viral genomes from replication intermediates. Additionally, NS3 contains RNA triphosphatase activity essential to RNA 5’ capping in conjunction with the viral NS5 protein (118).
**NS4A and NS4B**

Nonstructural proteins NS4A and NS4B are small hydrophobic membrane-associated proteins of approximately 16 kDa and 27 kDa, respectively that are translated in the lumen of the endoplasmic reticulum (14). Little is known about the functions of these proteins. The amino terminal 49 residues of NS4A are non-membrane associated and located in the cytoplasm; this region is followed by four hydrophobic regions that act as transmembrane segments and a carboxyl tail in the lumen of the ER. NS4A is localized to endoplasmic-reticulum derived structures containing viral dsRNA, NS2A, and other nonstructural proteins, suggesting its involvement in the viral replicase complex (70, 79). Protein NS4B is also localized to regions of RNA replication; it has been shown in vitro to release NS3 from single-stranded RNA, thereby aiding in the helicase activity of NS3 (111).

**NS5**

NS5 is the largest (104 kDa) and most conserved flavivirus protein. The amino terminal and central domains function as methyltransferases, in complex with NS3 to form the 5’ RNA cap structure required for recognition by host cell translational machinery (38). The carboxyl domain functions as an RNA-dependent RNA polymerase (RdRp), required for replication of the viral genome (7). The RdRp domain of NS5 is able to specifically block IFN-alpha signaling by binding to STAT-2 and inhibiting its phosphorylation (77).
B. Viral Replication Cycle

Dengue viruses infect dendritic cells, macrophages, monocytes, and endothelial cells of the lung, liver, and kidney (10, 51, 59, 108, 128). Dengue virus entry is facilitated by binding of DC-SIGN dendritic cells (108), however a specific DENV receptor is as yet unidentified. Following binding to a putative receptor, virus enters cells through clathrin-dependent endocytosis and is transported to cellular endosomal compartments (112). Maturation and acidification of endosomes triggers conformational changes in viral structural proteins allowing rearrangement of E protein dimers into trimeric spikes that insert into the host cell membranes to mediate fusion (81), uncoating of the nucleocapsid, and release of the viral genome (82). The plus sense RNA is quickly recognized by host cell transcription factors and translated into a single large polyprotein across the surface of the endoplasmic reticulum (8). Cleavage by viral NS2B-NS3 and host proteases results in the production of 3 structural and 7 nonstructural proteins (Figure 1).

Replication complexes composed of host and viral proteins form on the surface of intracellular membranes (82). The NS3 helicase aids in unwinding secondary structures at the 3’-end of the viral genome (65) in preparation for synthesis of a minus sense intermediates by NS5 RdRp. These negative sense intermediates function as templates for the synthesis of progeny viral genomes (14). Following transcription, the NS3 triphosphatase and NS5 methyltransferase decorate the plus sense RNA genomes with a 5’-cap structure, essential for protection from
degradation by host cell exonucleases, as well as for the initial recognition by host cell factors required for initiation of translation.

Newly synthesized RNA is packaged by the capsid protein to form a nucleocapsid which in turn buds into the lumen of the ER to acquire its lipid bilayer. Immature virus contains prM/E heterodimers arranged as trimeric spikes. Glycosylation and maturation of the structural proteins occurs as virus is transported across the Golgi and trans-Golgi network (Figure 6). Proteolytic cleavage of prM by host cell furin results in dissociated of the pr peptide during release, triggering further conformational changes and secretion of mature, infectious, fusion-competent particles (Figure 6) approximately 500Å in diameter.

An estimated 3 to 4-fold difference in virus particle concentration can be detected by biochemical assays compared to infectivity assays (113). This difference is attributed to the secretion of both immature or partially immature virions and subviral particles in addition to the mature infectious virus. The relative structures of immature or partially mature particles are vastly different than those of mature virus due to the presence of prM/E spikes (Figure 7), resulting from incomplete cleavage of prM during maturation (134). As such, immature virus is non-infectious (19). Subviral particles (approximately 315Å in diameter) have a smooth outer surface, contain only viral M and E proteins (119), and lack a nucleocapsid core and/or viral RNA. Recombinant expression of prM and E proteins in mammalian cells results in the production of subviral particles that have been used to study mechanisms of virus synthesis, secretion, and fusion (72, 101).
**Figure 6: Schematic diagram of the dengue replication cycle.** Membrane bound virions are internalized by receptor-mediated endocytosis into endocytic vesicles. Following fusion with host cell membranes, viral RNA is released into the cytoplasm and translated as a large polyprotein. Cleavage by host and viral proteases, along with RNA replication occurs on intracellular membranes, with virus assembly occurring on the surface of the endoplasmic reticulum. Virus maturation occurs as it is transported through the trans-Golgi network. Mature virions are released by exocytosis. Adapted from: (82).
Figure 7: CryoEM Images of West Nile Virus. Some immature (red arrows) and partially immature particles (green arrows) are frequently found in preparations of mature virus. Fully mature particles are marked with blue arrows. The same type of partially immature virus is usually also present in mature DENV samples. The scale bars represent 500 Å. Adapted from: (19)
C. Epidemiology

According to the World Health Organization (WHO), approximately 2.5 billion people are at risk of infection with any of the four serotypes of dengue. Each year between 50-100 million infections occur in over 100 dengue endemic countries (125). Transmission is confined to tropical and subtropical regions of the globe, principally Southeast Asia, Australia, and South and Central America through the bite of its arthropod vectors, the *Aedes aegypti* and *Aedes albopictus* mosquitoes (Figures 8, 9). Rarely, transmission of DENV has also been documented through congenital and mucosal routes as well as through organ transplantation and blood transfusion (74, 126).

Prior to WWII dengue was reported in only 9 countries worldwide, and epidemics due to dengue were reported infrequently (44, 125). Several epidemiological factors have contributed to the status of dengue as an emerging infectious disease including the increase in global travel and trade to Asia and Southeast Asia following World War II which aided in the spread and co-circulation of all four serotypes in endemic nations (14). The rapid rise in rural-to-urban migration in developing countries, combined with inadequate infrastructure for disposal of waste products, resulted in increased breeding grounds for the mosquito vectors (125). The importance of vector-control measures in limiting the spread of disease is illustrated by the re-emergence of dengue in South and Central America following the end of mosquito control measures in 1970 (Figure 9) (43).
Over half of all infections with dengue virus are either mild or asymptomatic (15, 30). In other cases, especially within pediatric populations, infection results in either dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) (57). To date, vaccines or specific treatments for disease caused by dengue are unavailable. For these reasons, dengue is a leading cause of childhood hospitalization and morbidity in Southeast Asia, accounting for an estimated 500,000 cases of DHF/DSS and 22,000 deaths each year (43, 125).

Infection with one of the four circulating DENV serotypes results in lifelong protection from reinfection with a homologous serotype, and short-term protection from infection with a heterologous serotype (14). Risk factors for the development of severe disease include the age and immune status of a patient, the infecting strain and serotype of virus, and the order of infection. Dengue is generally a childhood disease, affecting mainly infants between 4-9 months old and children between 5-9 years of age (46). Disease in infants is correlated with a decrease in levels of passively transferred maternal antibodies (18, 58). In older children and adults, severe disease is correlated with sequential infection by heterologous virus serotypes (46). For example, in 1997 the city of Santiago de Cuba experienced a DENV2 epidemic resulting in 5,208 laboratory confirmed cases: 5,003 DF and 205 DHF/DSS cases. Over 98% of DHF/DSS cases and 92% of DF cases were due to secondary infection in DENV1 immune individuals, while dengue naive individuals experienced relatively little apparent disease (45).
Genotypic differences within a serotype are associated with differences in DENV pathogenicity. Phylogenetic analyses of DENV2 epidemics in the Americas identified the Southeast Asian genotype as responsible for four different DHF epidemics, while the American genotype was associated only with DF epidemics (45, 94). Studies conducted in non-human primates have also demonstrated strain-dependent variability in both viremia and the magnitude of the immune response following infection with different dengue genotypes (75).
Figure 8: **Worldwide distribution of dengue.** Total Number of Dengue cases worldwide reported to the World Health Organization (1995-2005). Adapted from (124).
Figure 9: Dengue cases in the Americas reported to the World Health Organization. Total number of dengue cases in the Americas. Adapted from (124).
D. Clinical Manifestations

The incubation period of dengue virus, in cases of DF, is between 4-7 days followed by the rapid onset of a high grade fever lasting approximately 7 days (109). An initial rash, described as flushing of the face, neck, trunk, and/or legs is present in over 80% of cases (49). Following defervescence, the rash can develop into macular regions with petechiae and islands of sparing (49, 88). DF is commonly a benign illness but can also be characterized by retro-orbital pain, headache, myalgia, anthralgia, and leukopenia giving it the name “break-bone” fever (125). Hemorrhagic manifestations include gingival bleeding and blood in the urine or stool. In many cases anorexia and severe abdominal pain are also reported (86).

Because many DF symptoms are similar to disease caused by other viruses or bacteria, laboratory confirmation of dengue infection is a requisite of accurate diagnosis. In Southeast Asia, acute undifferentiated febrile illness is a leading cause of hospitalization and is attributed to several pathogens (107). Illness due to dengue specifically is determined serologically by either IgM/IgG ELISA, hemagglutination inhibition assay, and/or detection of viral genomes by RT-PCR (30, 49).

Dengue hemorrhagic fever and dengue shock syndrome are severe systemic manifestations of disease following dengue infection. The initial febrile period and symptoms are similar to that of DF. The main distinction of DHF is plasma leakage as evidenced by either an increase in hematocrit, a decrease in hematocrit following fluid replacement therapy, and/or pleural effusion, ascities, and hypoproteinemia. Other DHF symptoms include: rash, headache, myalgia, leukopenia,
thrombocytopenia, petechiae, and gastrointestinal, mucosal, or gingival bleeding (86, 88). Rapid diagnosis and adequate treatment of DHF symptoms, including fluid and electrolyte replacement often lead to a quick and spontaneous recovery. If left untreated severe circulatory system failure can occur. Symptoms of DSS include a weak and rapid pulse, cold or clammy skin, and restlessness. Patients experiencing DHF/DSS either die within 24 hours, or recover within 2-3 days (87).

E. Humoral Immune Response

DENV infection results in the production of antibodies directed against several viral antigens, notably the major structural proteins prM and E (24). Many of these antibodies are highly cross-reactive among the four serotypes. Interestingly prM antibodies show little cross-reactivity (17) with related flaviviruses while anti-E antibodies are strongly cross-reactive with several Flaviviruses including TBE, JE, WN, and DENV (102). These differences are attributed to relative levels in sequence conservation in prM and E, 35% and 50% respectively compared to JEV (24). Over 90% of the humoral response to E is directed against a single cryptic antigenic determinant, the flavivirus fusion peptide, a conserved immunodominant epitope present in all Flaviviruses (21, 42, 63). Many strongly cross-reactive antibodies recognize either immature virus (prM), partially digested virus, or incompletely processed or assembled virus released from lysed cells (102). Antibodies directed against either prM (24) or the fusion peptide (102) are often either weakly or non-neutralizing, and serve to enhance infection (discussed below).
The envelope glycoprotein contains the principal antigenic epitopes inducing virus-neutralizing antibodies following infection (14). Domains I and III contain predominately subcomplex and serotype-specific epitopes whereas domain II contains mainly cross-reactive determinants (60, 98). Domain I and II antibodies, albeit weakly neutralizing, cluster to six antigenic epitopes shown in Figure 10: the lateral ridge of dI, a linker region between dI and dIII, a hinge between dI and dII, the lateral ridge, central interface, dimer interface, and fusion loop of dII (85). Type-specific mAbs directed against linear epitopes are predominantly either weakly or non-neutralizing or recognize solvent-inaccessible determinants (78, 85) whereas strongly neutralizing antibodies are directed against discontinuous amino acid residues in conformationally dependent epitopes. Importantly, strongly neutralizing monoclonal antibodies directed against DENV, JEV, WNV and YF have identified a serotype-specific epitope on the upper lateral ridge of dIII (56, 69, 104, 115). Weakly neutralizing and non-neutralizing antibodies reactive with two or more dengue serotypes also recognize epitopes on dIII (71, 97, 104). A recently described antibody raised against DENV2 dIII has been found to bind with nanomolar affinity to a conserved dIII epitope and mediate neutralization of all four serotypes (90). While dIII-specific antibodies detected in convalescent human sera constitute less than 10% of the anti-E humoral immune response, their levels correlate with in vitro neutralization of homologous virus, suggesting a strong role of anti-dIII antibodies in the protective immune response (22, 117).
Figure 10: Location of mAb epitopes and informative sites on DENV E protein.

(A) The top image depicts the major antigenic sites on domains I (red) and II (yellow). The bottom image displays the location of informative sites on domains I and II (pink). (B) An enlarged view of domain III (blue) displaying antigenic and informative sites. The left image displays the lateral ridge and A strand epitopes. The right image displays the domain III informative sites (pink). Adapted from (116).
**F. Pathogenesis**

1) **Antibody Dependent Enhancement**

The observation that the majority of DHF/DSS cases occur in two populations, either infants experiencing primary infection between 5-9 months of age, when levels of maternally transferred antibodies have declined to below protective levels, or in children and adults experiencing secondary infection, suggests a role for anti-dengue antibodies in the immunopathogenesis of disease (46).

Antibody dependent enhancement (ADE) has been proposed to explain this phenomenon whereby pre-existing anti-dengue antibodies mediate increased uptake and infection of susceptible cells. While primary DENV infection confers lifelong immunity from reinfection with the same viral serotype, the immune response to a secondary heterotypic infection results in the expansion of weakly or non-neutralizing cross-reactive antibodies able to form infectious immune complexes. These DENV-Ab immune complexes facilitate binding to and infection of Fc-gamma receptor expressing cells, notably monocytes and macrophages (Figure 11). Thus ADE is thought to enhance the infectivity of heterotypic virus in dengue-immune individuals, ultimately increasing viral load and influencing clinical disease severity (41, 114). Indeed, evaluation of plasma viremia levels in dengue patients has demonstrated an association between continued viral replication or delay in clearance during defervescence with the onset of DHF compared to DF (121). Studies in dengue endemic areas have shown a direct relationship between preexisting dengue immunity and increased disease severity in children and adults (15, 46).
Recent evidence suggests a role for weakly neutralizing anti-prM antibodies in mediating enhanced infection of susceptible cells through an increased uptake of immature dengue virions (24). Once internalized, processing by host cell furin protease yields mature infectious, fusion competent virus able to infect and results in infection of susceptible cells (96). The ability of anti-prM antibodies to bind host cell proteins (47) coupled with their ability to transform non-infectious virions into infectious particles suggests a role for ADE during either secondary heterotypic infections or in primary infections in infants born to dengue immune mothers.

2) Cell Mediated Immunopathogenesis

The major clinical distinction between DF and DHF/DSS is evidence of plasma leakage mediated by changes in vascular endothelial cells. Although poorly permissive to direct infection (4), endothelial cells are susceptible to activation by cytokines produced during enhanced infection of bystander cells such as peripheral blood monocytes (3). Infection of monocytes and macrophages results in the presentation of viral antigens to memory CD4+ and CD8+ cells and secretion of cytokines that act directly on endothelial cells, inducing plasma leakage (Figure 11). Indeed, sera recovered from patients with severe disease have high levels of proinflammatory (IFN-γ and TNF-α) (36) and vasoactive (VEGF-A) (110) cytokines, responsible for the widening of blood vessels, compared to those experiencing DF. Anti-inflammatory cytokines IL-6 and IL-10 have also been detected in DHF/DSS
patient sera (84). The “cytokine storm” experienced by patients with severe disease is thought to be a result of both humoral and cell-mediated immunity.

The concept of original antigenic sin suggests that cross-reactive memory T-cells generated during primary DENV infection are preferentially activated during secondary heterologous infection compared to higher avidity serotype-specific naïve T-cells. Several DENV T-cell epitopes have been identified on both structural (C and E) and more conserved non-structural viral antigens including NS2B, NS3, NS4A, NS4B, and NS5 (76). T-cell lines generated from patients experiencing primary infection have demonstrated the ability to respond to homologous and heterologous virus serotypes or viral antigens through the production of pro-inflammatory cytokines associated with plasma leakage including IFN-γ, TNF-α, and TNF-β as well as IL-2 and MIP-1β (36, 62). Additionally, cytotoxic T-cells directed at DENV antigen have been shown to mediate lysis of both cognate and bystander cells (36). In this manner preferential proliferation and expansion of low avidity memory CD4+ and CD8+ cells contribute to less efficient viral clearance, increased viral replication, and an altered cytokine profile in patients experiencing DHF/DSS compared to DF.
Figure 11: **Immunopathogenesis of severe dengue disease.** ADE of infection in FcγR bearing cells allows for increased cellular infection. Dengue infected monocytes and macrophages present viral antigens to memory CD4 and CD8 T-cells which were sensitized during a previous infection. Memory T-cells require lower levels of stimulation to become activated than naïve T-cells, leading to proliferation and release of proinflammatory cytokines such as TNFα and IFNγ that act directly upon vascular endothelial cells and result in plasma leakage. Adapted from: (122).
II. Current Dengue Vaccine Strategies

Flavivirus vaccines against Yellow Fever (39) and Japanese Encephalitis (34) have been developed and licensed for use in endemic populations. Although over 2 billion people each year are at risk for infection with DENV, there is as yet no available vaccine. Obstacles to the development of a successful vaccine include the co-circulation of four antigenically distinct serotypes and the possibility for enhanced disease severity following sequential heterologous infections. For these reasons, the ideal vaccine candidate would elicit long lasting protective immunity against all four serotypes of DENV. Such strategies in development include live attenuated viruses, DNA vaccines, recombinant subunit vaccines, and purified formalin inactivated virus.

A. Live Attenuated Virus (LAV)

The most extensively evaluated candidate dengue vaccines are live attenuated viruses developed by either serial passage in non-human cell lines or recombinant DNA technology. LAV vaccines offer several advantages over non-replicating vaccines including the ability to closely mimic natural infection potentially inducing humoral and cellular immunity against all viral antigens. A major challenge in LAV vaccine development lies in the formulation of a tetravalent vaccine inducing protective immunity against all four serotypes.

Beginning in 1981, attenuation of DENV strains through serial passage in primary dog kidney (PDK) or African green monkey kidney (AGMK) cell lines has been pursued by both Mahidol University (Bangkok, Thailand) and the Walter Reed Army Institute of Research (WRAIR, Washington, DC) (9, 28). These viruses have
been evaluated as both monovalent and tetravalent formulations in clinical trials. While monovalent vaccine candidates induced seroconversion against homologous virus, administration of tetravalent formulations demonstrated varying degrees of reactogenicity, viremia, and seroconversion to each component attenuated strain (29, 54, 55). Problems encountered by Mahidol University in tetravalent vaccine development include the preferential replication and seroconversion to a single component virus (DENV3) due to competitive viral interference or under-attenuation (55). These vaccine constructs are no longer being evaluated. Problems involving a balance of attenuation and immunogenicity have also been encountered by the WRAIR tetravalent vaccine candidates. Under-attenuation of the DENV1 component virus and over-attenuation of the DENV4 component virus in 16 different vaccine formulations resulted in an unbalanced immune response with the highest and lowest levels of seroconversion to DENV1 and DENV4 respectively (29). Under-attenuation of the DENV1 component virus correlated with both reactogenicity and seroconversion (106). To date over 18 different WRAIR tetravalent formulations have been evaluated in Phase I and II clinical trials (105).

The long record of safety, efficacy, and durable immunity associated with the Yellow Fever 17D (YF17D) vaccine strain has prompted the development of DENV vaccines engineered onto a YF vaccine platform. Chimeric YF17D/DENV vaccine candidates against all four dengue serotypes were developed using recombinant DNA technology by substitution of YF17D prM/E genes with corresponding regions from each DENV serotype. Phase 1 evaluation of ChimeriVax-DEN2 in YF-naive
volunteers, demonstrated 100% seroconversion following a single dose of live attenuated vaccine (LAV). Tetravalent formulations of ChimeriVax-DEN vaccines evaluated in three separate Phase II clinical trials demonstrated seroconversion rates ranging from 88-100% against all four serotypes in both flavivirus-immune and naïve populations following the administration of 3 doses (64). These vaccines are promising candidates for tetravalent immunization.

Live attenuated virus vaccine candidates have been developed utilizing recombinant DNA technology to delete a 30-nucleotide stem-loop structure in the 3’untranslated region (3’-UTR) of each virus resulting in attenuated DENV1, DENV2, and DENV4 Δ30 strains (12, 25). Surprisingly removal of the 3’UTR failed to attenuate DENV3 compared to the parental strain prompting evaluation of alternative constructs (11). Chimeric viruses utilizing rDEN4Δ30 as a backbone were generated by substitution of prM/E genes with those of either DENV1, or DENV2, or DENV3. In phase 1 clinical trials, 95-100% seroconversion has been demonstrated following administration of a single dose of either rDEN1Δ30, rDEN2/4Δ30, or rDEN4Δ30 (25-27). These vaccines were found to be safe, well tolerated, and able to induce measurable immune responses against each corresponding serotype in healthy dengue-naïve volunteers. However 10/20 volunteers that received the rDENV4Δ30 vaccine developed an asymptomatic maculopapular rash. Efforts at decreasing reactogenicity in human volunteers have resulted in additional modifications of the Δ30 vaccine strains (27, 127). Evaluation of dengue 3 candidate vaccines is currently
underway in Phase 1 clinical trials with the ultimate goal of developing a tetravalent vaccine suitable for use in human subjects.

**B. Alternative Approaches**

While the above-described dengue vaccine candidates are being evaluated in clinical studies in human subjects, potentially safer and more effective next generation dengue vaccines are also in several stages of development. Non-replicating vaccines are unlikely to cause dengue-like illness following administration. However, the induction of long-lasting immune responses against all serotypes remains a major challenge.

The US Navy Medical Research Center (NMRC) has developed a naked DNA vaccine encoding the prM/E of DENV-1. Intramuscular delivery of three or four 1 milligram (mg) doses of vaccine protected 4/8 monkeys from viremia when challenged with homologous virus 4 months following vaccination (91). A Phase 1 clinical trial of dengue-1 prM/E DNA vaccine is underway to evaluate safety and reactogenicity in healthy flavivirus-naïve human adults.

A second approach, pursued by Hawaii Biotech, involves the administration of recombinant subunit vaccines composed of the amino terminal 80% of the E glycoprotein from all four dengue serotypes formulated in combination with DENV2 NS1 recombinant protein (95). The “80% E” entity, as it is called, lacks only the carboxyl terminal transmembrane anchor domain of the E glycoprotein. Preclinical evaluation in mice and non-human primates has demonstrated the induction of
protective immune responses following tetravalent immunization (20). A phase 1 clinical trial evaluating DENV1 80%E is currently underway.

A third approach, developed by WRAIR, involves the administration of purified formalin-inactivated virus (PIV). Administration of DENV2 PIV elicits neutralizing antibodies following administration of two doses in rhesus macaques. However, a recent study showed that only one out of fifteen animals (7%) was protected against viremia following challenge with near wild type virus two months following immunization (95). Clinical trials have yet to be done to evaluate the safety and immunogenicity of PIV in humans.
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† Data based on search of www.ClinicalTrials.gov website.
* Evaluation of monovalent vaccine formulations.

Table 1: Tetravalent dengue vaccine approaches.
III. References


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