

CHARACTERIZATION OF DENGUE VIRUS INTERACTIONS WITH HOST CELLS

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ABSTRACT

**Kari Ema Hacker: CHARACTERIZATION OF DENGUE VIRUS INTERACTIONS WITH
HOST CELLS**

(Under the direction of Aravinda de Silva)

Dengue virus (DENV) is an emerging pathogen of global importance. The causative agent of dengue fever and dengue hemorrhagic fever (DHF), DENV is spread to humans via the bite of an infected *Aedes aegypti* mosquito. With the rise of massive urban centers in tropical regions of the world and the increasing range of the mosquito vector, dengue virus has become endemic in over 100 countries resulting in explosive epidemics of DHF.

In this dissertation, we characterized the interactions of DENVs with host target cells, specifically human dendritic cells and monocytes. We report that viruses derived from mosquito and mammalian cells were able to interact with human dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), a dengue attachment factor, via a high mannose glycan on the viral envelope protein (E). The second glycan on E differed depending on the cell type in which the virus was grown. Mosquito-derived dengue had a paucimannose at this position whereas this sugar was complex in mammalian-derived virus.

Beginning in 1989, DHF emerged in Sri Lanka and has continued to cause serious epidemics annually. The emergence of DHF in Sri Lanka appears to be associated with the replacement of native viruses with a new genotype of dengue. In this dissertation, we determined that sixteen conserved amino acids differentiated the Sri Lankan pre- and post-DHF viruses. We tested viruses from each group and found that DHF-associated DENV3 do

not replicate in DC-SIGN expressing cells as well as viruses isolated after the emergence of DHF.

In order to identify the amino acid residue(s) responsible for this growth difference, we developed a dengue virus type 3 subtype II reverse genetics system. We were able to isolate recombinant virus and determined that it grows identically to the parental virus *in vitro*. We also generated chimeric viruses expressing the structural genes of one virus and the non-structural genes from the other virus. The chimeras were viable and we are currently characterizing their growth kinetics *in vitro*. Using this reverse genetics system, we can determine which amino acid differences are important in generating the kinetic growth difference in DC-SIGN expressing cells.

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LIST OF ABBREVIATIONS

| | |
|-----------|---------------------------------------------------------|
| ADE | Antibody dependent enhancement |
| APC | Antigen presenting cell |
| cEDIII | Consensus E domain III |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell-specific ICAM3 grabbing non-integrin |
| DENV | Dengue virus |
| DF | Dengue fever |
| DHF | Dengue hemorrhagic fever |
| DIC | Disseminate intravascular coagulation |
| D-MEM/F12 | Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 |
| DNA | Deoxyribonucleic acid |
| DSS | Dengue shock syndrome |
| E | Envelope |
| ELISA | Enzyme-linked immunosorbent assay |
| E-MEM | Miminal Essential Media with Earl's salts |
| ER | Endoplasmic reticulum |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| (pr)M | (pre-)Membrane |

| | |
|-----------------|----------------------------------------------------|
| MHC | Major histocompatibility |
| μg | Microgram |
| μL | Microliter |
| mL | Milliliter |
| MOI | Multiplicity of infection |
| MΦ | Macrophage |
| NH ₄ | Ammonium chloride |
| NIH | National Institutes of Health |
| ng | Nanogram |
| nm | Nanometer |
| NOD | Non-obese diabetic |
| NS | Non-structural |
| PCR | Polymerase chain reaction |
| PBS | Phosphate buffered saline |
| PIV | Purified inactivated virus |
| RNA | Ribonucleic acid |
| SCID | Severe combined immunodeficiency |
| SNP | Single nucleotide polymorphism |
| STAT | Single transducers and activators of transcription |
| TAP | Transport associated with antigen processing |
| TNF-α | Tumor necrosis factor α |
| UTR | Untranslated region |
| VEE | Venezuelan equine encephalitis virus |

| | |
|-----|---------------------------|
| VRP | VEE replicon particle |
| WHO | World Health Organization |
| WNV | West Nile virus |
| YF | Yellow fever virus |

CHAPTER 1. BACKGROUND AND SIGNIFICANCE

DENGUE VIRUS

Dengue virus (DENV) is a member of the family *Flaviviridae*, genus flavivirus. As such, it is a positive sense RNA virus of approximately 50nm in diameter (19, 25). DENVs are composed of four serologically distinct viruses, designated DENV1, DENV2, DENV3 and DENV4. These viruses evolved in the forests of Africa in sylvatic cycles with non-human primates (61). DENVs were first isolated during World War II from American servicemen stationed in southeast Asia and western Pacific Ocean islands (61). Despite the relatively recent isolation of the virus, DENV has been infecting humans for centuries. The first definitive case description of DENV disease is from the summer of 1780 in Philadelphia (179). Ancient medical writings from China suggest that dengue may have been causing disease as far back as 1100 years ago (61).

VIRION STRUCTURE AND COMPOSITION

The DENV particle is composed of five components: a ~10,700 nucleotide RNA, capsid protein, membrane (M) protein, envelope (E) protein, and a host-derived lipid bilayer. Viral particles appear as smooth surfaces of DENV E dimers arranged in a head-to-tail fashion parallel to the viral membrane (116). On one particle, ninety E dimers are arranged in a herring-bone pattern of icosahedral symmetry. As the major surface protein, many groups have focused on E in their studies of viral particles. The crystal structure of E revealed each monomer consists of three domains believed to play different roles in the virus life cycle (147, 148). E is a glycoprotein and contains two potential glycosylation sites at N-67 and N-153. Some of the biologic functions attributed to E include receptor binding, induction of antibody responses, viral fusion and viral assembly (25).

The other proteins comprising the viral particle help package the RNA and aid in assembly. Capsid is approximately 11kD and possesses positively charged basic residues (130). This protein encapsidates viral RNA and interacts with the genetic material through charged residues. Membrane is the mature form of pre-membrane (prM). This glycoprotein acts as a chaperone for, and is important in the folding of, E (130). During viral egress through the trans-Golgi network, host furin cleaves prM to M. Following cleavage, viral particles are considered mature (130).

The DENV RNA is composed of a 5' untranslated region (UTR), a large open reading frame coding for one polyprotein, and a 3' UTR (130). This RNA has a type-I 5' cap of m⁷GppA but lacks a polyA tail (25). The 5' UTR contains structures important in mediating translation of viral proteins and serves as a site of initiation of positive strand RNA synthesis (130). Additionally, the 5' UTR interacts with host factors. Directly downstream of the 5' UTR is one long open reading frame coding for ten distinct viral proteins (19). The structural genes are encoded in the 5' third of the genome and the remainder of the genome codes for the non-structural proteins (25). Following the stop codon is a highly structured 3' UTR essential for RNA replication and protein translation (130). The stem loop structures formed by intra-strand base-pairing within the 3' UTR interact with elongation factors essential for translation. Additionally, cyclization motifs interact with the 5' UTR and play important roles in replication. The 3' UTR has also been postulated to interact with regions of capsid, leading to viral RNA packaging (130).

DENV NON-STRUCTURAL PROTEINS

There are seven DENV non-structural proteins that have essential roles in the virus life cycle. NS1 is found inside of, secreted from, and on the surface of infected cells (130).

The cell-surface NS1 is GPI anchored and may play a role in signaling (130). NS1 is essential for viral replication and co-localizes to sites of RNA replication (130). Infected individuals develop a strong immune response to NS1 but the function of secreted protein remains unknown. NS2A is a small protein that coordinates the shift between viral replication and packaging (130). NS2B is the viral serine protease co-factor and interacts with NS3. NS3 possesses serine protease, helicase and NTPase activities and is involved in polyprotein processing and RNA replication.(130). The roles of NS4A and NS4B in infected cells are unknown. Along with NS2A, these proteins have been implicated in interferon antagonism but no role in viral replication or translation has been identified (152). NS5 is the viral RNA-dependent RNA polymerase. The amino-terminal of this protein has cap-processing activity and the C-terminal end possesses RNA-dependent RNA polymerase activity (130).

DENV LIFE CYCLE

DENV is an Arbovirus, meaning that it requires a blood-sucking arthropod to complete its life cycle. DENVs two hosts are humans and mosquitoes (63). Since it is an Arbovirus, DENV is able to replicate in human and mosquito cells. Upon encountering a cell, the virus particle binds at target cell surfaces through interactions between viral glycoproteins and cellular receptors (130). Following receptor engagement, DENV enters cells via receptor-mediated endocytosis and clathrin coated pits (130). The decreasing pH of pre-lysosome endosomal compartments triggers an irreversible rearrangement of E proteins, resulting in the formation of E trimers that mediate fusion between the viral and endosomal membranes (151). Following fusion, viral RNA is released into the cytoplasm. Translation of the viral genome begins immediately on the rough endoplasmic reticulum, leading to

rearrangement of cytoplasmic membranes in the perinuclear area of the cell. Following translation of NS5 and other viral replicase complex proteins, the viral genome replicates in vesicle packets, smooth structures of 70 to 200nm vesicles (130). Genome packaging is coupled to replication and assembly of viral particles occurs during budding into the ER (130). From the ER, viral particles exit cells by passing through the exocytic pathway where envelope glycan are processed and the heterotrimers of prM and E rearrange to form mature virions (238).

HUMAN DISEASE

Humans infected with dengue exhibit a number of clinical syndromes ranging from asymptomatic infection to dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (diagramed in Figure 1.1). As discussed in the section below, there are a number of host factors that influence the outcome of disease, including age, ethnicity and DENV immune status.

DENGUE FEVER

The age of an individual influences their clinical outcome following infection with DENV. Children are often asymptotically infected with DENV but may demonstrate several clinical syndromes. Infants and young children most often present with an undifferentiated febrile illness accompanied by a maculopapular rash (72, 232). As individuals age, they are more likely to become ill following a DENV infection, with 80% of adults developing clinical symptoms of disease (76). Older children and adults typically present with classic DF. First described as “breakbone fever” by Benjamin Rush during an epidemic in Philadelphia in the summer of 1780 (179), DF is characterized by an acute,

sudden onset saddleback fever, severe headache, nausea and vomiting, myalgias, retro-orbital pain, an early maculopapular rash, low grade thrombocytopenia and hepatomegaly (72, 90, 232). Some DF patients may exhibit hemorrhagic tendencies, including epistaxis and gastrointestinal bleeding (179, 232). Patients ill with DF will recover in two to seven days and suffer no short- or long-term sequelae of illness (178).

DENGUE HEMORRHAGIC FEVER

Dengue hemorrhagic fever (DHF) cases progress similarly to DF cases for the first two to seven days of illness. Following DF symptoms, patients experience a rapid drop in body temperature and abruptly become more ill (72). Defervescence is accompanied by signs of vascular leakage and circulatory disturbance (72, 232). The hallmark of DHF is increased vascular permeability, and patients experience thrombocytopenia, hemoconcentration, hemorrhage and shock (72, 232). Signs of shock include cool, blotchy and congested skin, circumoral cyanosis and increased pulse. Patients with increased vascular permeability who develop shock are assigned a diagnosis of dengue shock syndrome (DSS). The baseline vascular permeability of children is significantly higher than that of adults and most patients presenting with DHF and DSS are between the ages two and eight (9). DHF and DSS are usually of short duration with patients dying within 24 hours of developing symptoms or recovering within two days (232).

Atypical Manifestations of Dengue

Although most individuals who contract DENV and display clinical symptoms present with DF or DHF/DSS, a portion of patients display atypical manifestations of dengue (reviewed in 68). Unusual disease symptoms seen during dengue infection include neurological signs, hepatitis and GI involvement, cardiovascular manifestations, acute renal

failure, respiratory distress, lymphoreticular complications, and musculoskeletal involvement.

Neurological Manifestations

Most of the atypical dengue cases that present with neurologic manifestations display encephalopathy. Encephalopathy is characterized by decreased sensitivity, convulsions, nuchal rigidity, pyramidal signs, papilloedema and behavioral changes (68, 117). In Vietnam, as many as 1 in 200 DHF patients exhibit signs of encephalopathy (20). In addition to encephalopathy, neurologic diseases exhibited during dengue infection include encephalitis (134), neuropathy (34, 187, 188), Guillain-Barré syndrome (189, 209, 210), myelitis (118), and cerebral ischemia (32). Some patients experience neurological sequelae following dengue illness, including amnesia, dementia, manic psychosis, Reye's syndrome and meningoencephalitis (68).

It is largely unknown why some patients experience neurologic manifestations of dengue. Physicians hypothesize that the increased vascular permeability and capillary fragility seen in DHF may lead to intracranial hemorrhage, cerebral edema, hyponatremia, or cerebral anoxia (68). If a patient goes into shock, important organs may fail leading to an increase in toxic substances in the blood which influence central nervous system (CNS) function (68). There is some evidence that DENV may enter the CNS of patients (10, 134, 144, 169). Once in the CNS, DENV may infect neurons (10) or trigger infected macrophages to interact with neurons and induce cell damage (144).

Hepatic and Gastrointestinal Manifestations

A number of hepatic and gastrointestinal complications can be seen during DENV infection, including hepatitis, fulminant hepatic failure, acalculous cholecystitis, pancreatitis,

parotitis and diarrhea (reviewed in 68). Patients with hepatic manifestations present with acute abdominal pain, jaundice, hepatomegaly and elevated aminotransferase levels (68).

WORLD HEALTH ORGANIZATION DIAGNOSTIC CRITERIA

The World Health Organization (WHO) has strict criteria for diagnosing and classifying dengue disease. DF is considered probable if the patient has an acute illness with two or more of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, leucopenia AND supportive serology by ELISA or illness concurrent with a confirmed dengue case. DF is confirmed in a lab by one of the following: virus isolation through cell culture or mosquito inoculation; viral antigen detection with ELISA, immunohistochemistry or immunofluorescence; a four-fold increase in antibody titer; or viral RNA detection by PCR.

DHF diagnosis requires fever, hemorrhagic tendencies, thrombocytopenia¹, and evidence of plasma leakage due to increased vascular permeability. According to the WHO, hemorrhagic tendencies are present if patients exhibit one of the following: a positive tourniquet test²; petechia, echymoses, and/or purpura; bleeding from mucosal surfaces, the gastrointestinal tract and/or injection sites; hematemesis and/or malena. An increase in hematocrit equal to or 20% above normal, a decrease in hematocrit following column replacement, and signs of fluid accumulation (pleural effusions, ascites or hypoproteinemia) are considered evidence of plasma leakage.

¹ The WHO characterizes DHF thrombocytopenia as less than 100,000 platelets/mm³.

² A tourniquet test is a measure of capillary fragility and is considered a measure of a patient's hemorrhagic tendency. It is performed by inflating a blood pressure cuff on the upper arm of the patient to midway between systolic and diastolic pressures for five minutes. After five minutes, the cuff is removed and the number of petechiae is counted. A tourniquet test is considered positive if there are 20 or more petechiae in a square inch area of the patients arm (104).

The WHO classifies dengue hemorrhagic fever into four grades of severity. Grades I and II are DHF while III and IV are DSS. DHF grade I is characterized by a severe fever with non-specific constitutional symptoms. The only required manifestation of hemorrhage is a positive tourniquet test or easy bruising. A patient is diagnosed with grade II DHF if they exhibit spontaneous bleeding in addition to a positive tourniquet test or easy bruising. Patients receive the more serious diagnosis of dengue shock syndrome if they exhibit signs of circulatory failure (grade III) or profound shock characterized by an undetectable blood pressure or pulse (grade IV).

Some patients with DF may show signs of hemorrhage including epistaxis, gingival bleeding, GI bleeding, hematuria and menorrhagia. However, in the absence of signs of hemoconcentration and vascular permeability, these patients are not to be diagnosed with DHF. Instead, they are assigned the diagnosis of DF with atypical hemorrhage.

Many physicians who study dengue and treat patients feel that the WHO diagnostic criteria vastly underestimate the occurrence of DHF (7). The diagnostic criteria were developed in the mid-1960's in response to outbreaks of DHF/DSS in southeast Asia and they may not be appropriate for diagnosing modern disease in all geographical areas (175). In a number of retrospective chart studies, the sensitivity of the WHO diagnostic criteria approaches only 80%, suggesting that a vast number of DHF/DSS cases are under-diagnosed and under-reported (7, 175, 193). Physicians have suggested a number of reasons for this discrepancy and offered ways to improve the WHO guidelines.

The WHO guidelines are unknown or mistakenly applied by many physicians in countries inexperienced with dengue (175). Although the hallmark of severe disease is increased vascular permeability, many physicians are uninformed and only diagnose patients

with DHF if frank hemorrhage is present (175). One difficulty in diagnosing severe disease is the reliance of the WHO criteria on the tourniquet test (TT). As described above, the TT is an uncomfortable test that requires five minutes to perform. Many extremely ill and irritable young patients are unable to remain inactive for a sustained period so this test is omitted (7, 175). The TT may be difficult to interpret in certain patients. Petechiae are difficult to see on dark skinned patients and poorly perfused children may have a false negative result (7, 175). In addition to poor sensitivity, the TT has poor specificity and may fail to differentiate between DF and DHF/DSS patients (74). If repeated more than once during hospitalization, up to 90% of DF patients can have a positive test (7). Because a diagnosis of Grade I DHF is dependent upon a positive TT, up to 20% of DHF cases may be misdiagnosed as DF if doctors fail to perform the test or it yields a false negative (175). A WHO diagnosis of DHF/DSS requires many different and repeated lab values that may be difficult to perform in resource poor hospitals overwhelmed with dengue patients (175). In order to diagnose increased vascular permeability, a physician must determine that the patient's hematocrit is more than 20% above baseline, or visualize signs of fluid accumulation. Without baseline hematocrit values, physicians must obtain a convalescent hematocrit value before assigning a firm diagnosis (76, 175). Additionally, an increased hematocrit may be missed if the patient begins rehydration therapy prior to development of DHF/DSS (7, 175). Without a measured increase in hematocrit physicians must depend on signs of fluid accumulation to diagnose increased vascular permeability. The type of test ordered and the timing of the test may aid or limit this finding. Small pleural effusions may be missed by chest X-ray or may appear only following IV fluid replacement (7). The final important characteristic of DHF/DSS according to the WHO criteria is thrombocytopenia, defined as fewer than 100,000 platelets

per μL . This value is outdated and modern physicians diagnose thrombocytopenia as fewer than 150,000 platelets per μL . Platelets fail to drop below $100,000/\text{mm}^3$ in many dengue patients and thrombocytopenia may be transient and missed if measurements are not taken frequently (7, 76). Finally, vascular permeability is a hallmark of DHF/DSS in children. According to one study, healthy children have higher vascular permeability compared to adults (9). Therefore, signs of vascular permeability may be more subtle or absent in adult patients. Based on these limitations in the WHO diagnostic criteria, the WHO should re-evaluate their guidelines. In their examination, the WHO should consider redefining thrombocytopenia as $<150,000$ platelets per μL , standardizing or removing the TT as a criteria for Grade I DHF, and adding additional measurements to incorporate the level of treatment a patient has received at the time of diagnosis (175).

DENGUE TREATMENT

The WHO has developed guidelines to treat the most common clinical manifestations of dengue infection. Because there are no antivirals effective against dengue, their recommendation is supportive care (233). Despite the lack of a cure, mortality rates due to DHF/DSS are reduced from greater than twenty percent when untreated to one percent with proper medical care as outlined below (233).

For classic DF, doctors should recommend that their patients rest and take oral fluids (88, 176). DF patients may exhibit signs of dehydration due to fever, vomiting, or diarrhea and should replace lost fluids with oral electrolyte replacement solutions (232). Doctors can treat symptoms of DF with Tylenol and antiemetics (232). Aspirin is not recommended in

dengue patients due to its anticoagulation effects and the risk of Reyes Syndrome in children (88, 176). DF patients are advised to seek immediate medical attention if they develop signs of more severe disease, including hemorrhage, shock, or mental status changes (88). Hospitalization and admission to the intensive care unit is recommended if patients present with signs of severe dehydration or shock (88, 232)³.

The mainstay of DHF/DSS treatment is intravenous fluid in the form of isotonic solutions or Ringers for twenty-four hours or until the patient's hematocrit drops (88, 232). Restoring fluids in DHF/DSS patients can be tricky as there is a fine balance between too much fluid replacement and not enough (176). Fluid overload can result in massive effusions, respiratory compromise, and congestive heart failure. Too little fluid replacement can lead to worsening shock, acidosis, and disseminated intravascular coagulation (DIC) (176). While hospitalized, physicians should continuously monitor the patient's blood pressure, hematocrit, platelet count, urinary output, and mental status. Changes in any of these can indicate worsening shock and a poor prognosis (176). If any metabolic or electrolyte imbalances appear, physicians should correct them as acidosis can lead to DIC (232). If DIC does occur, heparin therapy is indicated (88). Patients may also be transfused if they lose excessive amounts of blood due to hemorrhage (232). Hospitalization is recommended for at least twenty-four hours following defervescence and patients should not be sent home until they meet the following criteria: return of appetite, clinical improvement, no respiratory distress, stable hematocrit, platelets greater than 50,000/mm³, and good urine

³ Severe dehydration is characterized by a greater than 10% loss of body weight. Signs of shock include increased heart rate, increased capillary refill time (>2 seconds), cool, mottled or pale skin, decreased peripheral pulses, mental status changes, oliguria, a sudden increase in hematocrit or continued high hematocrit despite fluid replacement, narrowed pulse pressures (<20 mm Hg), and hypotension (232).

output (176, 232). DSS patients should remain hospitalized for at least two days following their recovery from shock (232).

DENV TRANSMISSION

Dengue is an Arbovirus that requires a blood-sucking arthropod to complete its life cycle (63). DENV has completely adapted to humans and is maintained in a mosquito-human-mosquito transmission cycle in tropical and subtropical cities (63). The primary and most efficient DENV vector is *Aedes aegypti* (63, 90). *Aedes aegypti* is a domesticated mosquito that has adapted to feed entirely on humans (135). Once a mosquito has fed on a viremic human, the virus replicates in the arthropod mid-gut and disseminates to the salivary glands within twelve to twenty-seven days (206). Following dissemination to the salivary glands, female *Aedes* mosquitoes are infected with, and able to transmit, dengue for the remainder of their lives (63, 206). *Aedes* mosquitoes lay their eggs in clean, standing water that collects in containers around homes (135). These mosquitoes are unique in that they feed on more than one person per gonadotropic cycle and will resume feeding on a second individual if interrupted (87, 135). It has been postulated the DENV infected mosquitoes may feed on humans longer than non-infected mosquitoes, increasing the risk of virus transmission (161). Secondary DENV vectors include *Aedes albopictus* and *Aedes polynesiensis* (135).

DENGUE EPIDEMIOLOGY

Within the past forty years, DENV has emerged as an important global human pathogen. Factors responsible for the emergence of this disease include: increased population size, uncontrolled urbanization, increased transportation, and decreased public health control (157). Prior to 1980, epidemics of DHF were confined to a limited number of countries in southeastern Asia. Today, over 100 nations report DENV disease. These countries are located in Africa, Latin America, the eastern Mediterranean, southeast Asian and the Western Pacific (71, 233). Dengue transmission is heaviest between 35°N and 35°S latitude, the northern and southern most boundaries of the vector, *Aedes* mosquitoes (61).

DENGUE PATHOGENESIS

Once introduced into a human host through the bite of an infected mosquito, DENV replicates at the site of the bite or regional lymph node (72). The initial target cells of DENV are skin Langerhan's cells and dendritic cells (DC) (91, 140, 234). DC are potent antigen present cells (APC) that express a variety of pathogen recognition receptors; one of these, dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN) serves as an attachment factor for DENV (133, 153, 162, 220). DENV replicates with DC, macrophages ($M\Phi$), and other monocytes, seeding a serum viremia (6, 11, 133, 145, 153, 162, 220, 228). From the serum, DENV can enter a number of organs and tissues.

Risk factors for the development of severe disease suggest mechanisms of disease pathogenesis. These risk factors include: i) pre-existing DENV immunity, ii) viral genotype, and iii) sequence of infecting serotypes (71, 72). Based on these observations, it has been

proposed that viral virulence and aberrant host immune responses are responsible for the development of severe disease.

VIRAL VIRULENCE

One hypothesis on disease pathogenesis suggests that some viruses are more virulent and that severe disease is associated with these specific viruses (177). All four serotypes of DENV have been isolated from DHF/DSS patients, showing clearly that one serotype is not more virulent than the other three (72). Within each serotype, viruses separate into different genotypes based on nucleic acid sequence differences between strains (172). Specific genotypes of DENV2 and DENV3 are associated with the emergence of DHF in new geographical areas (64, 142, 173, 174). A new DENV2 genotype was introduced to the Caribbean from southeast Asia in 1981 resulting in a DHF epidemic that continues in Latin America today (174). Similar observations have been made regarding the introduction of new DENV3 genotype into Latin America and Sri Lanka (64, 142). The newly introduced DENV2 and DENV3 were associated with DHF in their geographic regions of origin and appear to have replaced the less virulent native DENV3 viruses in Latin America and Sri Lanka (142, 173, 174).

Viruses associated with severe disease have altered growth characteristics compared to viruses circulating prior to the emergence of DHF/DSS. The southeast Asian DENV2 replicates more efficiently in primary human dendritic cells and *Aedes aegypti* mosquitoes (39). In these systems, more virus is released during infection with the southeast Asian viruses compared to American viruses (39). This increased efficiency may lead to higher serum and salivary gland titers. The viruses introduced into Sri Lanka and associated with DHF replaced native DENV3 viruses within a short period of time (142). The invasive

viruses infect similar percentages of *Aedes aegypti* mosquitoes but replicate to higher titers in the midgut and disseminate more efficiently to the salivary glands suggesting they may have a fitness advantage in mosquitoes (85). An *in vitro* phenotype in human cells has not yet been characterized.

HOST IMMUNITY

The observation that DHF/DSS occurs primarily in children experiencing a secondary DENV infection suggests that the host immune response plays a role in disease pathogenesis (72). Halstead first proposed that DHF was “due to a self-destructive immune response sensitized by the first [DENV] infection” (72). Evidence supporting his hypothesis included: i) most all patients with DHF/DSS have detectable DENV IgG when they present to the hospital, ii) severe disease is seen only in areas where two or more serotypes are simultaneously or sequentially endemic, and iii) severe disease is restricted to local residents whereas visitors present with DF (84). In the DENV field, pre-existing host immunity to DENV is generally accepted to play a role in disease pathogenesis. However, there are conflicting opinions as to whether humoral or cell-mediated immune responses contribute to disease pathogenesis.

Humoral Immune Responses and Antibody Dependent Enhancement

In 1970, Halstead first proposed a role for anti-DENV antibody in the development of severe disease (79). He observed that shock during a primary infection was rare and only occurred in patients younger than one year. It was later shown that Thai infants developed DHF during a primary infection when the titers of their maternally derived anti-DENV antibody fell below neutralizing levels (110). *In vitro*, the maternal antibodies were able to neutralize or enhance DENV infection of mouse macrophages at low antibody concentrations

(110). Kliks *et al* were able to demonstrate a relationship between the neutralizing or enhancing activity of pre-infection sera and disease severity in Thai children (111). In this study, the presence of enhancing antibody in pre-infection sera was a strong risk factor for the development of severe disease (111).

Animal studies have supported these findings in Thai children. Non-human primates experiencing a secondary infection had higher serum titers than primarily infected animals (82, 139). Additionally, passively transferred antibodies enhance viral titers in DENV2 and DENV4 infection (54, 73).

Based on the data discussed above, antibody dependent enhancement (ADE) has been proposed as a mechanism for the development of severe disease. Following a primary DENV infection, individuals develop cross reactive immune responses of two to three months in duration (183). Cross-reactive antibodies wane and a strong homotypic neutralizing antibody response to the homologous serotype persists for the life of the patient (74, 183). When a patient is infected with a second DENV serotype, antibodies from the primary infection may bind to but not neutralize virus. In this situation, circulating APCs expressing Fc receptors may bind antibody-antigen complexes and internalize virus (reviewed in 75). After entering APC via ADE, DENV may replicate to high titers, trigger cytokine production, or stimulate immune signaling (75).

Despite enhancement data in humans and collaboration in animals, some DENV researchers are not convinced that ADE plays an important role in the pathogenesis of DHF and DSS. At least one group has been unable to replicate data correlating *in vitro* enhancement with an increased risk of developing severe dengue disease (126). It is possible that each cohort of dengue patients may differ in the contributory role of ADE in

their disease outcome or that ADE will differ depending on the infecting virus. It is also possible that the different *in vitro* enhancement assays led to differing results.

Cell Mediated Immunity

The second explanation for immune-mediated development of severe disease involves T cell-mediated immunity. Since the onset and resolution of increased vascular permeability in DHF/DSS is rapid, some groups suspect that cytokines released by activated T cells trigger the development of severe disease (178). There is strong evidence that a high level of T cell activation is associated with DHF (156). Viral antigens presented to memory CD4+ and CD8+ T cells stimulate them to proliferate and release proinflammatory cytokines (reviewed in 156). The cytokines produced include TNF- α and IFN- γ . High levels of these cytokines have been documented in DHF patients, lending credence to a role for activated memory T cells in disease pathogenesis (224).

It is probably an interplay of viral virulence, humoral adaptive immunity and cell mediated immunity that leads to the development of DHF/DSS. The pre-existing immunity towards dengue probably creates an environment in which the virulent DENVs trigger a pathogenic adaptive immune response that results in DHF/DSS rather than subclinical disease.

HUMAN GENETIC RISK FACTORS

Variance between individuals in clinical outcomes of a secondary infection with the same dengue viruses has led some groups to postulate that human genetics affect disease progression. This phenomenon was first observed in the absence of severe disease in specific ethnic groups (reviewed in 36). An important example of these ethnic differences in disease

susceptibility is illustrated in the resistance of African blacks to the development of dengue illness (36). In western Africa, multiple DENV serotypes co-circulate but there are no documented cases of DHF in native Africans. Additionally, dengue infection in Haiti is symptomatic in only white visitors, despite evidence of multiple dengue infections in Haitian children of African descent (83). Because of the presumed link between genetic background and susceptibility to developing severe disease, many genetic studies have investigated the correlation of gene polymorphisms with susceptibility to and protection from developing severe dengue disease (reviewed in 36). Since DHF and DSS manifestations are believed to be due to aberrant immune stimulation, most of these studies have investigated the role of innate and adaptive immunity genes in susceptibility to severe disease.

INNATE IMMUNE FACTORS

Important components of the innate immune response to dengue include monocytes and dendritic cells, serum complement, and natural antibodies. Dengue initially infects monocytes and dendritic cells during human infection and may antagonize their ability to produce interferon and cytokines, as well as their ability to communicate with adaptive immune cells. Complement can coat virus in the presence or absence of antibody, leading to neutralization (reviewed in 16). Dengue has been shown to co-localize with complement on the surface of circulating cells (17, 180). Natural antibodies can bind to dengue and cause agglutination.

DC-SIGN, a C-type lectin expressed on human dendritic cells, serves as an attachment factor for DENV (133, 153, 220). DC are important antigen presenting cells that respond to viral infection and bridge the innate and adaptive immune responses. Infection by DENV may modulate innate immune responses and communication between DC and T cells.

Therefore, Sakuntabhai *et al* investigated whether polymorphisms in DC-SIGN correlate with disease severity (185). In Thai children, one allele, DCSIGN1-336, did affect disease severity (185). A G at position 336, located in the DC-SIGN promoter, changed multiple predicted transcription factor binding sites and decreased SP1 transcriptional activity compared to an A at this position. SP1 is a zinc-finger motif containing transcription factor that binds directly to DNA and enhances transcription of specific genes. In this population, the G allele was rare in DF patients compared to controls and was strongly associated with DHF (185). The authors postulated that the decreased transcription associated with the G allele might reduce DC-SIGN expression on the surface of DC, thus decreasing DENV infection of these cells (185). Instead, virus may be forced to enter cells via an alternate pathway, specifically Fc receptors via antibody dependent enhancement, resulting in DHF.

In addition to DC, monocytes are a major target of DENV infection. Therefore the role of glucose-6-phosphate dehydrogenase in dengue disease has been investigated (26). Glucose-6-phosphate dehydrogenase is an enzyme located in the cytoplasm of all cells that is involved in mopping up free radicals that result in oxidative stress (26). Specific glyucose-6-phosphate dehydrogenase alleles result in decreased enzyme activity and confer increased susceptibility to bacterial infections (137, 226) and increased severity of viral infections (55) due to leukocyte dysfunction (40, 57, 226). Because dengue targets monocytes, it was investigated whether glucose-6-phosphate dehydrogenase deficiency affects disease outcome (26, 219). Such studies determined that red blood cell glucose-6-phosphate dehydrogenase deficiency is associated with an increased risk of developing DHF in Thai children (26, 219). Additionally, PBMCs from deficient patients were more readily infected with DENV2 than monocytes from normal controls (26). Because glucose-6-phosphate dehydrogenase

deficiency leads to leukocyte dysfunction, cells from affected patients probably don't respond as efficiently to DENV infection, leading to increased viral replication (35). Increased replication in these cells likely results in high serum viremia which has been associated with DHF (5).

Complement activation has been implicated in the development of severe dengue disease (5). In Thai patients, high levels of activated complement in acute phase sera were strongly correlated with the development of DHF (5). Three pathways can lead to the activation of complement: the mannose binding lectin, the classical antibody dependent, and the alternative (16). The mannose binding lectin (MBL) recognizes carbohydrate patterns on microorganisms and activates complement (16). Additionally, once this lectin recognizes carbohydrate patterns, it can promote complement independent opsonophagocytosis, modulate inflammation, and promote apoptosis (5). MBL deficiency has been associated with increased susceptibility to HIV and other viral infections, and protection from intracellular bacterial diseases (5). Because MBL deficiency could limit complement activation, the serum levels of MBL and MBL2 genotype have been investigated for a correlation to dengue disease severity in a Brazilian cohort (1). In this population, an allele associated with low serum level of MBL appeared protective against thrombocytopenia but not disease development (1). Patients with the wildtype MBL2 allele may have been more susceptible to thrombocytopenia because high levels likely promote binding to viral carbohydrates resulting in complement activation and platelet destruction (1).

The role of TNF- α , a cytokine associated with the development of severe disease (160, 224), in susceptibility to dengue disease has been examined in a Venezuelan cohort (52). In this group, the frequency of the TNF α -308A allele was increased in DHF patients.

This allele is a strong transcriptional activator of TNF- α expression (52). This study supports previous studies examining cytokine levels and suggests that high levels of TNF- α contribute to the development of DHF and DSS.

A final component of the innate immune response that has been examined for a role in dengue infection is natural antibodies. Individuals make antibodies to ABO blood antigens. Patients who express none or one blood group antigens make antibodies to the carbohydrate residues associated with the other blood group antigens they lack. These antibodies may recognize DENV carbohydrates and play a role in innate protection from disease. Thus, the role of blood group in the development of severe dengue disease was examined (103). In a Thai cohort, individuals with blood group AB were more likely to develop severe DHF (grade 3 vs grades 1 and 2) (103). Thus, the AB blood groups may be a risk factor for the development of severe disease.

The studies summarized above show that a number of different genes involved in innate immunity may be important in the development of DHF and DSS. It is too soon to determine which components of the human innate immune system are the most important contributors to the pathogenic immune response to DENV in DHF and DSS. It is clear from the studies above and previous studies that the innate immune genes contribute to pathogenesis in the context of a secondary infection.

ADAPTIVE IMMUNE FACTORS

The adaptive immune system plays an important role in dengue pathogenesis. Because secondary infection greatly increases the risk of developing severe disease, it appears there is a role for pathogenic adaptive immunity. Both B and T cells have been examined for a role in the development of DHF/DSS (reviewed in (119)). Because cytotoxic

T cells may play a role in the development of DHF/DSS, major histocompatibility (MHC) class I alleles have been examined. Additionally, the role of MHC II and antibody subclass has been examined.

The vitamin D receptor is expressed on immune cells and is associated with the stimulation of cell mediated immunity, decreased IgG production, and decreased lymphocyte proliferation (131). Thus it was examined whether SNPs in the gene encoding the vitamin D receptor play a role in the outcome of dengue disease. One SNP, a **tt** at position 352 of the receptor, results in an enhanced cell mediated immune response and has been associated with enhanced viral clearance and resistance to intracellular bacteria (131). In a Vietnamese cohort, the **t** SNP is associated with protection against severe DHF (131).

Different HLA class I alleles have been correlated with different cytotoxic T cell responses to dengue (53). A number of groups have examined whether specific class I MHC alleles are associated with severe dengue disease (reviewed in 36). In Thai populations, some alleles of HLA-A and HLA-B appear protective against DHF and others appear to increase susceptibility to severe disease (reviewed in 27). Currently, no single HLA allele is consistently associated with DENV disease severity indicating that processing and presenting of endogenous dengue antigens and the T cell repertoire is a complex association and may be specific to certain ethnicities (36).

A final component of the cellular adaptive response is TAP-1 and TAP-2. TAP proteins, or transporter associated with antigen processing, translocate peptides from the cytosol into the endoplasmic reticulum where the peptides associate with Class I (211). A recent study, performed in a south Indian population, found that three specific TAP alleles may influence primary dengue infection (211). Specifically, the frequencies of isoleucine at

position 333 and asparagine at position 637 of TAP1 were decreased in primary DHF and DSS patients compared to DF patients. Additionally, the frequency of valine at 379 of TAP2 was lower in primary DHF patients compared to DF patients (211). Thus, it appears that homozygous patterns of isoleucine at TAP1³³³, valine at TAP2³⁷⁹, and asparagine at TAP1⁶³⁷ are protective against the development of DHF.

Only a single study has been conducted to examine the role of MHC class II alleles in the development of dengue disease (122). In previous studies, HLA-DRB1 has been implicated in the pathogenesis of several diseases (122). Therefore, the role of HLA-DRB1 alleles was examined for a role in dengue pathogenesis in a Mexican population. The frequency of HLA-DRB1*04 was decreased in DHF patients compared to DF patients, indicating that this allele is negatively associated with DHF risk in this population (122). From this study it appears that HLA-DRB1*04 may act as a resistance factor against DHF.

Immunoglobulin classes and Fc receptors have been associated with severe dengue disease. This association may be due to the ability of certain IgG subclasses to bind to specific Fc receptors, resulting in antibody internalization. If IgG is bound to DENV, the resultant ADE of virus infection may influence disease outcome (reviewed in 75). IgG1, a complement fixing antibody subclass, has been associated with the development of severe dengue disease (114, 221). The levels of this antibody subclass are greatly increased in the acute phase serum of DHF and DSS patients compared to DF patients who have high levels of IgG2 (114, 221). Loke *et al* investigated the role of Fc_γ receptor allele IIA in the development of DHF/DSS in Vietnamese patients (131). They found that homozygosity for arginine at position 131 of IIA appears protective against the development of DHF, and that an arginine at this position of the receptor is associated with decreased opsonization of IgG2

antibodies (131). Thus, homozygosity for this allele may result in less antibody dependent entry of DENV into host cells.

As with innate immunity, it is not clear from the studies presented above which adaptive immune system genes play a significant role in the development of DHF. Genes may play different contributory roles in different ethnic or racial groups or upon infection with different viruses. It is clear that much more work must be done to understand the role that host genetics play in the development of DHF and DSS.

ANIMAL MODELS

One of the difficulties of studying dengue pathogenesis is the lack of a good animal model that recapitulates human disease. Currently there are two types of animals used to study dengue - non-human primates and mice. Neither is a complete model that reproduces human disease. DENV cannot productively infect all non-human primates and does not cause symptomatic disease in species that do develop viremia. Immunocompetent mice do not usually develop viremia following infection with DENV strains isolated from human patients. Clinical isolates of DENV can infect various strains of immunocompromised mice, but cause neurologic disease instead of DF or DHF seen in humans (reviewed in 235). Hemorrhagic disease or shock syndrome has been observed in two mouse models but both are contrived. AG129 mice deficient in interferon signaling exhibit signs of shock following intravenous infection with a mouse adapted DENV2, D2S10 (163, 200). Immunocompetent C57BL/6 mice develop hemorrhage within days of intradermal inoculation with extremely high doses of DENV2 16681 (28, 236).

NON-HUMAN PRIMATE MODELS

DENV naturally infects only humans and non-human primates (206). Despite circulating naturally in a jungle sylvatic cycle in Asian and African primates (172, 227), there is no evidence that DENV causes disease in non-human primates (reviewed in 235). Non-human primates do develop viremia (81) and are the closest model to human infection; therefore, rhesus macaques are used to test vaccine candidates.

Chimpanzees

Although closely related to humans, chimpanzees do not serve as a good model for DENV infection (191). Following intradermal or subcutaneous inoculation, single strains of DENV1, DENV2, DENV3 or DENV4 failed to cause overt, febrile or hematologically detectable disease (191). Following primary infection, chimpanzees developed low-level viremia within three days of inoculation and displayed neutralizing antibody responses within eight weeks (191). Individual animals did develop some clinical abnormalities. Some chimpanzees exhibited a skin rash at the injection site, many developed small, palpable lymph nodes, and one had a slightly enlarged spleen (191). However, no chimpanzees developed thrombocytopenia. Chimpanzees were infected with a heterologous DENV serotype twenty-six months following primary infections to simulate secondary infection. Individuals infected with DENV1 or DENV4 developed low level viremia, but those infected with DENV2 or DENV3 did not have detectable virus in their sera (191). Factors such as animal size and cost have precluded further characterization of dengue infection in chimpanzees.

Macaca mulatta

The majority of the work characterizing DENV infection of non-human primates has been through infection of rhesus macaques (*Macaca mulatta*). Following subcutaneous inoculation of juvenile macaques with DENV, individuals did not develop symptomatic disease (81). Despite inguinal and axillary lymphadenopathy and viremia, monkeys did not exhibit changes in behavior or appetite. Viremia developed between two and four days post infection and lasted for three to six days (81). Laboratory tests were performed to determine if the macaques developed characteristics of human disease. Some monkeys exhibited leucopenia with relative lymphocytosis but none displayed thrombocytopenia or hypoproteinemia (81). Additionally, no monkeys showed signs characteristic of DHF/DSS. During this primary infection, the distribution of the virus was limited (139). Prior to the development of viremia, DENV was detected only at the injection site and in the regional lymph node. At the time of viremia, virus was slightly more widespread and was located in skin, lymph nodes and other reticuloendothelial system organs (139). Once viremia had subsided, DENV was detected in the gastrointestinal tract and the respiratory tract in addition to lymph nodes and peripheral leukocytes (139). In all of these organs, in situ staining revealed that DENV antigen was restricted to lymphocytes and macrophages (78). DENV-infected macaques were tested for antibody responses to the infecting and heterologous serotypes. Regardless of viremia levels, all animals developed neutralizing antibodies specific to the infecting serotype (81). Based on these studies, it was concluded that rhesus macaques have viremia kinetics and antibody responses similar to DENV-infected humans.

In an attempt to develop a model of DHF/DSS, DENV-immune macaques have been inoculated with heterologous serotypes of DENV (82). In all secondary infections except for

DENV3 monkeys developed delayed onset viremia compared to primary infection with the same serotype. Secondary infection with DENV3 was unique in that monkeys had peak viremia greater than that seen in primary DENV2 infection (82). Additionally, some monkeys exhibited thrombocytopenia. In all secondarily infected macaques, virus recovery from tissues was increased compared to primary infection (139). One DENV4-immune animal was of particular interest because it showed lab values consistent with those seen in human DSS patients (82). Following DENV2 infection, this animal developed leukocytosis, thrombocytopenia and hypoproteinemia, and exhibited increased prothrombin time as well as elevated hematocrit (82).

One other characteristic significant to the rhesus macaque model is the phenomenon of antibody dependent enhancement during DENV infection (54, 73). Subneutralizing quantities of DENV immune sera (73) or monoclonal antibodies (54) can be intravenously administered to dengue naïve macaques, rendering their sera enhancing *in vitro*. Following infection with either DENV2 or DENV4, monkeys developed increased viral titers compared to control animals (54, 73). The CH₂ domain of the Fc region of IgG appears responsible for mediating this phenomenon (54).

Because of the similarity of the rhesus macaque infection kinetics and immune response compared to human DENV infection, their small size and ease of care, and the enhancement phenomenon similar to human infants (111, 205), rhesus macaques are considered to be a reasonable model of DENV infection. Following primary infection with all four serotypes, animals remain susceptible to the remaining three serotypes (77). Therefore, rhesus macaques are considered a good model to test the ability of a DENV vaccine to produce neutralizing or enhancing antibodies. However, this model is not without

limits. Primarily, animals do not develop symptoms of illness during primary or secondary infection. Thus, the only way to differentiate neutralization or enhancement of a virus is through measurement of serum virus titers. One of the reasons for the difference in human and macaque clinical profile in response to DENV may be due to a different innate immune response mounted against the virus. There has been preliminary work demonstrating that macaques produce different cytokines in response to primary DENV infection compared to humans. Five days post infection these animals produce a potent antiviral response characterized by the induction of interferon-stimulated genes, but absent of IFN- α , IFN- β , IFN- γ and TNF- α (190). In humans, there is potent upregulation of these genes, particularly TNF- α (160, 224).

MOUSE MODELS

There are three types of mouse models of DENV infection: immunocompetent mice, immune deficient mice, and human chimeric mice (reviewed in 218, 235). All of these models have limitations and none closely mimic human disease. Human clinical isolates of DENV do not infect immunocompetent mice very well and these mice do not display symptoms consistent with human disease (reviewed in 218). An ideal mouse model would be physiologically relevant in route of infection, immune status of the host, and clinical signs of disease. A model in which some animals develop a DF-like disease and other animals exhibit signs of DHF/DSS, particularly following a secondary heterologous infection, is desired and is being pursued.

Immunocompetent Mouse Models

The ability of DENV to infect four immunocompetent mouse strains has been investigated. Initial studies with DENV and the albino swiss mouse involved intracranially

inoculating suckling or weaning mice with human clinical isolates and passaging them until mice developed paralysis (37, 184). Once adapted to mouse brains, these viruses were no longer able to infect and cause disease in humans (184). More recently, dengue has been studied predominantly in two mouse strains: Balb/c, and C57BL/6.

Balb/c mice have been tested as a model for DENV infection (4, 154). These mice developed paralysis at four days post infection when inoculated intraperitoneally with a mouse-adapted DENV2 (4). On necropsy they showed thrombocytopenia, decreased hematocrit, hemorrhagic necrosis of the spleen, and liver damage (4). This pathology was TNF- α mediated and could be delayed by administering an anti-TNF- α monoclonal antibody (4). When Balb/c mice were intraperitoneally inoculated with a human DENV isolate, the mice developed no clinical signs of disease (154). The human DENV did appear to target the liver of these mice, since hepatic injury was seen from two to seventeen days post infection and mice exhibited elevated liver enzymes. Additionally, the mice developed low-levels of viremia (154). A Balb/c mouse model has not been further explored as a model for human DENV infection as liver and CNS involvement are not prominent characteristics of primary human infection.

The other immunocompetent mouse strain that has been investigated as a mouse model for DENV infection has demonstrated some promise. DENV2 16681, a Taiwanese isolate, is able to infect C57/BL6 mice and induce hemorrhage (28, 236). Three days following intradermal inoculation of three billion infectious virions, mice develop high serum viremia and hemorrhage at various sites (28). In addition to hemorrhage at the site of inoculation, mice exhibit bleeding at lymph nodes and in the gastrointestinal tract (28). The sites of gross hemorrhage exhibit red blood cell extravasation and endothelial cell apoptosis

microscopically (28). Hemorrhage in this model is dependent on endothelial cell apoptosis induced by TNF- α and free radical production (236). The route of viral inoculation appears important in the C57BL/6 model since intravenous inoculation results in liver injury rather than hemorrhage (29). While promising, this model is not without limitations. Hemorrhage in DENV-infected people is exhibited primarily during secondary infection of a DENV-immune individual with a heterologous serotype. In this model, hemorrhage is seen during a primary infection. Additionally, increased vascular permeability is the hallmark of DHF, not hemorrhage (233). Therefore, in an ideal model mice would show signs of fluid accumulation and shock instead of bleeding. Another concern with the model is the enormous infectious dose required. As the viral dose decreases, mice exhibit signs of neurological disease rather than hemorrhage (28).

All of the mouse models of DENV infection in immunocompetent mice are limited in their applicability to human disease. The Albino Swiss and Balb/c models require mouse adaptation of DENV strains and exhibit clinical symptoms irrelevant to human disease. While human isolates of dengue can infect A/J mice, infected individuals do not develop viremia, an important component of human disease, and the symptoms mice exhibit do not resemble human disease. While the most promising of the four immunocompetent mouse models, C57BL/6 mice are not ideal. An infected C57BL/6 mouse exhibits hemorrhage following intradermal inoculation but a high infectious dose is necessary and hemorrhage occurs within three days of a primary infection. In order to more accurately resemble human disease, an infected mouse should develop fever and viremia following a primary infection and hemorrhage should occur during a secondary infection as serum virus titers fall.

Immune-Deficient Mouse Models

Many labs have attempted to develop a mouse model of DENV infection in immune-deficient mice since human isolates of DENV fail to replicate in immunocompetent mice and these mice fail to exhibit symptoms of DF and DHF/DSS. Mice deficient in the adaptive and the innate immune system have been infected with DENV. None of these immune-deficient mice exhibit signs consistent with human disease and the lack of important immune components confuses data interpretation.

Very little work has been performed to investigate DENV infection of mice deficient in adaptive immune components. Severe combine immunodeficient (SCID) mice, lacking both T and B cells, are resistant to intraperitoneally inoculated dengue and do not develop signs of disease or viremia (129). If the virus is injected intracranially, these same mice develop paralysis and die despite showing no clinical, pathological, or laboratory abnormalities (2). μ MT mice, deficient in B cells, do not show signs of infection and virus cannot be recovered from extraneural or neural tissues (199). Mice deficient in CD4⁺ or CD8⁺ T cells survive DENV infection and virus is not recovered from their tissues (199). RAG knockout mice, deficient in both B and T cells, are extremely susceptible to infection with a mouse-adapted DENV1 isolate. These same mice are able to limit virus replication when infected with a non-mouse adapted DENV2 (199). The resistance of the RAG knockout mice to DENV infection indicates that B and T cells are not required to limit DENV infection.

A/J mice, which lack the complement component C5, intravenously infected with PL046, a Taiwanese DENV2 isolate, develop paralysis and die within eight to fourteen days of infection (96, 198). DENV2 was not recovered from serum but virus was detected in the

central nervous system and the liver (96). In addition to paralysis, mice developed thrombocytopenia and leukopenia (198). DENV2 infected mice had high numbers of activated B and T cells in their spleens and produced copious amounts of IFN- γ (198). Because A/J mice do not develop high serum viremia and their major symptom of disease is paralysis, they are not a good model to study human DENV disease.

To determine if transgenic mice deficient in the IFN response were susceptible to DENV, mice deficient for IFN- α/β (A129), IFN- γ (G129), and both IFN- α/β and IFN- γ (AG129) receptors were intraperitoneally and intravenously inoculated with DENV (102, 192, 199). Mice deficient in either IFN- α/β or IFN- γ receptors did not exhibit symptoms of disease when infected intraperitoneally (102). When infected intravenously, these same mice developed high levels of viremia and G129 mice succumbed to infection (199). AG129 mice infected via both routes developed a dose-dependent paralysis following infection with mouse adapted and human isolated DENV1 and DENV2 (102, 199). Infected mice exhibited a dose-dependent viremia, an enlarged spleen, an inflammatory cytokine response and convalescent IgG (102, 192). Infectious DENV was found in the CNS (199) and NS1 was detected in serum (192). Additionally, viral antigen was detected in dendritic cells and macrophages of the spleen and lymph nodes respectively (6, 121).

STAT1-deficient mice, which are less immunocompromised than AG129, have been intravenously infected with DENV1 and DENV2 (201). These mice are resistant to DENV disease and control virus load early during infection. This model of DENV infection has also demonstrated that CLEC5A, a C-type lectin, plays a role in DENV-induced vascular leakage and hemorrhage (31).

In order to develop a model of DENV infection that more closely models severe human disease, one group alternately passed a human DENV2 isolate between *Aedes C6/36* cells and AG129 mice. The resultant virus, D2S10, causes a lethal infection of AG129 mice between three and five days post infection but is unable to infect wildtype mice (200). Infected AG129 mice exhibit hunching, fur ruffling and lethargy, but not paralysis. An important component of this DENV model is increased vascular permeability (200). The microvasculature of the liver and small intestine display increased permeability and damage at a cellular level (200). The increased vascular permeability in AG129 mice following infection with D2S10 is dependent on TNF- α in the serum (200). The ability of D2S10 to cause vascular permeability is mapped to two mutations in the viral envelope at positions 124 and 128 (163, 200). In D2S10, substitutions at these positions result in a more negatively charged external surface of E. This change in charge limits the ability of DENV to bind to heparin sulfate, and viral clearance from the peripheral circulation is delayed (163).

AG129 mice infected with D2S10 represent a model that mimics human DSS, but this model has two major drawbacks. First, it does not allow for analysis of an immunocompetent host response to DENV. Second, the virus has been adapted via serial passage between mosquitoes and a host so that it may induce disease in a mouse-specific manner.

Human Chimera Mouse Models

Since most human isolates don't easily infect immunocompetent mouse strains, a few groups have attempted to infect humanized mice with clinical DENV strains. In these models, human cells are transplanted into immunocompromised mice lacking an adaptive immune system (reviewed in 235). DENV appears to infect the transplanted human cells,

resulting in viremia and signs of clinical illness. Despite moderate success with infection and disease development, human chimera mouse models are fraught with several limitations. Due to the absence of an intact immune system in these mice, there is limited crosstalk between the mouse's innate immune response and the adaptive immune response developed in human cells. Additionally, these mice have proven extremely difficult to work with. Transplantation of these mice with human cells tends to be laborious and engraftment rates vary. Furthermore, these mice are often sickly and fail to thrive.

Because some human DF and DHF/DSS patients exhibit signs of liver involvement, two groups have attempted to transplant SCID mice with human liver cell lines. Mice transplanted with the human hepatocarcinoma cell line HepG2 were intraperitoneally infected with DENV2 (2). Early during infection, virus was detected in the liver and in serum. These mice lost weight and developed hind limb paralysis within two weeks of infection. In addition to manifesting physical signs of infection, mice exhibited marked thrombocytopenia, elevated hematocrit, and increased prothrombin time (2). In addition to these laboratory abnormalities, HepG2 engrafted mice had hemorrhaging in the liver and small intestine. These findings suggest that this mouse model shows DHF/DSS-like manifestations (2).

Since monocytes and dendritic cells appear to be the primary target of human DENV infection, two groups have infected CD34⁺ hematopoietic stem cell engrafted mice with DENV (8, 120). Non-obese diabetic (NOD)/SCID mice transplanted with these stem cells have circulating DC (8) and display symptoms of DF following DENV2 infection. Between two and eight days post infection, infected mice experienced an increase in body temperature, visible skin rash and marked thrombocytopenia (8). In addition to these clinical

signs, engrafted mice had virus in their spleens, liver and skin (8). All mice recovered from DENV infection within three weeks of inoculation. $RAG^{-/-}\gamma_c^{-/-}$ engrafted with $CD34^{+}$ hematopoietic stem cells (RAG-hu) are reconstituted with human T and B cells, DC, and $M\Phi$ (120). Following intraperitoneal and subcutaneous inoculation with a DENV2 cocktail, RAG-hu mice supported productive viral infection and exhibited viremia for up to three weeks following infection (120). These mice displayed a high fever for up to two weeks following infection but did not show other signs of DF or DHF/DSS (120). The RAG-hu mouse model of DENV infection is intriguing because these mice developed human neutralizing IgM and IgG responses to DENV (120). Thus, this model may potentially provide an avenue to study human immune responses to primary DENV infection.

DENGUE PREVENTION

PREVENT TRANSMISSION

The most effective way to decrease the spread of dengue is through control of the vector, *Aedes* mosquitoes (90, 233). Environmental management and chemical eradication are means by which the mosquito vector can be controlled (233). The most cost effective way to decrease dengue transmission is to eliminate *Aedes* breeding sites in and around the home (61, 88). *Aedes* mosquitoes breed in clean water and thus larval reduction can be achieved by eliminating water containers that serve as larval habitats (61). The most effective way to implement this environmental management is through community-based efforts involving health education and community outreach (61). While this approach may be slow in eliminating mosquito populations, it is the most sustainable. In the recent past, vertical paramilitary organizations were successful in nearly eliminating *Aedes* mosquitoes

and drastically reducing dengue transmission in Latin America (61, 93). Once success was achieved, resources were reallocated and dengue has returned (61). Chemical methods of vector elimination are expensive and may adversely affect human health. Therefore, chemical spraying should be used only during an outbreak to curb transmission.

VACCINATION

Due to the current inability to reduce dengue transmission through vector control, a vaccine is needed to prevent transmission and disease in endemic areas (196). Currently there is no licensed dengue vaccine for use in humans. Adaptive immunity contributes to resolution of infection and has a role in preventing reinfection. Unfortunately, adaptive immunity also plays an important role in the enhancement of disease severity as discussed above (231). Therefore, immunization against dengue must address issues of protective immunity and the role of pathogenic immunity (231).

There are many challenges to the development of an effective dengue vaccine (reviewed in 69, 231). Currently, we do not entirely understand immunity to DENV and the correlates of protective immunity are unknown. Due to the risk of more severe disease with a heterologous secondary infection, an effective vaccine must induce protective immunity against all four serotypes of DENV (80). In endemic areas, a dengue vaccine must be able to overcome pre-existing immunity from either passively transferred maternal antibodies or a previous dengue infection (231). Additionally, an effective vaccine must induce long-term humoral and cell-mediated immunity (69). Thus, the ideal dengue vaccine would meet the following criteria (reviewed in 69, 231): be free from significant reactogenicity, induce lifelong immunity against infection by any of the four dengue serotypes, be suitable for use in infants, not increase the risk of DHF/DSS from concomitant or subsequent dengue

exposure, induce long-term humoral and cell-mediated immunity, and be economical to produce with minimal or no repeat immunizations. Current efforts to develop a dengue vaccine can be divided into four groups: live attenuated virus vaccines, chimeric vaccines, inactivated virus vaccines, and subunit/vectored vaccines.

Live Attenuated Virus Vaccines

Most of the groups working towards a dengue virus vaccine are focusing on the use of live attenuated virus. This form of vaccine is preferred because it can produce a durable humoral and cell-mediated immune response to both structural and non-structural genes (231). Because the virus is alive, attenuated virus vaccines have the potential to produce clinical disease if underattenuated or revert to wildtype. Thus, live attenuated virus vaccine candidates must be tested extensively in humans and meet specific criteria. It is preferred that the attenuated virus be genetically stable and the attenuating mutations understood (69). A live attenuated virus should be limited in its ability to replicate systemically, producing little to no viremia (231). However, in a tetravalent formulation, each component of the vaccine must be able to replicate sufficiently to produce a balanced immune response (69). To date, four different live attenuated dengue vaccines have been developed with varying degrees of success. Three groups have passaged clinical isolates of DENV in tissue culture until the virus has become attenuated. The fourth group has relied on rationally designed genetic attenuation.

The first live attenuated tetravalent dengue vaccine was developed in a joint effort between Mahidol University in Thailand and Sanofi Aventis. They passaged a clinical isolate of each of the four serotypes in cell culture until they isolated viruses that were attenuated in mouse and monkey models (231). An unsuccessful Phase I clinical trial was

initiated in Thailand and this vaccine has since been abandoned due to safety concerns. Despite limited replication in mice and monkeys, it was found that this vaccine was under-attenuated upon vaccination of Thai adults and children (181, 182, 186). In these clinical trials, up to ninety percent of patients complained of systemic symptoms, and numerous patients developed clinical dengue fever (181, 182). Additionally, the tetravalent formulation induced an imbalanced immune response (181, 182).

The United States Army and GlaxoSmithKline are jointly developing a live attenuated tetravalent dengue vaccine. Their vaccine is based upon viruses attenuated by passage in primary dog kidney cells with terminal passages in primary rhesus lung cells (231). The monovalent and tetravalent vaccines have been evaluated in rhesus macaques and are attenuated in their ability to produce viremia (47, 106, 215). Additionally, vaccinated macaques developed decreased viremia following challenge with wildtype virus (47, 106, 113, 215). In response to the tetravalent formulation, macaques developed a slightly unbalanced antibody profile to the four serotypes, suggesting low levels of interference (215). Based on these promising results in primates, multiple formulations of a tetravalent vaccine have been tested in humans in Phase I/II clinical trials (204, 214). In these trials, all patients reported mild systemic symptoms following their first dose of the vaccine. Following the second vaccine dose, all patients developed tetravalent neutralizing antibodies, however the response was unbalanced (204, 214).

The third live attenuated dengue vaccine generated by serial passaging is at a much earlier stage of development than the ones described above. This virus, PDK-53, is a monovalent DENV2 vaccine attenuated through mutations outside of the structural genes. It

has currently been tested in mice and monkeys and been deemed safe and immunogenic (167).

The final live dengue vaccine, in development by the National Institutes of Health (NIH), is based on attenuating genetic mutations (231). DENV4 Δ 30 was isolated in a 3'UTR deletion screen designed to identify potential vaccine candidates with reduced replication in monkeys (141). In a Phase I clinical trial, volunteers vaccinated with this virus exhibited low levels of viremia and high levels of neutralizing antibodies (46). Based on the promising attenuation with a thirty nucleotide deletion in the 3' UTR of DENV4, the NIH developed a tetravalent vaccine by deleting the same thirty nucleotides of DENV1, DENV2, and DENV3 (15). DENV3 Δ 30 was found to be under-attenuated in SCID-Huh7 mice and rhesus macaques (14, 15). In its current form, the tetravalent formulation consists of DENV1 Δ 30, DENV4 Δ 30, DENV2 Δ 30 and DENV3/4 Δ 30 (15). DENV3/4 Δ 30 is a chimera, replacing the prM and E genes of DENV4 Δ 30 with those of DENV3. Following vaccination with this tetravalent formulation, macaques exhibited a balanced antibody response to all four serotypes and protection from challenge (15). This tetravalent vaccine has not yet been tested in humans.

Although live attenuated tetravalent dengue vaccines are in advanced clinical trials, they are not perfect. Thus far, it has proven difficult to produce a safe vaccine that induces a balanced immune response in humans. The two most promising vaccines, produced by the US Army and the NIH, both have limitations. Following vaccination with the US Army formulation, volunteers complained of side effects and some patients developed symptomatic dengue. Additionally, the attenuating mutations of the four viruses are unknown. The tetravalent vaccine in development by the NIH looks promising in non-human primates, but

ultimately the effectiveness of the vaccine will be determined in human clinical trials, and by the ability of the vaccine to elicit a balanced immune response. It remains unclear if live attenuated vaccines will be able to induce immunity in the face of pre-existing DENV or other flavivirus immunity.

Chimeric Virus Vaccines

Two groups are attempting to generate a dengue vaccine by constructing chimeric viruses between dengue and other flaviviruses. In both of these systems, ChimeraVAX and RepliVAX, the prM and E of Yellow Fever virus (YF) and West Nile virus (WNV) respectively have been replaced with those of DENV.

ChimeraVAX, developed by Acambis and now licensed to Sanofi, is based upon the Yellow Fever 17D vaccine. YF17D has been used in humans for years and is considered relatively safe and effective. In ChimeraVAX, the structural genes prM and E of YF have been replaced with the structural genes of DENV (67). Non-human primates vaccinated with two doses of the tetravalent formulation of ChimeraVax produced neutralizing antibodies towards all four DENV serotypes (67). In Phase I clinical trials, vaccinated individuals exhibited few adverse effects and had broad humoral and cell-mediated immune responses to all four DENV serotypes following boosting (70). This vaccine is entering Phase III clinical trials this year and is a leading dengue vaccine candidate.

RepliVaxD2, uses a single-cycle encapsidation defective West Nile Virus system, in which prM and E of WNV have been replaced by those of DENV (217). In this system, the WNV RNA genome contains a truncated capsid gene and cells cannot package the genome without capsid supplied in *trans*. Virions containing the defective RNA can infect normal cells and these cells will release subviral particles containing the DENV prM and E (217).

Since the RNA genome cannot spread cell-to-cell, the vaccine is very safe. Following optimization of subviral particle secretion in cell culture, AG129 mice were vaccinated with the WNV/DENV chimera, RepliVaxD2 (217). These mice exhibited dose-dependent DENV2 neutralizing antibody responses and were protected from DENV2 challenge (217).

ChimeraVAX and RepliVax are promising DENV vaccine technologies but both require further testing. RepliVax has only been tested in a mouse model and may not act similarly in mice and primates. ChimeraVAX appears safe in preliminary studies but larger trials will be necessary to confirm safety and balanced immunity.

Whole Inactivated Virus, Subunit and Vected Vaccines

In order to overcome some limitations of live attenuated and chimeric vaccines, groups are developing vaccines based on inactivated virus, viral subunits or other viral vectors. Inactivated virus and viral subunit vaccines are extremely safe and should produce balanced immune responses to all four serotypes. Unfortunately, they produce immunity only to structural genes and require adjuvants to produce robust immune responses (231). Vected DENV vaccines are safe and should be able to overcome pre-existing immunity in endemic regions (231).

Purified formalin-inactivated whole DENV2 has been tested in non-human primates alone and in combination with other subunit vaccines (166, 167, 207). Following immunization with purified inactivated virus (PIV) alone, macaques developed high neutralizing antibody titers and were protected from challenge with DENV2 (166, 167, 207). When administered with a DENV DNA vaccine, PIV was less effective at inducing a protective immune response (207).

Regions of DENV2 E have been tested in vaccine formulations with limited success (167, 207). The amino 80% of E given with various adjuvants was able to induce high levels of neutralizing antibodies but was unable to protect rhesus macaques from viremia following challenge (167). *E. coli*-expressed E domain III fused to mannose binding protein produced poor antibody responses in rhesus macaques when administered alone (207). When recombinant protein was combined with a DNA vaccine expressing prM and E, vaccinated monkeys produced neutralizing antibodies but were not protected from viremia following virus challenge (207).

A number of groups have attempted to develop DNA vaccines to express E of DENV (3, 12, 127, 170, 207). prM and E expressed from a CMV promoter and administered intradermally have produced conflicting results in non-human primates. DENV2 prM and E DNA was able to induce long-lasting immunity and protection from virus challenge in rhesus macaques (207). *Aotus nancymae* monkeys were partially protected from DENV3 challenge following immunization with a plasmid expressing DENV3 prM and E (12). The final DNA vaccination approach immunized Balb/c mice with a plasmid expressing a E domain III consensus sequence (cEDIII) designed to represent all four serotypes of DENV (127). Serum from mice vaccinated with three doses of cEDIII could neutralize all four serotypes of DENV (127).

Venezuelan Equine Encephalitis (VEE) vectors have been engineered to express DENV antigens, and VEE replicon particles (VRP) have been administered to mice and monkeys (30, 230). DENV VRPs contain the full-length genome of VEE with the structural genes of VEE replaced with prM and E of DENV. These particles are single-cycle vectors that enter target cells and express high levels of prM and E, resulting in the formation of

DENV subviral particles (230). DENV1 VRPs have been administered to cynomolgous macaques in combination with a DNA vaccine designed to express the same antigens. A DNA prime and boost followed by a VRP boost resulted in complete protection from DENV challenge (30). When a DENV2 VRP was administered to DENV2 immune Balb/c mice, the vaccine induced neutralizing antibodies in young mice and protected from a lethal intracranial challenge (230).

Replication-defective recombinant adenovirus vectors expressing DENV structural genes have been tested in mice and non-human primates (92, 99, 171). In the initial experiments mice were vaccinated with Adenovirus vectors designed to express the ectodomain of DENV2 E (99). These mice developed neutralizing antibody and Th1 immune responses against DENV2 (99). Following the development of adenovirus vectors that can accommodate larger transgenes, a new tetravalent DENV vaccine was developed (92, 171). This vaccine is composed of two bivalent vectors that contain prM and E of either DENV1 and DENV2, or DENV3 and DENV4 (92, 171). As single bivalent vaccines, these vectors were able to induce potent neutralizing antibody responses against all four DENV serotypes and broadly cross-reactive cellular immune responses (92). Based on success in mice, this vaccine was tested in rhesus macaques as a formulation of both bivalent vectors (171). After administration of two doses, animals exhibited high neutralizing antibody titers to all four DENV serotypes, were protected from challenge with DENV 1 and DENV3, and were partially protected from challenge with DENV2 and DENV4 (171). Vaccinated animals did not develop a detectable cell mediated immune response (171).

Subunit vaccines are not as immunogenic as live attenuated vaccines and may not stimulate long-term immunity. Oftentimes, subunit vaccines must be administered with an

adjuvant to boost the immune response. Additionally, antivector immunity is a concern with Adenovirus vectored vaccines and VRP. Thus far, the Adenovirus immune status has not affected immune responses to Adenovirus vectored vaccines in macaques (171). VEE immunity is of little concern since VEE in humans is extremely rare in dengue endemic areas (230). Only time will tell how subunit vaccines will fare in inducing a balanced immune response and overcoming pre-existing vector immunity.

OVERVIEW OF THIS THESIS

The goal of this dissertation was two-fold. The first goal was to compare the abilities of mosquito- and mammalian-derived DENV to interact with and infect human DC-SIGN expressing monocytes. The second goal was to characterize two groups of viruses in their ability to infect via DC-SIGN.

Unlike Alphaviruses, mosquito- and mammalian-derived DENV infect similar numbers of DC-SIGN expressing monocytic cells and replicate with similar kinetics. In order to discern why these viruses infect similarly, we characterized the E glycans and determined that both mosquito- and mammalian-derived DENV have high mannose glycans. The high mannose glycan of virus produced in both cell types is located at position N-67 and interacts with DC-SIGN, mediating infection. The second E glycan at position N-153 differs in mosquito- and mammalian-derived DENV. In mosquito cells, this glycan is processed down to three mannose residues during viral egress. Mammalian cells are able to add sugar residues to proteins during egress and the N-153 is a complex sugar. We were able to determine that when expressed as the ectodomain alone, E does not contain any high

mannose glycans. Membrane anchoring of E packs many dimers into a small area and likely hinders access of processing enzymes to this glycan.

In the second chapter, we sequenced the entire genomes of viruses isolated in Sri Lanka prior to and following the emergence of DHF. We were able to compare the sequences of these viruses to DHF-associated viruses isolated in Africa and Latin America. Based upon these sequences, we were able to identify coding and UTR changes conserved in DHF-associated viruses. We compared the abilities of Sri Lankan pre- and post-DHF viruses to infect DC-SIGN expressing human monocytes, and found that post-DHF viruses may replicate less well in mammalian cells than the pre-DHF viruses. By comparing one representative virus from each group, we were able to show that the ‘good’ virus enter cells and progresses past fusion faster.

In the third portion of this dissertation, we developed a reverse genetics system for DENV3, genotype II. We were able to show that the recombinant virus behaved similarly to the parental virus in cell culture. We were also able to construct chimeric viruses by assembling the full-length virus with a coding region from another virus inserted from E through NS3. Now that we’ve developed a reverse genetic system, we can use it to identify residues in DENV3 that are important for replication and growth *in vitro*.

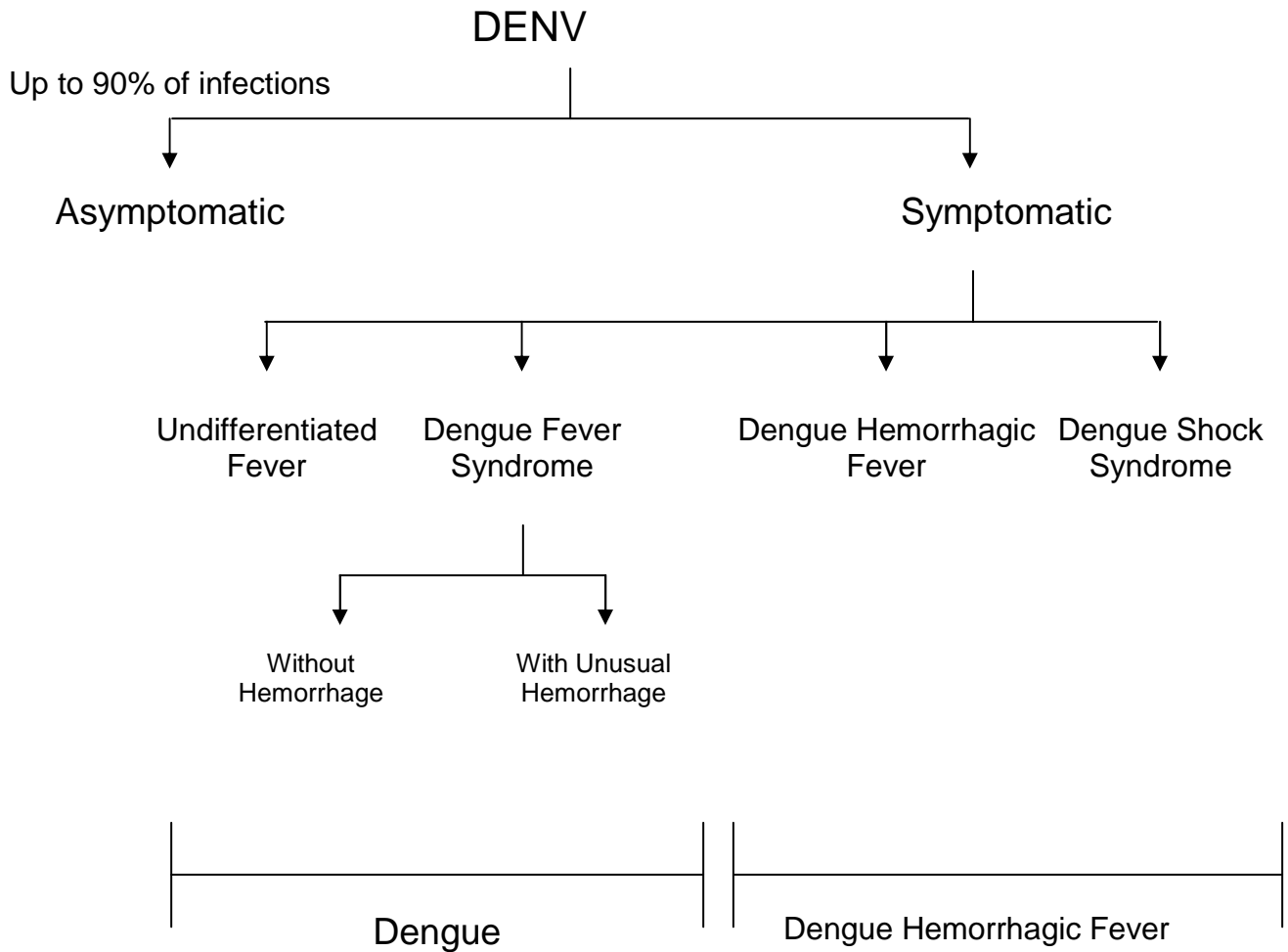


Figure 1.1 Clinical manifestations of DENV infection in humans

Upon infection with DENV, 90% of people do not develop symptoms. In the remaining 10%, dengue disease can manifest itself as a number of different syndromes. Patients may develop an undifferentiated fever, dengue fever with or without hemorrhagic manifestations, dengue hemorrhagic fever, or dengue shock syndrome.

**CHAPTER 2. N-LINKED GLYCANS ON DENGUE VIRUSES GROWN
IN MAMMALIAN AND INSECT CELLS**

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ABSTRACT

In this study we compared the ability of mosquito and mammalian-cell derived dengue virus (DENV) to infect human dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) expressing cells and characterized the structure of envelope (E) protein N-linked glycans on DENV derived from the two cells types. DENVs derived from both cell types were equally effective at infecting DC-SIGN-expressing human monocytes and dendritic cells. The N-linked glycans on mosquito cell-derived virus were a mix of high mannose and paucimannose glycans. In virus derived from mammalian cells, the N-linked glycans were a mix of high mannose and complex glycans. Our results indicate that N-linked glycans are incompletely processed during DENV egress from cells, resulting in high mannose glycans on viruses derived from both cell types. Studies with full-length and truncated E protein demonstrated that incomplete processing was most likely a result of the poor accessibility of glycans on the membrane-anchored protein.

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INTRODUCTION

Dengue viruses (DENVs) are enveloped, positive-sense RNA viruses of the flavivirus genus that are transmitted via the bite of *Aedes* mosquitoes. Each year, over 2.5 billion people are at risk of contracting dengue, 100 million people develop symptomatic infections, and up to 2.5% of dengue hemorrhagic fever (DHF) patients die (62, 64, 232, 233). Despite the global public health importance of dengue, the cell biology of DENV is poorly understood. Vector-borne viruses such as dengue must productively infect cells of arthropod and mammalian origin. As the post-translational protein processing machinery is different in insect and mammalian cells, the structure of virion glycoproteins produced in the two hosts may be different. Recent work with a wide variety of viruses has shown that protein glycosylation can influence viral virulence (reviewed in 223). Recent studies with alphaviruses, which are also transmitted by arthropod vectors, have demonstrated that membrane protein N-linked oligosaccharides on membrane proteins are differentially processed by enzymes in insect and mammalian cells (195). Structural differences in the glycans derived from insect and mammalian cells influence the ability of the viruses to infect target cells (112, 194, 195). In this study we characterized the N-linked glycans on the envelope protein of DENVs grown in different cells types and assessed the functional consequences of these differences.

The DENV particle is made up of three structural proteins designated: envelope (E), membrane (M) and capsid (C) proteins (23, 116). E is the major membrane glycoprotein on the surface of the virion responsible for virus attachment and fusion (23). Human dendritic cells (DCs) are a target of DENV infection (91, 140, 234). The infection of DCs is mediated by the binding of DENV to dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN),

a C-type lectin that preferentially binds to terminal mannose sugars on glycans (21, 22, 49, 153, 220). DENV E protein has two potential N-linked glycosylation sites at N-67 and N-153, whereas most other flaviviruses have only a single N-linked glycosylation site at N-154 (24, 89). DC-SIGN binds to DENV via the glycan at N-67 on E protein (133, 149, 153, 162, 220).

Virions produced in the mosquito vector and the human host may have structurally different N-linked glycans because insect and mammalian cells process glycans differently during exocytosis (100). Both hosts transfer a lipid-linked oligosaccharide, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, to asparagine residues co-translationally in the endoplasmic reticulum (ER) (98). Insects and mammals have mannosidases and glucosidases capable of trimming mannose and glucose residues during exocytosis. In mosquito cells, proteins can make it through the exocytic pathway maximally (paucimannose) or minimally (high-mannose sugars) trimmed (95, 98, 100, 138). In either case, terminal mannose residues are present on N-linked oligosaccharides in insects. Mammalian cells have enzymes that can further process trimmed oligosaccharides by adding sialic acid, glucose, galactose and other sugars to produce complex N-linked oligosaccharides with no terminal mannose residues (100, 158). As DC-SIGN preferentially binds to terminal mannose residues, one would predict that viruses grown in insect cells would bind to this receptor and infect DCs better than viruses grown in mammalian cells. In fact, DC-SIGN can distinguish between mosquito- and mammalian-derived alphavirus E2 glycans, resulting in more efficient infection by mosquito-derived virus (112, 194, 195). Similarly, mosquito-derived West Nile virus, a flavivirus relative of dengue, also infects cells through DC-SIGN better than mammalian-derived virus

(43). The ability of DC-SIGN to preferentially interact with mosquito-derived WNV maps to the lectin carbohydrate recognition domains (43).

The glycans on DENV envelope have been roughly characterized for some serotypes. There is no consensus on the number and structure of the sugars added to E in mosquito or mammalian cells. Smith and Wright first reported that the mouse-adapted dengue type 2 (New Guinea C) has two N-linked glycans on the envelope glycoprotein. They also concluded that the sugars added were heterogeneous in structure and composition (208). Johnson *et al* confirmed the addition of two glycans to the envelope protein of dengue type 1 (Hawaii) grown in C6/36 cells, but found only a single glycan at position 67 in dengue type 2 (Jamaica) grown in the same cells (101). They concluded these sugars were high mannose based on their ability to bind ConA. More recently, the structure of glycans has been characterized on soluble E of DENV1, DENV2 and DENV3. In these systems, both glycans were processed to paucimannose or complex glycans in insect and mammalian cells respectively (133, 145, 148). Two recent papers have examined the phenotype of envelope glycan mutant DENVs and shown that the glycan at N-153 is not necessary for virus production and spread in either mosquito or mammalian cells (18, 145). N-67 was essential for virus spread in mammalian cells, which is consistent with a role for this glycan in binding to host cell receptors. There is disagreement over whether the glycan at N-67 is necessary for viral spread in mosquito cells (18, 149). Further studies are needed to characterize the structure and functional significance of N-linked glycans on the 4 serotypes of DENVs produced in mosquito or mammalian cells.

In this paper, we report that both mosquito- and mammalian-derived DENV infect DC-SIGN-expressing cells with similar efficiency. We used lectin blots and enzymes that

specifically cleave glycans of defined structure to characterize N-linked glycans on mosquito- and mammalian-derived DENV. All four serotypes of DENV grown in insect and mammalian cells had two N-linked glycans on E protein. In both cell types, one of the glycans had a high mannose structure indicating incomplete processing. The second sugar was either paucimannose or complex on virus produced in mosquito or mammalian cells, respectively. We propose that, unlike alphaviruses and other flaviviruses, DENVs derived from both hosts can efficiently infect DCs because of the presence of unprocessed, high mannose glycans on E protein.

MATERIALS AND METHODS

CELL LINES AND VIRUS STRAINS

C6/36 *Aedes albopictus* cells were obtained from the American Type Culture Collection (ATCC No: CRL-1660) and propagated in Minimal Essential Media with Earl's salts (E-MEM) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 10% fetal bovine serum (Gibco/Invitrogen) at 28 °C in 5% CO₂. Vero (African Green Monkey) Clone 81 cells were a gift from Robert Putnak at Walter Reed Army Medical Center, and were propagated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.2% sodium bicarbonate and 10% fetal bovine serum (Gibco/Invitrogen) at 37 °C, 5% CO₂. The DENVs used in this study were DENV1 West Pac 74, DENV2 New Guinea C and 16681, DENV3 H87 and CH53489, and DEN4 TVP 360.

VIRUS PROPAGATION AND PURIFICATION

DENV stocks were grown in either C6/36 mosquito cells or Vero cells. To generate stocks, seed virus was added to 80% confluent cells at a multiplicity of infection (MOI) of 0.01 in reduced serum media (E-MEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 2% fetal bovine serum for C6/36 cells and D-MEM/F-12 supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.2% sodium bicarbonate and 5% fetal bovine serum for Vero cells). After seven days, media was harvested from cells and clarified by centrifugation at 10,000 x g for 30 minutes. The virus containing supernatant was supplemented with 20% fetal bovine serum and stored at -80°C. For studies requiring purified and concentrated virus, the supernatant was centrifuged at 76,221 x g for 5 hrs to pellet virus under a 20% sucrose:PBS (w:v) cushion. Pelleted virus was resuspended in PBS, loaded onto a 15%-60% (v:v) continuous iodixanol gradient, and centrifuged at 29,331 x g for 154 minutes. Fractions containing purified virus were diluted in PBS and centrifuged at 76,221 x g for 5 hours to pellet the virus and remove the iodixanol. Purified virus was stored at -80 °C.

VIRUS GENOME QUANTIFICATION AND TITRATION

Viral genomes were quantitated by real-time PCR as previously described using primers to amplify the 3' UTR (94). To calculate MOIs for infection assays, virus titers were determined using flow cytometry in a method similar to the one described by Lambeth *et al* (124). Briefly, two thousand Vero cells were plated in 96-well plates and infected with known amounts of viral genomes. Twenty-four hours after infection, infected cells were stained and infection was determined by flow cytometry. Cells were fixed and permeabilized

and then stained with Alexa488-conjugated anti-flavivirus envelope antibody 4G2 (ATCC HB-112). Virus released into the supernatant of infected cells was quantitated on Vero cells in a 24-well format immunofocus assay modified from that described in AP61 cells (45). Subconfluent Vero monolayers were infected with serial dilutions of infected cell supernatants. Overlay media (Opti-MEM I (Gibco/Invitrogen) containing 0.8% methyl cellulose (Electron Microscopy Sciences)) was then added to plates and cells were incubated for five days at 37 °C, 5% CO₂. Monolayers were stained with 400 ng anti-flavivirus envelope antibody 4G2 (ATCC HB-112) followed by a 1:500 dilution of a peroxidase conjugated secondary antibody (Sigma-Aldrich) for 1 hour each at 37°C. Foci were visualized with peroxidase substrate. Titers were expressed as focus forming units per mL and were calculated by multiplying the average number of foci per well at a given dilution by the inverse dilution factor and dividing by the volume added to each well.

DENV INFECTION OF U937 CELLS EXPRESSING DC-SIGN

A human monocytic cell line (U937) constitutively expressing DC-SIGN was obtained from Mark Heise at the University of North Carolina at Chapel Hill (115). The parental and DC-SIGN transduced cells were maintained at 37 °C, 5% CO₂ in RPMI complete media (RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 50 mM 2-mercaptoethanol, and 10% fetal bovine serum (Gibco/Invitrogen)). For infection assays, virus was added to cells at MOIs of 0.001, 0.01, and 0.05. Cells and virus were incubated for two hours in the presence of media alone, 100 µg mL⁻¹ isotype control monoclonal antibody clone 20116 (R&D Systems mAB004), 100 µg mL⁻¹ anti-CD209 blocking antibody clone 120507 (R&D System mAB1612), 20 µg mL⁻¹ mannan, or 5 mM EDTA. After two hours, cells were

washed and resuspended in RPMI complete media. Twenty-four hours post-infection, cells were processed and stained for infection with 4G2 as described above. For the forty-eight hour DENV2 time course experiment, cell and supernatants were harvested at twelve-hour intervals. Cells were processed for FACS and supernatants were titered on Vero cells in the immunofocus assay described above.

ISOLATION AND INFECTION OF MONOCYTE-DERIVED DENDRITIC CELLS

Human dendritic cells were derived from peripheral blood monocytes as described by Moran *et al* (150). Briefly, buffy coats obtained from the American Red Cross were diluted 1:2 with PBS and peripheral blood monocytic cells were isolated by centrifugation over Ficoll-Hypaque (Sigma). Monocytes were enriched by adding 100 million cells to a tissue culture flask for 2 hours and removing the non-adherent cells. The adherent cells were cultured in complete RPMI media supplemented with 800 U mL⁻¹ GM-CSF and 500 U mL⁻¹ IL-4 (Peprotech). Fresh cytokines were added on day 3 and immature dendritic cells were harvested on day 6. Immature dendritic cells were infected with dengue virus at an MOI of 0.05 and processed for flow cytometry as described above for U937+DC-SIGN cells.

CHARACTERIZATION OF N-LINKED GLYCANS ON DENV

Purified DENVs were digested with EndoglycosidaseH or PNGaseF (New England BioLabs) according to the manufacturer's protocol with a minor modification. Instead of denaturation in the provided glycoprotein denaturation buffer containing DTT, viruses were denatured in 0.5% SDS. Treatment with these enzymes can determine whether glycans are high mannose or complex (136). Digested viral proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions and analyzed by Western or lectin blots. In Western blots, E protein was detected using anti-dengue

monoclonal antibody 4G2 as a primary antibody and HRP conjugated goat anti-mouse IgG as a secondary antibody. Glycosylated envelope was detected with Roche's DIG Glycan Differentiation Kit lectins *Galanthus nivalis* agglutinin (GNA) or *Datura stramonium* agglutinin (DSA) according to the manufacturer's protocol. Western and lectin blot films were scanned and images were processed with Adobe Photoshop.

DETERMINING THE ROLE OF PRM IN E PROTEIN N-LINKED GLYCAN PROCESSING

To study whether E packaging in viral or subviral particles effects the processing of N-linked glycans in E protein, we created constructs of DENV2 truncated ectodomain E and full-length E protein for expression using Venezuelan Equine Encephalitis replicons as previously described by White *et al* (230). Reverse transcriptase PCR was used to create full length E protein with prM (prM/E) and the soluble ectodomain of E with prM (prM/E85). The amplified DNAs were cloned into the multicloning sites of VEE replicon vectors provided by Nancy Davis of the Carolina Vaccine Institute. Full-length T7 transcripts were generated as previously described and electroporated into Vero 81 cells (44). Cells were plated into 6-well plates. At 4 hours post electroporation the cells were starved for 1 hour in cysteine- and methionine-free media (MP Biomedicals). At 5 hours post electroporation, cells were metabolically labeled with 100 μ Ci/well [35 S] methionine and cysteine (Promix amino acid mixture (GE Healthcare)) for 7 hours. At the end of labeling, the cells and media were harvested to examine cell associated and secreted E protein. The media was clarified by centrifugation at 10,000 x g for 10 minutes. Labeled cells were lysed in TNE NP-40 buffer containing protease inhibitors (10 mM Tris; 200 mM NaCl; 1 mM EDTA; pH 7.4 + 1% (v:v) NP-40). Cell associated and secreted E proteins were immunoprecipitated with 2 μ g 4G2 and Protein A Sepharose beads (Sigma). Purified proteins were digested with

endoglycosidases as described above, separated by SDS-PAGE under reducing conditions and visualized using a Phosphorimager or film.

RESULTS

INFECTION OF DC-SIGN-EXPRESSING MONOCYTIC AND DENDRITIC CELLS BY MOSQUITO- AND MAMMALIAN-DERIVED DENV

Studies with some mosquito-borne viruses have demonstrated that virions produced in insect cells infect dendritic cells better than virions produced in mammalian cells (43, 112, 194). In these studies the superior infectivity of mosquito-derived virus was linked to high mannose N-linked glycans present in mosquito- but not mammalian-derived virus (42, 43, 112, 194, 195). We performed experiments to determine if mosquito-derived DENV also infected monocytes and dendritic cells better than mammalian-derived virus.

Initial experiments were conducted with a human monocytic cell line (U937) engineered to stably express human DC-SIGN, a DENV attachment factor (115). Mosquito and mammalian-derived DENVs were used to infect DC-SIGN-expressing U937 cells (Figure 2.1a). Cells were harvested twenty-four hours post-infection following a single cycle of replication. We determined the efficiency of infection by measuring the percentage of cells expressing DENV E protein. Both mosquito- and mammalian-derived viruses of all four DENV serotypes infected similar numbers of cells (Figure 2.1a). To determine if this phenotype was dependent on the amount of virus used to infect cells or the time of testing, we performed dose-response experiments and growth curves with DENV2. At all MOIs tested, mosquito- and mammalian-derived DENV2 infected similar percentages of cells (Figure 2.1b). Additionally, similar percentages of DC-SIGN expressing U937 cells were

infected at all time points tested (Figure 2.1c) and similar amounts of virus were released into the supernatant (Figure 2.1d).

Interactions between DC-SIGN and its mannose ligand can be inhibited by DC-SIGN antibody, excess mannan or EDTA treatment (153, 220). To confirm that mosquito- and mammalian-derived DENV infection was DC-SIGN dependent, we infected parental U937 cells and DC-SIGN expressing U937 cells in the presence of inhibitors (Figure 2.2a). As expected the expression of DC-SIGN enhanced DENV infection (153, 220). Pre-incubation of cells with DC-SIGN blocking antibody ($100 \mu\text{g ml}^{-1}$), mannan ($20 \mu\text{g ml}^{-1}$) or EDTA (5 mM) for 30 minutes reduced infection of both mosquito- and mammalian-derived DENV (Figure 2.2a). These studies indicated that mosquito and mammalian cell derived DENVs use DC-SIGN and infect cells with similar efficiency.

Primary human myeloid DCs are susceptible to DENV infection and are likely to be an important target during natural infection (91, 140). Experiments were done to compare infection of these primary cells with mosquito- and mammalian- derived DENV. Myeloid DCs were obtained from 3 different donors and infected with DENV at an MOI of 0.05. Both mosquito- and mammalian-derived viruses infected similar proportions of primary DCs (Figure 2.2b). Moreover, DENV2 infection of primary DCs was reduced by DC-SIGN antibody, mannan and EDTA indicating that infection was dependent on DC-SIGN (Figure 2.2b). We conclude that unlike some other mosquito-borne viruses, insect- and mammalian-derived DENVs infect DC-SIGN-expressing cells with similar efficiency (43, 112, 194).

COMPOSITION OF N-LINKED GLYCANS ON DENV

DENV E protein possesses two conserved, potential N-linked glycosylation sites at N-67 and N-153 (23, 208). To determine why mosquito-derived DENV did not have an

advantage infecting via DC-SIGN compared to mammalian-derived virus, we compared the structures of the glycans added to E protein of DENV produced in the two cell types. Purified DENV2 and DENV3 were treated with PNGaseF or EndoH. PNGaseF removes all N-linked glycans, whereas EndoH only removes N-linked sugars containing more than three terminal mannose residues, typically found in immature N-linked glycans (136). Following treatment with PNGaseF, DENV grown in Vero and C6/36 cells had an electrophoretic shift of approximately four kilodaltons, corresponding to a loss of two glycans (Figure 2.3, labeled 0N) (208). When the viruses were treated with EndoH, DENV2 displayed differing digestion patterns compared to DEN1, DENV3 and DENV4. E protein in both mosquito- and mammalian-derived DENV1, DENV2 and DENV4 exhibited an electrophoretic shift corresponding to 2 kilodaltons, indicating the presence of one high mannose, EndoH sensitive glycan and another more processed glycan that was EndoH resistant (Figure 2.3, labeled 1N). DENV2 did not exhibit one distinct band when digested with EndoH. Instead, there were three bands corresponding to E: one migrated with undigested E (2N), one migrated with PNGaseF digested E (0N), and one migrated between the undigested and deglycosylated E (1N) (Figure 2.3). These results indicate that the glycans on DENV2 E are heterogeneous. A significant portion of E had at least one EndoH sensitive or high mannose glycan. Importantly, the digestion patterns exhibited by mosquito- and mammalian-derived DENV did not differ. Thus, both cell types produce virus in which at least one N-linked glycan on DENV is not fully processed during virus egress from cells. The resulting high mannose glycan is likely responsible for the ability of both mosquito -and mammalian-derived virus to efficiently infect DC-SIGN-expressing cells.

To further characterize the EndoH resistant and sensitive N-linked glycans on DENV, we used lectins with defined specificity for terminal sugar residues in lectin blotting assays (Figure 2.4). The two lectins used were *Galanthus nivalis* agglutinin (GNA) and *Datura stramonium* agglutinin (DSA). GNA specifically recognizes terminal mannose linked to mannose, whereas DSA recognizes Gal(1-4)GlcNAc present in complex N-linked glycans (41, 197). In insect cells, both unprocessed (EndoH sensitive) and fully processed (EndoH resistant) N-linked glycans should contain terminal mannose residues because insects do not have Golgi enzymes for the addition of terminal sugars to produce complex sugars (100). As expected, when insect-derived DENV2 was analyzed by lectin blotting, E protein bound to GNA but not DSA. The GNA binding was lost following treatment with PNGase, which removed all N-linked glycans. The EndoH resistant glycans bound to GNA but not DSA confirming that they have a paucimannose structure.

When DENV2 E protein from mammalian-derived virus was tested for lectin binding, both lectins bound to E protein indicating that a high mannose and a complex glycan were present on the protein (Figure 2.4). Following EndoH treatment GNA binding was lost while the DSA binding remained, confirming that the virus had a heterogeneous population of both high mannose and complex glycans. The lectin binding studies also confirmed that irrespective of host, DENV contains one high mannose N-linked glycan. The second glycan was a complex sugar or a paucimannose sugar in virus derived from mammals and mosquitoes respectively.

ROLE OF E PROTEIN MEMBRANE ANCHOR IN N-LINKED GLYCAN PROCESSING

DENVs assemble on the ER membrane. The virions containing a lipid envelope with prM and E proteins and a capsid with the RNA genome bud into the lumen of the ER and the

virus particles are secreted out of the cell. E and prM expressed without other viral proteins are secreted out of cells as subviral particles that bud into the ER (51). To determine if membrane anchoring and/or viral particle formation was responsible for incomplete processing of DENV N-linked glycans, a Venezuelan Equine Encephalitis (VEE) replicon particle (VRP) protein expression system was used to express prM with full-length E and prM with just the ectodomain of E (prM/E85) (44). Full-length E protein is secreted out of cells as a subviral particle, whereas E85 is secreted as a soluble protein (230). The constructs were expressed in Vero cells, metabolically labeled with [³⁵S], and the glycans on intra- and extracellular E were characterized by glycosidase digests (Figure 2.5). Full-length E and E85 had two glycans added as indicated by an electrophoretic shift of four kilodaltons following PNGaseF digestion (Figure 2.5). All intracellular forms of E were sensitive to EndoH indicating that the majority of this protein was in the ER and had not progressed through the Golgi (Figure 2.5a). Full-length E expressed with prM and secreted to the media as subviral particles exhibited an EndoH digestion pattern identical to whole virus (Figure 2.3) and consisted of a mixed population of EndoH resistant and sensitive glycans. In contrast, ectodomain of E (E85) expressed with prM was completely EndoH resistant following secretion from cells indicating that both glycans were fully processed into complex sugars (Figure 2.5b). These findings indicate that incomplete N-linked glycan processing of DENV is a result of the membrane anchor and/or secretion as a subviral particle.

DISCUSSION

In this study we compared the ability of DENVs derived from mosquito and mammalian cells to utilize DC-SIGN as an attachment factor for infecting monocytic cells.

Our results demonstrate that viruses derived from both cells types were equally effective at infecting DC-SIGN-expressing human monocytes and DCs. We also characterized the structure of N-linked glycans on DENVs grown in insect and mammalian cells. Two N-linked glycans were added to E protein in both cells types. In virus derived from mammalian cells, the N-linked glycans were a mix of high mannose sugars and complex sugars. The N-linked glycans on mosquito-derived virus were a mix of the high mannose sugars and paucimannose sugars. The carbohydrate recognition domains of human DC-SIGN preferentially interact with high mannose glycans when compared to single mannoses and complex carbohydrates (50, 146). We propose that the presence of unprocessed, high mannose sugars in both mosquito- and mammalian-derived virus is responsible for the ability of these viruses to infect DC-SIGN-expressing cells with similar efficiency. We chose Vero cells as a representative cell line to produce DENV since it is commonly used in vaccine studies to grow virus (reviewed in 231). DENV produced in other mammalian cell types can also infect DC-SIGN expressing U937 cells, including virus produced in human monocytic cells (data not shown).

Previous studies with alphaviruses and West Nile virus have demonstrated that mosquito-derived virus infects DC-SIGN-expressing cells better than mammalian-derived virus (43, 112, 194). The superior infectivity of mosquito-derived virus was attributed to the presence of terminal high mannose glycans in mosquito- but not mammalian-cell derived virus. Our results demonstrate that this phenomenon cannot be generalized to DENV because both mosquito- and mammalian- derived viruses had incompletely processed, high mannose glycans and were able to efficiently infect DC-SIGN-bearing cells. In studies with alphaviruses and West Nile virus, the superior infectivity of mosquito-derived virus has also

been attributed to the ability of mosquito- but not mammalian-grown virus to suppress type I interferon responses (194, 202). The mechanism by which mosquito-derived virus suppresses this innate immune response is currently unknown. Confirming previous reports, we were unable to detect type I interferon by bioassay in either our mosquito- or mammalian-DENV infected cultures at twenty-four hours (data not shown) (33, 155, 213). Further studies are needed to explore if mosquito- and mammalian-derived DENVs differ in their ability to suppress host innate anti-viral responses.

Previous studies have come to different conclusions about the number and structure of N-linked glycans on DENV E protein. Smith and Wright reported that dengue type 2 E has two N-linked glycans and that the glycans were heterogeneous in structure (208). Johnson *et al* confirmed the addition of two glycans in dengue type 1 but found only a single glycan in dengue type 2 (101). Moreover, they concluded that the sugars were high mannose due to the binding of ConA. More recently, the structure of glycans has been characterized on recombinant soluble E expressed in insect, human and rodent cell lines; Both glycans are EndoH resistant, indicating heavy glycan processing (133, 145, 147, 148). Here we compared the number of glycans in multiple isolates of DENV belonging to all 4 serotypes and observed the presence of two glycans in all but one case (Figure 2.3 and data not shown). Thus, we propose that two N-linked glycans is the norm for DENV E. We also observed a mix of fully processed (paucimannose or complex) and unprocessed (high mannose) N-linked glycans for all 4 serotypes grown in both mosquito and mammalian cells. The presence of high mannose and complex glycans on mammalian-cell derived virus is not unique to DENV (128). We conclude that incomplete glycan processing is a general feature

of DENVs, resulting in terminal high mannose N-linked glycans being a part of the virion irrespective of the host cells used to propagate virus.

Two recent papers have examined the phenotype of mutant DENVs and demonstrated that the glycan at N-153 is not necessary for virus production and spread in mosquito or mammalian cells (18, 149). These studies demonstrated that N-67 is essential for virus spread in mammalian cells, which is consistent with a role for this glycan in binding to host cell receptors and in other steps of the viral life cycle. N-67 was not required for production of infectious virus from mosquitoes or mosquito cells, indicating a non-essential role for N-67 in the vector (18, 149). In this current study we did not determine the location of the unprocessed and processed N-linked glycans. We predict that the processed, complex (mammalian-derived virus) or paucimannose (insect-derived virus), glycan is at position N-153 because the West Nile virus glycan at the corresponding position is processed to an EndoH resistant form (86). Consequently, the second potential glycosylation site at N-67 is likely to contain the unprocessed, high mannose glycan we observed in dengue virions. This location is consistent with published data indicating this glycan binds to DC-SIGN (149, 162). A glycan at N-67 is present in dengue but not other flaviviruses. However, when an N-linked glycan was artificially added to this position of West Nile virus reporter particles, the glycan was unprocessed (high mannose) and mediated infection of DC-SIGN-expressing cells (42).

When expressing recombinant DENV E proteins, we observed incomplete processing of N-linked glycans only when the protein was membrane anchored. In E protein secreted from cells expressing DENV E ectodomain, both glycans were in a processed, EndoH resistant form. In contrast, when full length E protein, which is membrane anchored, was

expressed, the secreted protein had an EndoH digestion pattern identical to virus with some envelopes containing EndoH sensitive, high mannose glycans. Membrane anchored E protein and prM bud into the ER to form subviral particles that are secreted out of cells (51). We speculate that the membrane anchoring of these proteins may alter glycosidase processing of N-67 by placing structural constraints on cellular glycosidases, thereby limiting processing. When envelope is produced as a soluble form, as in prM/E85 VEE constructs, the protein passes through the host ER and Golgi as monomers or dimers that may be associated with prM (132). In this context, the glycans are likely readily accessible by host glycosidases. Previous work with Sindbis virus shows that the ability of host glycosidases to access specific locations determines the structure of envelope glycans (97). Our results have implications for the interpretation of DENV E protein crystal structures. The structures that have been solved for E to date are based on recombinant, soluble E protein secreted from *Drosophila* cells (147, 148). We demonstrated that glycans on the virion are processed differently by ER and Golgi enzymes compared to glycans on soluble recombinant proteins. If glycan structure is important for dimer formation or protein folding, the E structures based on secreted E ectodomain may be misleading.

Our results demonstrate that the heavily processed N-linked glycan (most likely at N-153) has a different structure in mosquito (paucimannose, with terminal mannose) and mammalian (complex, with no terminal mannose) cells. The differences between these structures may influence receptor interactions. Davis *et al* showed that envelope N-154 of WNV, which corresponds to N-153 of DENV, can mediate infection via DC-SIGNR(42). DC-SIGNR recognizes both high mannose and complex glycans (43). The recognition of complex glycans is mediated by binding to terminal N-acetylglucosamine (42). DENV

infection of myeloid cells can also be mediated by mannose receptor, which specifically recognizes glycans terminating in mannose, fucose and N-acetyl glucosamine (145). LSEctin can act as an attachment factor for viruses such as filovirus and SARS-CoV (56). Although the specificity of LSEctin is not known, mannan cannot inhibit binding to filovirus glycoproteins suggesting that it does not recognize high or terminal mannose glycans (56). Since mammalian- and mosquito-derived DENV contain different glycans at N-153 and since DC-SIGNR, LSEctin, and MR differentially recognize these glycans, there may be cell types expressing these lectins that are differentially infected by mosquito- and mammalian-derived DENV.

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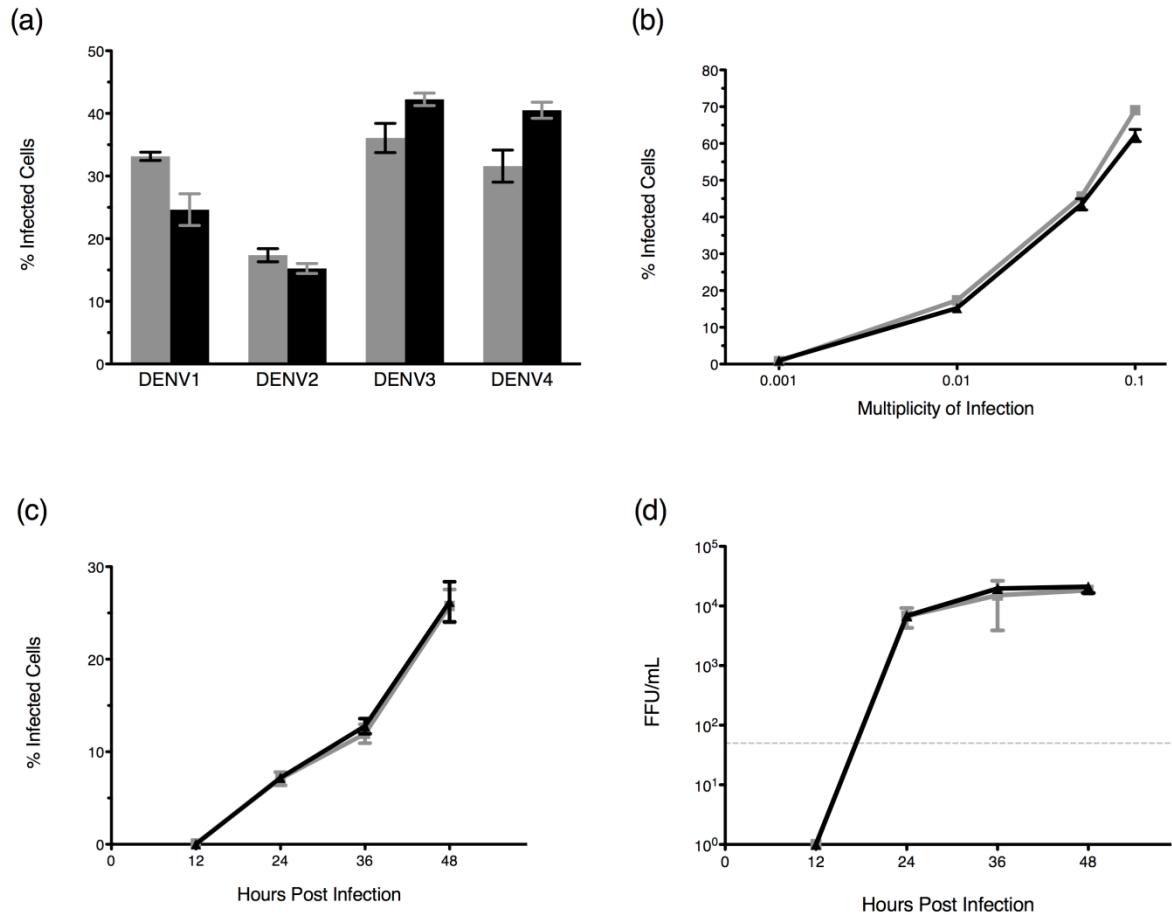


Figure 2.1 Infection of human U937 cells expressing DC-SIGN by mosquito and mammalian cell-derived dengue virus (DENV).

a) DC-SIGN-expressing U937 cells were infected with all four serotypes of DENV derived from insect cells (gray bars) or mammalian cells (black bars). DENV1 and DENV4 were added at an MOI of 0.001 while DENV2 and DENV3 were added at an MOI of 0.01. b) Mosquito-derived (gray squares) and mammalian-derived (black triangles) DENV2 were added to U937 cells expressing DC-SIGN at MOIs of 0.001, 0.01, 0.05 and 0.1. c) and d) DC-SIGN expressing U937 cells were infected with mosquito- (gray squares) or mammalian-derived (black triangles) at an MOI of 0.01. At 12-hour intervals for 48 hours, cells were harvested and stained for intracellular antigen (c) and supernatants were titers to determine virus release (d). All values represent average of experiments performed in triplicate and are representative of one of two experiments. Standard deviations are shown. The dotted gray line in (d) indicates the level of detection of our immunofocus titration assay.

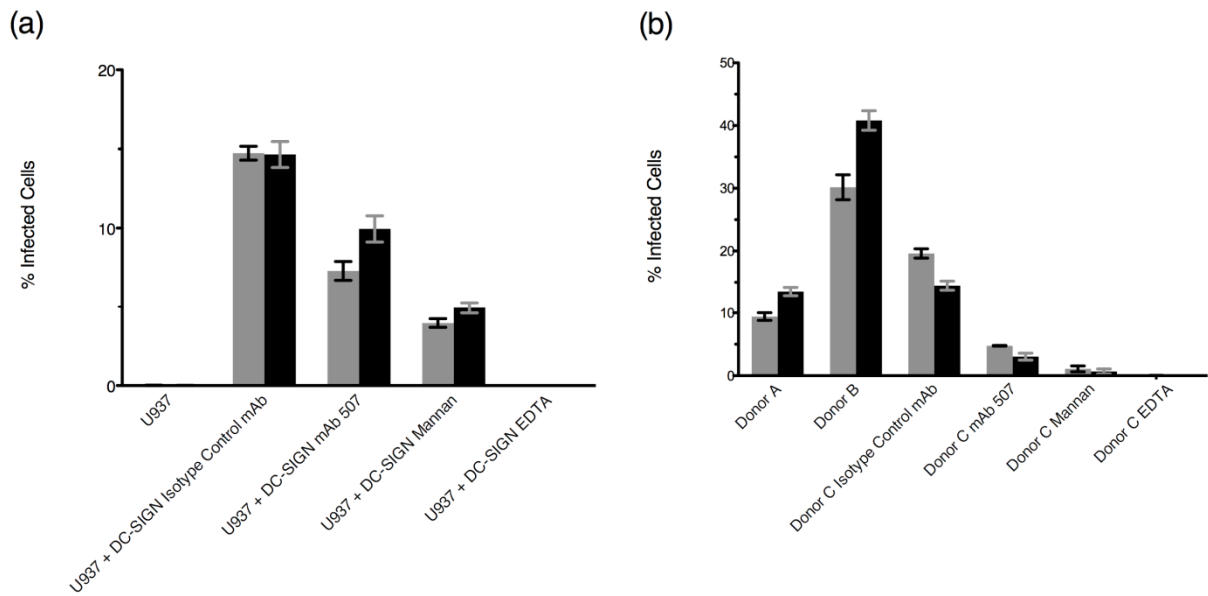


Figure 2.2 Infection of primary human myeloid dendritic cells (DCs) and DC-SIGN expressing monocytes by mosquito and mammalian cell-derived dengue virus (DENV) is DC-SIGN dependent.

DC-SIGN expressing U937 (a) and DC from three different donors (b) were infected with mosquito-derived (gray bar) or mammalian-derived (black bar) DENV2 at an MOI of 0.05. Percentages represent the averages of infections performed in triplicate with standard deviations indicated. Infections were done in the presence and absence of DC-SIGN antibody, mannan or EDTA. Cells were harvested twenty-four hours post infection and infected cells were detected by staining with an anti-E antibody followed by flow cytometry. Values represent average of experiments performed in triplicate and are representative of one of three experiments. Standard deviations are shown.

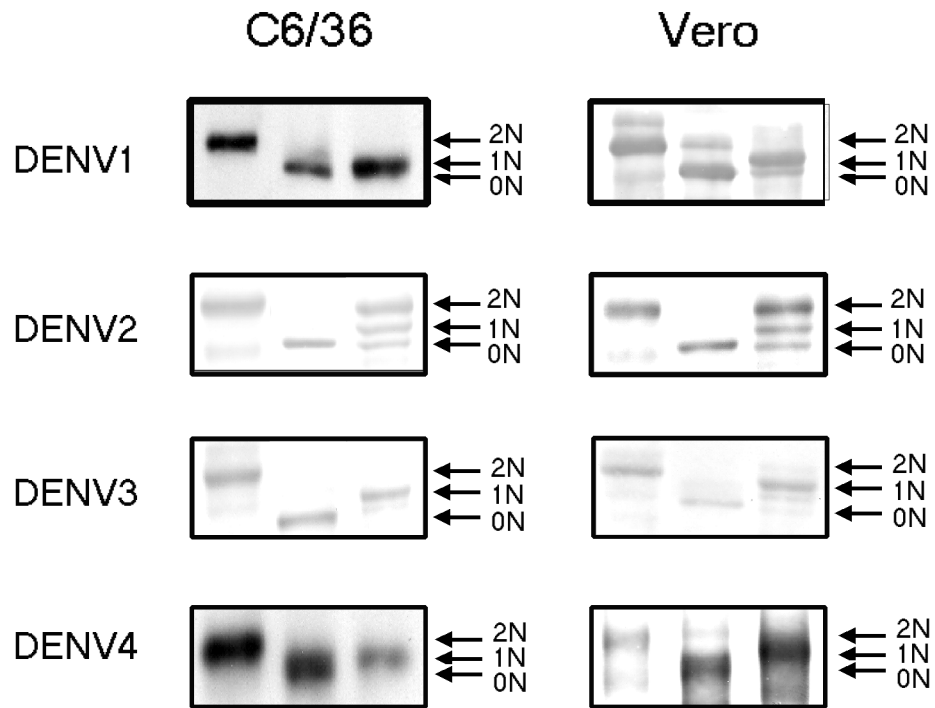


Figure 2.3 Dengue viruses grown in both insect and mammalian cells have high mannose N-linked glycans.

DENVs were grown in C6/36 (mosquito) and Vero81 (mammalian) cells. Purified virus was digested with PNGaseF (**P**), EndoH (**E**) or mock digested (**U**), and the relative gel mobility of E was determined by Western blot. Bands are labeled with 0N, 1N or 2N, corresponding to the number of N-linked glycans on E protein.

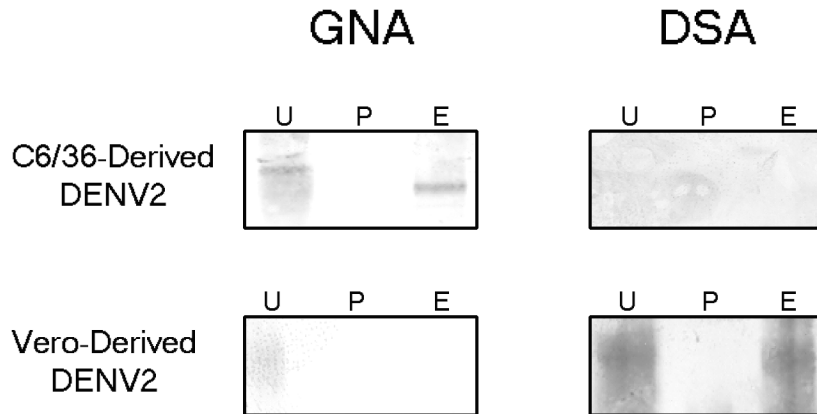


Figure 2.4 Mosquito-derived DENV has two terminal mannose sugars while mammalian-derived DENV has only one.

Purified DENV2 produced in C6/36 and Vero cells was digested with PNGaseF (**P**) or EndoH (**E**) and lectin blots were performed. GNA is a lectin that recognizes terminal mannose residues. Both mosquito and mammalian cell-derived DENV bound to this lectin indicating the presence of terminal mannose residues. Following digestion with EndoH (**E**), only the mosquito-derived virus bound to the lectin. These results are consistent with the mammalian derived virus having one EndoH sensitive high mannose glycan and one EndoH resistant complex glycan with no terminal mannose residues. The results are also consistent with insect derived virus having one EndoH sensitive high mannose glycan and one EndoH resistant paucimannose glycan with terminal mannose residues. DSA is a lectin that recognizes Gal-(1,4) GlcNAc, which is only found in complex sugars. Only the virus grown in Vero cells bound to this lectin.

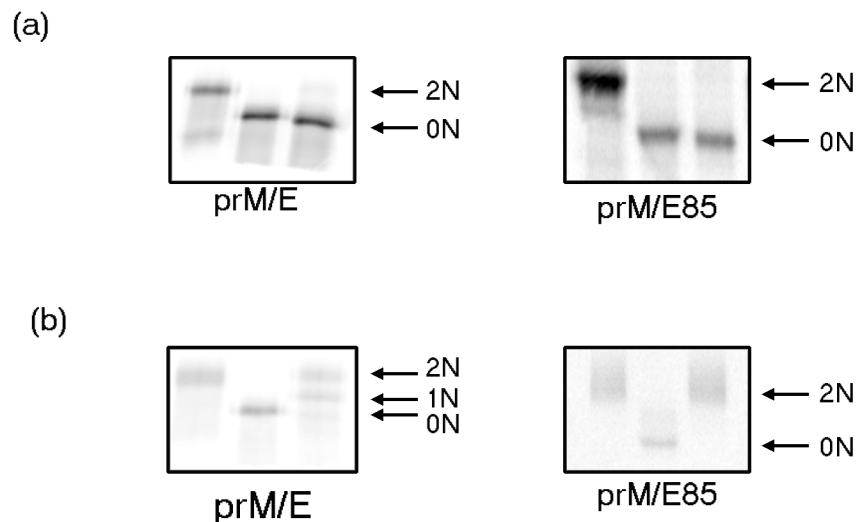


Figure 2.5 Role of the E protein membrane anchor in glycan processing.

DENV2 full-length (preM/E) and soluble (preM/E85) E were expressed in Vero cells using a VEE replicon particle (VRP) expression vector. Vero cells were electroporated with VRP RNA and radiolabeled with [³⁵S]-methionine and cysteine. Envelope protein was immunoprecipitated from cell lysates (**panel a**) and supernatants (**panel b**), digested with endoglycosidases, and separated by SDS-PAGE. Intracellular forms of E protein (**panel a**) were completely sensitive to EndoH (**E**) indicating that most of the protein had not proceeded beyond the endoplasmic reticulum. The secreted full length E protein (**panel b, preM/E**) was incompletely processed and consisted of a mix of EndoH (**E**) resistant and sensitive glycans. In contrast, soluble E (**panel b, preM/E85**), was fully processed and contained two glycans that were EndoH resistant.

**CHAPTER 3. CHARACTERIZATION OF SRI LANKAN CLINICAL
DENV3 ISOLATES**

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ABSTRACT

Prior to 1989, DHF/DSS was a rare disease in the small island of Sri Lanka. Beginning in 1989 and continuing through 2009, DENV infection frequently results in severe cases of DHF and DSS. Previous work analyzing nucleic acid sequences of viruses isolated between 1983 and 1997 suggested that the emergence of DHF in Sri Lanka was due to the introduction of a distinct clade of viruses that replaced viruses circulating prior to 1989 (142). In this study, we sequenced the entire genomes of viruses circulating in Sri Lanka prior to the emergence of DHF and viruses associated with DHF in Sri Lanka, east Africa and Latin America. Based on these full-length sequences, we confirmed the previous evolutionary studies. Additionally, we identified sixteen conserved amino acid substitutions and one 3'UTR change between the two virus groups. To determine if DHF-associated viruses may possess an *in vitro* growth phenotype, we performed infection assays and growth curves in mosquito and mammalian cell lines. Twenty-four hour infection assays suggested that Sri Lankan pre-DHF viruses may have a higher infectivity on human monocytic cells than post-DHF viruses. We performed kinetic growth experiments comparing one virus from each group and discovered that UNC3009 (pre-DHF) has a growth advantage over UNC3006 (post-DHF).

INTRODUCTION

Within the past forty years, dengue has emerged as a major human pathogen worldwide. The virus has been recognized as a human pathogen since the 18th century but it wasn't until recently that explosive epidemics of DHF have become common in tropical and subtropical regions surrounding the globe (63). During and after World War II, southeast Asia and western Pacific islands exhibited high rates of dengue transmission and thousands of DHF/DSS cases each year (60). Beginning in the 1980s, the DHF epidemic spread to India, China, the Caribbean and Latin American (60).

Despite evidence that pre-existing host immunity to dengue contributes to the development of severe disease, the emergence of DHF as a global epidemic is not all attributable to host factors alone. The dramatic expansion of DHF/DSS has been associated with urbanization and expanded vector habitats (60). Additionally, all four serotypes co-circulate in regions that used to only have one or two serotypes (63). Additionally, there are a number of examples where epidemics of DHF coincide with the introduction of a new virus into specific geographical areas (60, 66, 174). One such example is the emergence of DHF on the Indian subcontinent, specifically in Sri Lanka.

Prior to 1989, all four serotypes of DENV circulated in Sri Lanka but there were no regular epidemics of severe disease (143, 222). Between 1966 and 1980, there were fewer than ten cases of severe disease in Sri Lanka (143). Additionally, only 22 potential cases of DHF were documented between 1980 and 1985 (225). Beginning in the summer of 1989, a major DHF epidemic began in Sri Lanka. During the first year of the epidemic, there were 206 cases of clinically diagnosed DHF with a case mortality rate of 10% (225). In the two years that followed, there were 1,121 and 867 DHF cases (225).

Since 1991, huge DHF epidemics have continued to occur annually in Sri Lanka (105, 143) (figure 3.1). In order to elucidate a cause for the sudden emergence of DHF, the Sri Lankan Ministry of Health carried out seroprevalence studies and analyzed environmental factors, examining whether a change in DENV transmission could explain the emergence of DHF (143, 225). DENV transmission did not increase between 1966 and 1991; in 1966, 33% of the Colombo population had antibodies to DENV versus 48% in 1991 (225). Additionally, all four viruses circulated in Sri Lanka prior and subsequent to the emergence of DHF with DENV2 and DENV3 dominating (143). There was also no change in the density, species, or feeding frequency of mosquitoes in Colombo (225). Following analysis of isolates of all four serotypes, Messer *et al* discovered that a new group of DENV3 viruses within genotype III, designated IIIB, had replaced the group of viruses that existed prior to 1989 on Sri Lanka (IIIA) (125, 142). These post-DHF viruses are more closely related to DHF-associated viruses isolated in Africa and Latin America than IIIA viruses isolated in Sri Lanka prior to the emergence of DHF (142).

Since 2000, there have been enormous epidemics of DHF in Sri Lanka each year (Figure 3.1) (105). All four serotypes of DENV still circulate on the island and DENV2 and DENV3 predominate (105). Following phylogenetic analyses based on prM and E sequencing, it was discovered that a new clade of DENV3 viruses had emerged in Sri Lanka. These new viruses are closely related to DENV3 genotype IIIB viruses and likely arose by virus evolution on the island of Sri Lanka (105).

In this study, we sought to further characterize genotype IIIA and IIIB DENV3 isolated in Sri Lanka. First, we sequenced the entire genome of eight Sri Lankan DENV3 isolated between 1983 and 1997. We confirmed that these viruses formed two distinct virus

clades within genotype IIIB and that the post-DHF viruses share a common ancestor with viruses from east Africa and Latin America. We were also able to characterize amino acid substitutions that differentiated pre-DHF viruses from post-DHF viruses. These changes were located in E, NS1, NS3, NS4A and NS5. Finally, we sought to determine if there is an *in vitro* phenotype differentiating the pre-DHF viruses from the post-DHF viruses. We determined that the pre-DHF viruses infected slightly more DC-SIGN expressing U937 cells than post-DHF viruses. Cells infected with the pre-DHF viruses also released more infectious virus into the supernatant compared to those infected with post-DHF viruses. We focused on one virus from each group that displayed different replication kinetics in mammalian cells and determined that a replication kinetic difference is responsible for the phenotype in human monocytic cells.

MATERIALS AND METHODS

CELL LINES AND VIRUS STRAINS

C6/36 *Aedes albopictus* cells were obtained from the American Type Culture Collection (ATCC No: CRL-1660) and propagated in Minimal Essential Media with Earl's salts (E-MEM) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 10% fetal bovine serum (Gibco/Invitrogen). Cells were grown at 28 °C in 5% CO₂. A human monocytic, cell line (U937) constitutively expressing DC-SIGN was obtained from Mark Heise at the University of North Carolina at Chapel Hill and were maintained at 37 °C, 5% CO₂ in RPMI complete media (RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 50 mM 2-mercaptoethanol, and 10% fetal bovine serum (Gibco/Invitrogen)) (115). Vero

(African Green Monkey) Clone 81 cells were a gift from Robert Putnak at Walter Reed Army Medical Center, and were propagated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.2% sodium bicarbonate and 10% fetal bovine serum (Gibco/Invitrogen) at 37 °C, 5% CO₂.

Twelve virus strains were used in these studies. Eight were isolated from hospitalized patients in Sri Lanka between 1983 and 1997. The Sri Lankan clinical isolates are: UNC3001 (89SriLanka1; D2783), UNC3002 (83SriLanka2; D1306), UNC3006 (97SriLanka1; L57), UNC3008 (89SriLanka3; D2803), UNC3009 (89SriLanka2; D2863), UNC3010 (93SriLanka; D5241), UNC3011 (85SriLanka; 073), and UNC3013 (83SriLanka; D1266). One virus, UNC3028 (85Mozamb3; 1559), was isolated in Mozambique in 1985. All nine of these viruses were provided by Duane Gubler at the Centers for Disease Control, Puerto Rico. Three Latin American DENV isolates, UNC3017 (98Nicara1; 6845), UNC3018 (98Nicara3; 7071) and UNC3020 (94Nicara; 032267), were provided by Eva Harris at the University of California, Berkeley.

VIRUS PROPAGATION AND TITRATION

DENV stocks were grown in C6/36 mosquito cells. To generate stocks, seed virus was added to 80% confluent cells at a multiplicity of infection of 0.01 in reduced serum media (E-MEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 2% fetal bovine serum for C6/36 cells and D-MEM/F-12 supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.2% sodium bicarbonate and 5% fetal bovine serum for Vero cells). After seven days, media was harvested from cells and clarified by centrifuging at 10,000 x g for 30

minutes. The virus containing supernatant was centrifuged at 76,221 x g for 4 hrs to pellet virus under a 20% sucrose:PBS (w:v) cushion. Pelleted virus was resuspended in PBS containing 20% FBS and stored at -80 °C.

Virus preparations were titered on Vero cells in a 24-well format immunofocus assay modified from that described in AP61 cells (45). Subconfluent Vero monolayers were infected with serial 10-fold dilutions of infected cell supernatants in Opti-MEM I (Gibco/Invitrogen) supplemented with 1% penicillin/streptomycin (Gibco/Invitrogen) and 2% heat-inactivated FBS. Virus was allowed to adsorb for 2 hours at 37°C. Overlay media (Opti-MEM I containing 5% FBS, 1% penicillin/streptomycin and 0.8% methyl cellulose (Electron Microscopy Sciences)) was then added to plates and cells were incubated for five days at 37 °C, 5% CO₂. Cells were washed two times with 1X PBS (Cellgro) and fixed and permeabilized by incubating in 80% methanol:PBS (v:v) at room temperature for 20 minutes. Cells were then blocked with 5% milk:PBS (w:v) at room temperature for 10 minutes. Monolayers were stained with 400 ng anti-flavivirus envelope antibody 4G2 (ATCC HB-112) followed by a 1:500 dilution of a peroxidase conjugated secondary antibody (Sigma-Aldrich) for 1 hour each at 37 °C. Foci were visualized with 125 µL of TrueBlue Peroxidase Substrate (KPL, Inc). Titers were expressed as focus forming units per mL and were calculated by multiplying the average number of foci per well at a given dilution by the inverse dilution factor and dividing by the volume added to each well.

WHOLE VIRUS SEQUENCING, ALIGNMENT AND PHYLOGENETIC TREE CONSTRUCTION

Viral RNA was isolated from clarified infected cell supernatants with the QiaAmp Viral RNA Mini Kit (Qiagen) using the manufacturer's protocol as described by Messer *et al* (65, 142). Viral RNA was recovered and first strand synthesis was performed using

Superscript III reverse transcriptase (Invitrogen) and a reverse primer at the 3' terminus of the viral genome (5'-TACATGCCTTCAATGAAGAGATTCAGG-3'). The viral cDNA was amplified using the EXPAND High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) in six fragments using the primers listed in table A.1. These PCR fragments were then sequenced using primers on both strands, with 500bp coverage (table A.2). cDNA sequences were assembled and analyzed in ContigExpress (Gibco/Invitrogen). Whole genome sequences were aligned in Align X and translated in Vector NTI (Gibco/Invitrogen). Phylogenetic trees were constructed in Mega4.

INFECTION OF *Aedes C6/36* AND HUMAN MONOCYTIC CELLS

For infection assays, virus was added to 5×10^4 U937+DC-SIGN or C6/36 cells per well at a multiplicity of infection (MOI) of 0.05. Virus was adsorbed onto cells for two hours at 37 °C or 28 °C, 5% CO₂ in reduced serum media. After two hours, cells were washed with PBS to remove unbound virus. U937+DC-SIGN cells were resuspended in RPMI complete media and incubated at 37 °C. At 6-hour intervals post infection, infected cells and supernatant were harvested. E-MEM complete media was added to adherent C6/36 cells and the cells were incubated at 28 °C. At 12-hour intervals, cells and supernatants were harvested. Supernatants from U937+DC-SIGN and C6/36 cells were clarified and virus titers were determined on Vero cells in the immunofocus assay described above. Both cell types were stained and % infection was determined by flow cytometry. Cells were fixed and permeabilized and then stained with Alexa488-conjugated anti-flavivirus membrane antibody 2H2 (kindly provided by Robert Putnak, Walter Reed Army Medical Center).

AMMONIUM CHLORIDE ASSAY

DC-SIGN expressing U937 cells were infected with either UNC3006 or UNC3009 at an MOI of 1.0. Prior to infection, these cells were either incubated in 40mM ammonium chloride (NH₄), pH 7.4 or reduced serum RPMI media for forty-five minutes. Virus was adsorbed to cells as described above in the presence or absence of NH₄. Following a two-hour adsorption, cells were washed with PBS and resuspended in RPMI growth media or media containing 40mM NH₄. NH₄ was added to cells to attain a final concentration of 40mM at various timepoints post infection (15, 30, 45, 60 and 90 minutes, and 2, 4 and 22 hours post infection). At twenty-four hours post infection, cells were harvested and stained and % infection was determined by flow cytometry as described above.

RESULTS

WHOLE GENOME SEQUENCING OF DENV3 CLINICAL ISOLATES

Eight dengue serotype 3 viruses isolated in Sri Lanka between 1983 and 1997, one East African virus isolated in 1985, and three Nicaraguan viruses isolated in 1994 or 1998 were sequenced and analyzed in ContigExpress. The nucleic acid sequences of all eight Sri Lankan viruses are 99% identical (figure 3.2). Following sequencing, the 5' non-coding region, the complete polyprotein coding region and approximately one-third of the 3' non-coding region were aligned and a phylogenetic tree was constructed (figure 3.3). Phylogenetic trees depict evolutionary relationships between sequences. Our tree confirms previous trees based on prM and E sequencing. In trees based on structural gene sequences and the entire genome sequence, the pre-DHF and post-DHF viruses form two distinct evolutionary groups (figure 3.2 and 142). All four pre-DHF viruses sequenced show that

viruses sampled between 1983 and 1989 and not associated with DHF form a discrete evolutionarily distinct group of viruses. Three of the post-DHF viruses, UNC3001, UNC3006 and UNC3008, form a distinct second clade. UNC3010 appears between these post-DHF viruses and the pre-DHF viruses, despite being isolated in 1993 during an outbreak of DHF. As discussed in Messer *et al* (142), the two distinct groups suggest the introduction of new viruses into Sri Lanka and replacing previously circulating strains rather than evolution into more virulent strains.

In an attempt to discover the origin of DHF-associated viruses in Sri Lanka, the pre- and the post-DHF DENV3 were compared to viruses isolated in Mozambique in 1985 and Nicaragua in the 1990s. Figure 3.2 confirms that these DENV3 are closely related to the Sri Lankan post-DHF viruses (142). The East African isolate is most closely related, indicating that viruses from this area may have been introduced into Sri Lanka.

Following the confirmation of evolutionary relationships within DENV3 genotype III, we wanted to compare the polyprotein amino acid sequence of the pre- and post-DHF clinical isolates. Therefore, the coding regions of the eight viruses were translated into polyproteins in VectorNTI (Invitrogen/Gibco). The protein sequences of these eight viruses are also extremely similar (figure 3.4) and there are a number of conserved coding changes conserved within the two groups. One of these changes, a serine in pre-DHF virus and a proline in post-DHF viruses, occurs at positions 124 in domain II of envelope and is the only conserved structural change. The remaining fifteen conserved changes are found in the non-structural genes (highlight in figure 3.4). The majority of the non-structural changes are found in NS5, the viral RNA-dependent RNA polymerase. Interestingly, all but four of the conserved changes that distinguish the post-DHF viruses are also found in the Mozambique and

Nicaraguan isolates. This finding suggests that the Sri Lankan viruses have evolved since their introduction to the island. If conserved amino acid differences between pre- and post-DHF DENV3 are responsible for viral virulence, this finding also hints at residues important in pathogenesis.

In addition to the coding changes discussed above, there are conserved non-coding differences in pre- and post-DHF viruses found in the 3'UTR (figure 3.2). These changes are: C at nucleotide 10,307 of IIIA viruses changed to T in all DHF-associated viruses, G at nucleotide 10,436 of IIIA viruses and UNC3020 changed to A in all other viruses sequenced, and a T at nucleotide 10,594 changed to C in IIIB viruses (203).

PRE-DHF ISOLATES MAY DIFFERENTIALLY INFECT MAMMALIAN CELL LINES

In previous experiments with DENV2, the southeast Asian viruses associated with severe disease behave differently in cell culture experiments than less pathogenic American viruses (38, 39). This *in vitro* phenotype has been mapped to differences in E and the non-coding regions. Because our pre- and post-viruses differ in regions known to influence *in vitro* viral growth, we examined whether the IIIA and IIIB clinical isolates behave differently *in vitro*. To test this, we infected DC-SIGN expressing U937 cells with our Sri Lankan DENV3 at high and low MOIs. At twenty-four hours, there was a suggestion that pre-DHF viruses may infect a high percentage of cells than post-DHF DENV3 (figure 3.5a). To further examine and tease apart this phenotype, we performed growth curves in DC-SIGN expressing U937 cells, harvesting and titring supernatants every twelve hours post infection (figure 3.5b).

Unfortunately, we only have four Sri Lankan clinical DENV3 isolates in each group. Therefore, if the magnitude of the difference in growth is small, we do not have the statistical

power to conclude whether pre-DHF viruses enter cells and replicate faster than post-DHF viruses. Thus, we decided to focus on two viruses that firmly represent their groups, UNC3009 (IIIA) and UNC3006 (IIIB). UNC3009 infects DC-SIGN expressing U937 and primary DC extremely well while UNC3006 is a poor infector of these same cells (figure 3.6a and data not shown). After performing detailed growth curves with UNC3009 and UNC3006, we learned that the UNC3006's growth defect is seen only in mammalian cells (figure 3.6). UNC3006 and UNC3009 infect an equal percentage of C6/36 mosquito cells at all time points tested and an equal amount of virus is released into the supernatant of infected cells (figure 3.6b).

Based on these growth curves performed in DC-SIGN expressing U937 cells, there appears to be a kinetic difference between UNC3009 and UNC3006. UNC3009 is able to replicate more quickly than UNC3006. To determine if the structural difference at position 124 of the envelope enables the virus to enter cells more quickly than UNC3006, we performed an ammonium chloride fusion inhibition experiment with these two viruses (figure 3.7). In human monocytic cells, ammonium chloride increases the pH of intracellular compartments. Since DENV undergoes a low-pH mediated fusion event to enter the cytoplasm of cells, ammonium chloride will prevent the fusion of DENV and thus a productive infection by any virus upstream of endosomal escape. All virus particles that have progressed past fusion will be able to continue the life cycle, resulting in a productive DENV infection. In this entry inhibition time course experiment, NH_4Cl was added to cells every fifteen minutes for the first hour of infection, at ninety minutes post infection, and at two, four and twenty-two hours post infection. Interestingly, our DENV3s were slow to enter cells, taking at least ninety minutes for fifty percent of infectious virus to progress past fusion (figure 3.7). In multiple

experiments, fifty percent of UNC3009 progressed past fusion by ninety minutes post infection. It was not until four hours post infection that fifty percent of UNC3006 had escaped the endosome. Based on the NH_4Cl entry inhibition experiments we performed there may be a small difference in the speed at which UNC3009 and UNC3006 enter cells. We do not know if this short delay in UNC3006's entry could have a downstream effect resulting in a significant replication defect.

DISCUSSION

In this chapter, we analyzed two groups of viruses isolated from patients in Sri Lanka. The viruses in one group, DENV3 genotype IIIA, were isolated prior to the emergence of DHF and are not associated with severe disease. The second group, DENV3 genotype IIIB, is associated with the emergence of DHF in Sri Lanka (142). Earlier genetic and evolutionary studies of these viruses focused only on the structural genes, specifically prM and E. In this study, we sequenced the entire virus and repeated evolutionary analyses. The relationships based upon the previous sequencing were confirmed in our whole genome analysis. We also translated the whole genome sequences of IIIA and IIIB viruses and analyzed amino acid positions that differ between the two groups. We found sixteen conserved amino acid difference between pre- and post-DHF viruses. Only one of these conserved changes was in the structural genes and fifteen were in the non-structural proteins. Finally, we performed infection assays with pre- and post-DHF viruses that suggested pre-DHF viruses infect more mammalian cells. This higher infectivity may be due to faster replication kinetics associated with quicker entry into target cells.

The phylogenetic trees constructed in this study confirm findings by Messer *et al* (142). We found that the pre- and the post-DHF viruses form two distinct clades of viruses. The post-DHF viruses are more closely related to an east African DENV3 isolate than they are to the pre-DHF viruses. These results lend credence to the hypothesis proffered by Messer *et al* that DHF emerged in Sri Lanka following the introduction of genetically distinct DENV3 from east Africa or India (142).

Following whole genome sequencing of the pre- and post-DHF clinical isolates, we analyzed the polyprotein amino acid sequences and identified positions that differ between the two groups. Because UNC3010 does not cleanly group with either IIIA or IIIB viruses, we excluded it from our analysis. There are only sixteen amino acid differences conserved between the pre- and the post-DHF viruses. Only one of these changes, S124P in domain II of E, occurs in the structural proteins. This change is surface exposed and is located in the vicinity of the fusion peptide. Because a serine to proline is a drastic amino acid difference, it is possible that this change could influence the structure of the virion, interactions with host cell receptors, or fusion characteristics of E. With DENV2, mutations in this region have been associated with mouse adaptation and viral clearance (163). Changes in a different region of E are implicated with different infectivity of the American and southeast Asian DENV2s in monocytic cells (38, 39).

The conserved non-structural differences between the pre- and the post- viruses are located in NS1 (4), NS2A (1), NS3 (1), NS4A (2), and NS5 (7). Of these changes, four (including the one difference in NS2A) are seen only in the DHF associated viruses in Sri Lanka and not in those isolated in east Africa or Latin America. NS1 plays an important role in viral replication and co-localizes to sites of RNA replication (130). Recent work has

suggested that NS1 may also play a role in signaling during viral infection (reviewed in 58). NS3 is the viral serine protease that co-translationally cleaves the polyprotein, producing active individual proteins (130). The role of NS4A during viral replication and translation is unknown. Currently, its only known role is as a potential interferon antagonist (152). NS5 is the viral RNA-dependent RNA polymerase. The amino-terminal of this protein has cap-processing activity and the C-terminal end possesses RNA-dependent RNA polymerase activity (48, 130). Only one of the changes conserved among all DHF associated viruses was located in the polymerase domain (48). The other conserved differences are located in the nuclear localization signal (374) and the methyltransferase domain (50, 253 and 270) (48). Additionally, there were two conserved 3'UTR nucleotide changes found in viruses associated with DHF: C 10,307 T and G 10,436 A. The non-coding regions of DENV RNA play an important role in polyprotein translation and viral replication (130). Specifically, the basepairing between regions of the 3' UTR forms stem loop structures that interact with elongation factors essential for translation and play a role in RNA encapsidation (130).

During our comparison of the IIIA and IIIB DENV3 Sri Lankan isolates, we performed experiments that suggested the pre-DHF viruses might infect more cells than post-DHF DENV3. Results with representative viruses from these two groups, UNC3009 and UNC3006, suggested this infectivity difference is because UNC3009 replicates faster than UNC3006. Amino acid differences in E, NS1, NS3 and NS5, and nucleotide changes in the 3'UTR could influence various stages of the viral lifecycle, resulting in more efficient infection of pre-DHF viruses (UNC3009) compared to post-DHF viruses (UNC3006). Specifically, the S124P change in E could influence receptor binding or viral fusion. The two conserved NS4A differences could alter the host response to DENV3 infection, resulting

in different outcomes of infection. NS1, NS3 and NS5 changes could alter the efficiency of protein translation, serine protease cleavage or RNA replication. Finally, nucleotide changes in the 3'UTR could alter stem loop structures and result in decreased polyprotein translation or RNA encapsidation.

Experiments performed with UNC3006 and UNC3009 to determine why UNC3009 infects more cells faster than UNC3006 have so far been inconclusive. Thus far, it does not appear that viral binding to host cell receptors differs between these two viruses. Additionally, a host cell interferon response does not appear to be responsible for UNC3006's *in vitro* growth defect. In order to more definitively answer why UNC3009 (and potentially pre-DHF clinical isolates) infects mammalian cells more efficiently than UNC3006 (post-DHF clinical isolates), we would like to utilize viral genetics and identify the protein responsible for *in vitro* phenotype. In the next chapter we describe the construction of a DENV3 infectious to identify genetic determinants responsible for phenotypic differences between DENV3 strains.

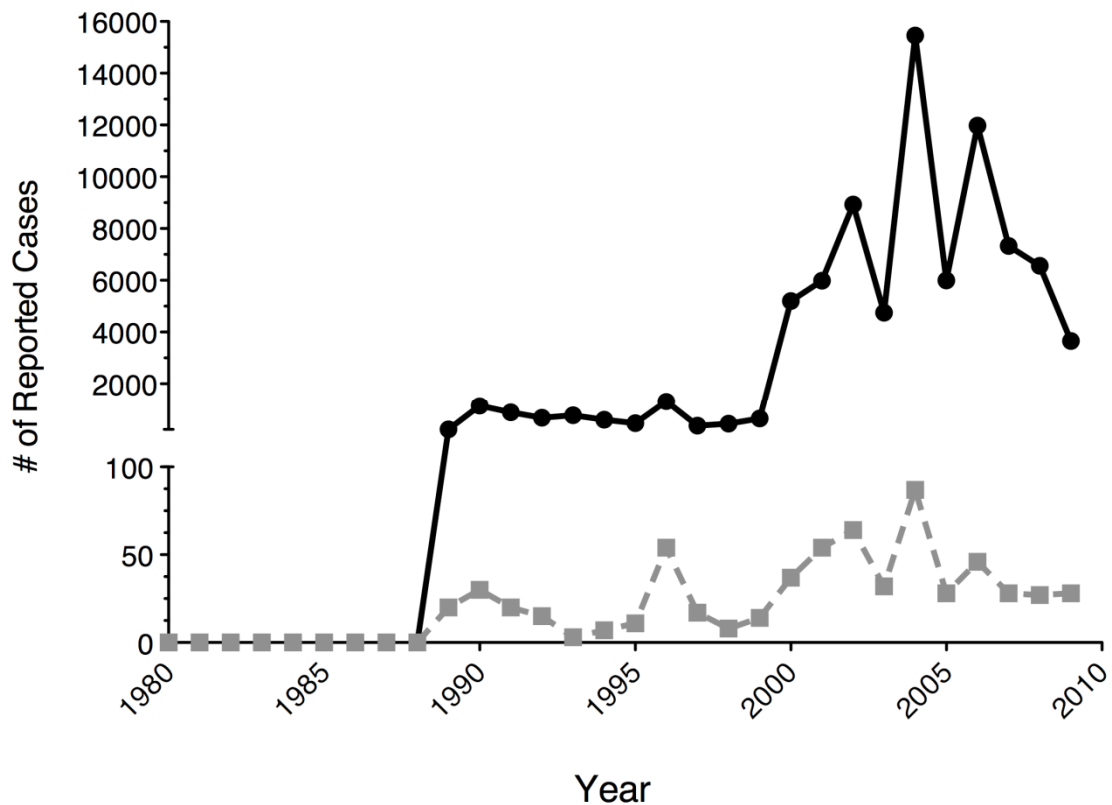


Figure 3.1 Emergence of DHF in Sri Lanka.

The number of cases of DHF/DSS (black circles) and the number of deaths due to DENV (gray squares), as reported to the Sri Lankan Ministry of Health, are shown above. The data representing 2009 is only through April 21, 2009. Figure is adapted from (143) and data provided by the Sri Lankan Ministry of Health Epidemiology Unit.

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UNC3001 AGTTGTTAGTCTACGTGGACCGACAAGAACAGTTTCGACTCGGAAGCTTGCTTAACGTAGTGCTAACAGTTTTTTATTAGAGAGCAGATCTCTGATGAAC [ 100]
UNC3002 ..... [ 100]
UNC3006 ..... [ 100]
UNC3008 ..... [ 100]
UNC3009 ..... [ 100]
UNC3010 ..... [ 100]
UNC3011 ..... [ 100]
UNC3013 ..... [ 100]

UNC3001 AACCAACGGAAGAAGACGGGAAAACCGTCTATCAATATGCTGAAACGCGTGAGAAAACCGTGTGCAACTGGACCACAGTTGGCGAAGAGATTCTCAAAG [ 200]
UNC3002 .....A.....T..... [ 200]
UNC3006 .....T..... [ 200]
UNC3008 .....T..... [ 200]
UNC3009 .....A.....T..... [ 200]
UNC3010 .....T..... [ 200]
UNC3011 .....A.....T..... [ 200]
UNC3013 .....A.....T..... [ 200]

UNC3001 GACTGCTGAACGGCCAGGGACCAATGAAATTGGTTATGGCGTTCATAGCTTTCCTCAGATTTCTAGCCATTCCACCAACAGCAGGAGTCTTGCTAGATG [ 300]
UNC3002 ..... [ 300]
UNC3006 ..... [ 300]
UNC3008 ..... [ 300]
UNC3009 ..... [ 300]
UNC3010 ..... [ 300]
UNC3011 ..... [ 300]
UNC3013 ..... [ 300]

UNC3001 GGGAACCTTCAAGAAGTCGGGAGCCATTAAGGTCCTGAAAGGCTTCAAGAAGGAGATCTCAAACATGCTGAGCATAATCAACAAACGGAAAAAGACATCG [ 400]
UNC3002 .....G.....A.....A [ 400]
UNC3006 .....A.....A.....G..... [ 400]
UNC3008 .....A.....A.....G..... [ 400]
UNC3009 .....G.....A..... [ 400]
UNC3010 .....A.....G.....T..... [ 400]
UNC3011 .....G.....A.....A [ 400]
UNC3013 .....G.....A..... [ 400]

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Figure 3.2 Nucleic acid sequence alignment of eight DENV isolated from hospitalized patients in Sri Lanka.

UNC3002, UNC3009, UNC3011, UNC3013 were isolated in or before 1989 and are not associated with DHF. The other four viruses, UNC3001, UNC3006, UNC3008 and UNC3010, were isolated in or after 1989 and are closely related to DENV3 viruses that continue to circulate in Sri Lanka. The sequence of UNC3001 is listed as the consensus and periods indicate identity. Dashes represent missing sequence data. All nucleic acid changes are represented with letters below the consensus.

UNC3001 CTCTGTCTCATGATGATATTGCCAGCAGCACTTGCTTTCCACTTGACTTCACGAGATGGAGAGCCGCGCATGATTGTGGGGAAGAATGAAAGAGGAAAAAT [500]
 UNC3002 [500]
 UNC3006 [500]
 UNC3008 [500]
 UNC3009 [500]
 UNC3010 [500]
 UNC3011 [500]
 UNC3013 [500]

UNC3001 CCCTACTTTTTAAGACAGCCTCTGGAATTAACATGTGCACACTCATAGCCATGGACTTGGGAGAGATGTGTGATGACACGGTCACCTTACAAATGCCCCCA [600]
 UNC3002 .T.....A..... [600]
 UNC3006 [600]
 UNC3008 [600]
 UNC3009 .T.....A..... [600]
 UNC3010C.....T..... [600]
 UNC3011 .T.....A..... [600]
 UNC3013 .T.....A..... [600]

UNC3001 CATTACCGAAGTGGAGCCTGAAGACATTGACTGCTGGTGCAACCTTACATCAACATGGGTGACTTATGGAACGTGCAATCAAGCTGGAGAGCATAGACGC [700]
 UNC3002A.....G.....C..... [700]
 UNC3006A..... [700]
 UNC3008A..... [700]
 UNC3009A.....T.....G..G.....C..... [700]
 UNC3010A.....C..... [700]
 UNC3011A.....G.....C..... [700]
 UNC3013A.....G.....C..... [700]

UNC3001 GACAAAAGATCAGTGGCGTTAGCTCCTCATGTCGGCATGGGACTGGACACACGCCAACCAACCTGGATGTCGGCTGAAGGAGCTTGGAGACAAGTCGAGA [800]
 UNC3002G.....C.....T.....T..... [800]
 UNC3006 [800]
 UNC3008 [800]
 UNC3009G.....C.....T.....T..... [800]
 UNC3010G.....C.....C.....T.....A..... [800]
 UNC3011G.....C.....T.....T..... [800]
 UNC3013G.....C.....T.....T..... [800]

UNC3001 AGGTAGAGACATGGGCCCTCAGGCACCCAGGGTTCACCATACTAGCCCTATTTCTTGCCATTACATAGGCACCTTCCTTGACCCAGAAGGTGGTTATTTT [900]
 UNC3002T.....T.....C..... [900]
 UNC3006C..... [900]
 UNC3008T..... [900]
 UNC3009T.....T.....C..... [900]
 UNC3010T.....C..... [900]
 UNC3011T.....T.....C..... [900]
 UNC3013T.....T.....C..... [900]

UNC3001 TATACTACTAATGCTGGTCACCCATCCATGACAATGAGATGTGTGGGAATAGGAAACAGAGATTTTGTGGAAGGTCTATCAGGAGCTACGTGGGTTGAC [1000]
 UNC3002 C.....G.....T..... [1000]

UNC3006 [1000]
 UNC3008G..... [1000]
 UNC3009 C.....G.....T..... [1000]
 UNC3010 ...T..T.....G.....G..... [1000]
 UNC3011 C.....G.....T..... [1000]
 UNC3013 C.....G.....A.....T..... [1000]

UNC3001 GTGGTGC TCGAGCACGGGGGTGTGTGACTACCATGGCTAAGAACAAGCCACGCTGGATATAGAGCTTCAGAAGACCAGGCCACCCAACCTGGCGACCC [1100]
 UNC3002T.....T..... [1100]
 UNC3006 [1100]
 UNC3008 [1100]
 UNC3009T.....T..... [1100]
 UNC3010 [1100]
 UNC3011T.....T..... [1100]
 UNC3013T.....T..... [1100]

UNC3001 TAAGGAAGCTATGCATTGAGGGGAAAATTACCAACATAACAACCTGACTCAAGATGTCCTACCCAAGGGGAAGCGGTTTTGCCTGAGGAGCAGGACCAGAA [1200]
 UNC3002C..... [1200]
 UNC3006 [1200]
 UNC3008T..... [1200]
 UNC3009T.....C..... [1200]
 UNC3010 [1200]
 UNC3011C..... [1200]
 UNC3013C..... [1200]

UNC3001 CTACGTGTGTAAGCATAACATACGTAGACAGAGGCTGGGGGAACGGTTGTGGCTTGTGGCAAGGGGAAGCTTGGTAACGTGTGCGAAATTTCAATGCCTG [1300]
 UNC3002T.....A..... [1300]
 UNC3006T..... [1300]
 UNC3008 [1300]
 UNC3009T.....A..... [1300]
 UNC3010T.....A.....A..... [1300]
 UNC3011T.....A..... [1300]
 UNC3013T.....A..... [1300]

UNC3001 GAACCAATAGAGGGAAAAGTGGTGCATATGAGAACCTCAAATACACCGTCATCATTACAGTGCACACAGGAGACCAACACCGGTAGGAAATGAAACGC [1400]
 UNC3002 ...T..... [1400]
 UNC3006 [1400]
 UNC3008 [1400]
 UNC3009 ...T.....G..... [1400]
 UNC3010G..... [1400]
 UNC3011 ...T.....G..... [1400]
 UNC3013 ...T.....G..... [1400]

UNC3001 AGGGAGTCACGGCTGAGATAACACCTCAGGCATCAACCACTGAAGCCATCTTGCCTGAATATGGAACCCCTGGGCTAGAATGCTCACCACGGACAGGTTT [1500]
 UNC3002 [1500]
 UNC3006 [1500]

UNC3008 [1500]
 UNC3009 A T [1500]
 UNC3010 .A [1500]
 UNC3011 T [1500]
 UNC3013 T [1500]

UNC3001 GGATTTCAATGAAATGATCTTACTAACAATGAAGAACAAAGCATGGATGGTACATAGACAATGGTTTTTTGACCTACCTCTACCATGGACATCAGGAGCT [1600]
 UNC3002 C [1600]
 UNC3006 ... C . T [1600]
 UNC3008 [1600]
 UNC3009 C T [1600]
 UNC3010 C G [1600]
 UNC3011 C [1600]
 UNC3013 C [1600]

UNC3001 ACAACAGAAACGCCAACCTGGAACAGGAAGGAGCTTCTTGTGACATTCAAAAACGCACATGCGAAAAACAAGAAGTAGTCGTCCTTGGATCGCAAGAGG [1700]
 UNC3002 A T [1700]
 UNC3006 G [1700]
 UNC3008 T T [1700]
 UNC3009 A T [1700]
 UNC3010 A T [1700]
 UNC3011 A T [1700]
 UNC3013 A T [1700]

UNC3001 GAGCAATGCATACCGCACTGACAGGAGCCACAGAAATCCAAAACCTCAGGAGGCACAAGCATTGCGGGGCACCTAAAATGTAGACTTAAGATGGACAA [1800]
 UNC3002 A [1800]
 UNC3006 [1800]
 UNC3008 [1800]
 UNC3009 A [1800]
 UNC3010 [1800]
 UNC3011 A [1800]
 UNC3013 A [1800]

UNC3001 ATTGGAACCAAGGGGATGAGCTATGCAATGTGCACGAATACCTTTGTGTTGAAGAAAGAAGTCTCAGAAACGCAGCATGGGACAATACTCATTAAAGGTC [1900]
 UNC3002 G ... A ... [1900]
 UNC3006 [1900]
 UNC3008 [1900]
 UNC3009 A ... [1900]
 UNC3010 C T [1900]
 UNC3011 G ... A ... [1900]
 UNC3013 G ... A ... [1900]

UNC3001 GAGTACAAAGGGGAAGATGCACCTTGCAAGATTCTTTCTCCACAGAGGATGGACAAGGGAAAGCTCACAATGGCAGACTGATCACAGCCAACCCAGTGG [2000]
 UNC3002 [2000]
 UNC3006 [2000]
 UNC3008 [2000]

UNC3009 [2000]
 UNC3010C.....T..... [2000]
 UNC3011 [2000]
 UNC3013 [2000]

UNC3001 TGACTAAGAAGGAGGAGCCTGTCAATATTGAGGCTGAACCTCCTTTTGGGGAAAGTAATATAGTAATTGGAATTGGAGACAACGCCTTGAAAATCAACTG [2100]
 UNC3002 [2100]
 UNC3006 [2100]
 UNC3008 [2100]
 UNC3009 [2100]
 UNC3010C..... [2100]
 UNC3011 [2100]
 UNC3013 [2100]

UNC3001 GTACAAGAAGGGAAGCTCTATTGGGAAGATGTTGAGGCCACTGCCAGAGGTGCAAGGCGCATGGCCATCTTGGGAGACACAGCTTGGGACTTTGGATCA [2200]
 UNC3002G.....C..... [2200]
 UNC3006 [2200]
 UNC3008 [2200]
 UNC3009G.....C..... [2200]
 UNC3010G.....G..... [2200]
 UNC3011G.....C..... [2200]
 UNC3013G.....C..... [2200]

UNC3001 GTGGGTGGTGTCTGAACTCATTAGGC AAAATGGTGCACCAAATATTCGGAAGTGTTACACAGCCCTATTCAGTGGAGTCTCTTGGGTGATGAAAATTG [2300]
 UNC3002G.....T..... [2300]
 UNC3006 [2300]
 UNC3008 [2300]
 UNC3009G.....T..... [2300]
 UNC3010T.....T..G..... [2300]
 UNC3011G.....T..... [2300]
 UNC3013G.....T..... [2300]

UNC3001 GAATAGGTGTTCTTGACTTGGATAGGGTTGAATTCAAAAACACATCCATGTCATTTTCATGCATTGCGATAGGAATCATTACACTCTATCTGGGAGC [2400]
 UNC3002C..... [2400]
 UNC3006C..... [2400]
 UNC3008 [2400]
 UNC3009C..... [2400]
 UNC3010C..... [2400]
 UNC3011C..... [2400]
 UNC3013C..... [2400]

UNC3001 TGTGGTACAAGCTGACATGGGGTGTGCATAAACTGGAAGGCAAAGAAGTCAAAATGTGGAAGTGAATTTTCGTCACCAACGAGGTCCATACCTGGACA [2500]
 UNC3002 C..... [2500]
 UNC3006 [2500]
 UNC3008 [2500]
 UNC3009 C.....CA..... [2500]

UNC3010T. [2500]
 UNC3011 C..... [2500]
 UNC3013 C..... [2500]

UNC3001 GAGCAATACAAATCCAAGCAGACTCCCCAAAAGATTGGCGACAGCCATTGCAGGCGCTTGGGAGAATGGAGTGTGCGGAATTAGGTCAACAACCAGAA [2600]
 UNC3002A.....C..... [2600]
 UNC3006 [2600]
 UNC3008 [2600]
 UNC3009A.....C..... [2600]
 UNC3010A.....T..... [2600]
 UNC3011A.....C..... [2600]
 UNC3013A.....C..... [2600]

UNC3001 TGGAGAATCTCTTGTGGAAGCAAATAGCCAATGAACTGAACTACATATTATGGGAAAACAATATCAAATTAACGGTAGTTGTGGGCGATACAATTGGGGT [2700]
 UNC3002 [2700]
 UNC3006T.....G..... [2700]
 UNC3008 [2700]
 UNC3009 [2700]
 UNC3010C..... [2700]
 UNC3011 [2700]
 UNC3013 [2700]

UNC3001 CTTAGAGCAAGGAAAAAGAACTAACACCACAACCCATGGAGCTAAAATACTCATGGAAAACATGGGGAAAGGCAAAAATAGTGACAGCTGAAACACAA [2800]
 UNC3002G.....T.....G.....T..... [2800]
 UNC3006T..... [2800]
 UNC3008T.....G..... [2800]
 UNC3009G.....T.....G.....T..... [2800]
 UNC3010G.....G..... [2800]
 UNC3011G.....T.....G.....T..... [2800]
 UNC3013G.....T.....G.....T..... [2800]

UNC3001 AATTCTCCTTCATAATAGACGGGCCAAACACACCGGAGTGTCCAAGTGCCTCAAGAGCATGGAATGTGTGGGAGGTGGAAGATTACGGGTTCCGGAGTCT [2900]
 UNC3002T..... [2900]
 UNC3006 [2900]
 UNC3008 [2900]
 UNC3009T..... [2900]
 UNC3010T.....T..... [2900]
 UNC3011T..... [2900]
 UNC3013T..... [2900]

UNC3001 TCACAACCAACATATGGCTGAAACTCCGAGATGTGTACACCCAATATGTGACCATAGGCTAATGTCGGCAGCCGTCAAGGATGAGAGGGCCGTACACGC [3000]
 UNC3002G.....T.....T..... [3000]
 UNC3006G.....T..... [3000]
 UNC3008 [3000]
 UNC3009G.....T.....T..... [3000]
 UNC3010G.....T.....G..T..... [3000]

UNC3011G.....T.....T.. [3000]
 UNC3013G.....T.....A.....T.. [3000]

UNC3001 CGACATGGGCTATTGGATAGAAAGCCAAAAGAATGGAAGTTGGAAGCTAGAAAAAGCATCCCTCATAGAGGTGAAAACCTGCACATGGCCAAAATCACAC [3100]
 UNC3002T..... [3100]
 UNC3006T..... [3100]
 UNC3008A.....A..... [3100]
 UNC3009G.....T..... [3100]
 UNC3010G..... [3100]
 UNC3011T..... [3100]
 UNC3013T..... [3100]

UNC3001 ACTCTTTGGAGCAATGGTGTGCTAGAGAGTGACATGATCATCCCAAGAGTCTAGCTGGTCCTATTTGCAACACAACACTACAGGCCTGGGTACCCACCCC [3200]
 UNC3002 ..C.....C.....T..... [3200]
 UNC3006 [3200]
 UNC3008C..... [3200]
 UNC3009C.....T..... [3200]
 UNC3010C.....T.....A.....C..... [3200]
 UNC3011 ..C.....C.....T..... [3200]
 UNC3013C.....T..... [3200]

UNC3001 AAACAGCAGGACCCTGGCACTTAGGAAAATTGGAGCTGGACTTCAACTATTGTGAAGGAACAACAGTTGTATCACAGAAAACCTGTGGGACAAGAGGCC [3300]
 UNC3002 ...G..... [3300]
 UNC3006 [3300]
 UNC3008 ...G..... [3300]
 UNC3009 ...G..... [3300]
 UNC3010 ...G.....T..... [3300]
 UNC3011 ...G..... [3300]
 UNC3013 ...G..... [3300]

UNC3001 ATCATTGAGAACAACAACAGTGTGAGGGAAGTTGATACACGAATGGTGTGCGCTCGTGCACACTTCTCCCCTGCGATACATGGGAGAAGACGGCTGC [3400]
 UNC3002 [3400]
 UNC3006 [3400]
 UNC3008 [3400]
 UNC3009 [3400]
 UNC3010 ...C..... [3400]
 UNC3011 [3400]
 UNC3013 [3400]

UNC3001 TGGTATGGCATGAAATCAGACCCATCAGTGAGAAAAGAGAGAACATGGTAAAGTCTTTAGTCTCAGCGGGAAGTGAAAAGTTGGACAACCTTACAATGG [3500]
 UNC3002T.....C.....A..G..... [3500]
 UNC3006A..... [3500]
 UNC3008 [3500]
 UNC3009T.....C.....A..G..... [3500]
 UNC3010 [3500]
 UNC3011T.....C.....A..G..... [3500]

UNC3013C.....A..... [3500]
 UNC3001 GTGCTTGTGTTGGCAATCCTCTTTGAAGAGGTGATGAGAGGAAAATTTGGGAAGAAACACATGATTGCAGGGTTCTTTCACGTTTGTGCTCCTCT [3600]
 UNC3002T.....C..... [3600]
 UNC3006 [3600]
 UNC3008 [3600]
 UNC3009A..G.....T.....C..... [3600]
 UNC3010T...T..... [3600]
 UNC3011T.....C..... [3600]
 UNC3013T.....C..... [3600]
 UNC3001 CTCAGGGCAAATAACATGGAGAGACATGGCGCACACTAATAATGATTGGGTCCAACGCCTCTGACAGGATGGGAATGGGCGTCACCTACCTAGCTTTA [3700]
 UNC3002 ...G.....C.....A.....T..... [3700]
 UNC3006 [3700]
 UNC3008 [3700]
 UNC3009 ...G.....C.....A.....T..... [3700]
 UNC3010C... [3700]
 UNC3011 ...G.....C.....A.....T..... [3700]
 UNC3013C.....A.....T..... [3700]
 UNC3001 ATTGCAACATTTAAATCCAGCCATTCTGGCTTTGGGATTTTCTAAGAAAAGTACATCTAGAGAAAATTTATTGTTAGGAGTTGGGCTGGCTATGG [3800]
 UNC3002G.....T..... [3800]
 UNC3006 [3800]
 UNC3008 [3800]
 UNC3009G.....T..... [3800]
 UNC3010G.....T...C... [3800]
 UNC3011G.....T..... [3800]
 UNC3013 [3800]
 UNC3001 CAACAACGTTACAACCTGCCAGAGGACATTGAACAAATGGCAAATGGAATCGCTCTGGGGCTCATGGCTCTTAAACTGATAACACAATTTGAAACATACCA [3900]
 UNC3002C.G..... [3900]
 UNC3006 [3900]
 UNC3008C..... [3900]
 UNC3009C.G..... [3900]
 UNC3010 [3900]
 UNC3011C.G..... [3900]
 UNC3013C.G..... [3900]
 UNC3001 ATTATGGACGGCATTAGTCTCCTTAACGTGTTCAAATACAATTCTTACGTTGACTGTTGCCTGGAGAACAGCCACCCTGATTTGGCCGGAGTTTCGCTT [4000]
 UNC3002C.....GT..... [4000]
 UNC3006A..... [4000]
 UNC3008 .C..... [4000]
 UNC3009A...C.....C.....GT..... [4000]
 UNC3010 ...G.....T.....T..... [4000]
 UNC3011C.....GT..... [4000]
 UNC3013 .C.....C..... [4000]

UNC3001 TTACCAAGTGTGCCAGTCTTCGAGCATGAGGAAAACAGACTGGCTTCCAATGACAGTGGCAGCTATGGGAGTTCACCCCTACCACCTTTTATTTTAGCT [4100]
 UNC3002C.....T..... [4100]
 UNC3006C [4100]
 UNC3008 [4100]
 UNC3009C.....T..... [4100]
 UNC3010A.....C.....T.....C..... [4100]
 UNC3011C.....T..... [4100]
 UNC3013T..... [4100]

UNC3001 TGAAAGACACACTCAAAGGAGAAGCTGGCCACTGAATGAAGGGGTGATGGCTGTTGGGCTTGTGAGCATTCTGGCCAGTTCTCTCCTTAGAAATGATGT [4200]
 UNC3002G.....G..... [4200]
 UNC3006 [4200]
 UNC3008C..... [4200]
 UNC3009G.....C.....G..... [4200]
 UNC3010C..... [4200]
 UNC3011G.....G..... [4200]
 UNC3013C..... [4200]

UNC3001 GCCCATGGCTGGACCATTAGTGGCCGGGGCTTGCTGATAGCGTGCTACGTACATAACTGGCACGTGAGCAGACCTCACTGTGGAAAAGCAGCAGATGTA [4300]
 UNC3002C.....A.....G..... [4300]
 UNC3006G [4300]
 UNC3008T..... [4300]
 UNC3009A.....C.....A..... [4300]
 UNC3010A..... [4300]
 UNC3011C.....A.....G..... [4300]
 UNC3013C.....A..... [4300]

UNC3001 ACATGGGAGGAAGAGGCTGAGCAAACAGGAGTGTCACCACTTAATGATCACAGTTGATGATGATGGAACAATGAGAATAAAAGATGATGAGACTGAGA [4400]
 UNC3002 [4400]
 UNC3006C..... [4400]
 UNC3008 [4400]
 UNC3009 [4400]
 UNC3010G..... [4400]
 UNC3011 [4400]
 UNC3013 [4400]

UNC3001 ACATCCTAACAGTGCTTTTAAAAACAGCATTACTAATAGTATCAGGCATCTTCCATACTCCATACCCGCAACATTGTTGGTCTGGCATACTTGCCAAAA [4500]
 UNC3002T..... [4500]
 UNC3006T.....C..... [4500]
 UNC3008 [4500]
 UNC3009T..... [4500]
 UNC3010C.....T.....C..... [4500]
 UNC3011T..... [4500]
 UNC3013 [4500]

UNC3001 GCAAACCCAAAGGTCCGGCGTTCTGTGGGACGTACCCAGCCCCCAGAGACACAGAAAGCAGAAGTGGAAAGAAGGGGTTTATAGGATCAAACAGCAAGGA [4600]
 UNC3002A.....A.....C..... [4600]
 UNC3006A..... [4600]
 UNC3008A..... [4600]
 UNC3009A.....A.....T.....C..... [4600]
 UNC3010A.....T.A..... [4600]
 UNC3011A.....A.....C..... [4600]
 UNC3013A.....A..... [4600]

UNC3001 ATTCTTGGGAAAACCCAAGTAGGGTTGGAGTACAGAAAGAAGGAGTCTTCCACACCATGTGGCACGTACAAGAGGGGCAGTGTGACACATAATGGGA [4700]
 UNC3002 ..C..C..... [4700]
 UNC3006 ...T..... [4700]
 UNC3008 [4700]
 UNC3009 ..C..C.....C..... [4700]
 UNC3010 [4700]
 UNC3011 ..C..C..... [4700]
 UNC3013C..... [4700]

UNC3001 AAAGACTGGAACCAAACCTGGGCTAGCGTGAAAAAGATCTGATTTCATACGGAGGAGGATGGAGATTGAGCGCGCAATGGCAAAAAGGGGAGGAGGTGCA [4800]
 UNC3002T..A..... [4800]
 UNC3006T..... [4800]
 UNC3008A..... [4800]
 UNC3009T..A..... [4800]
 UNC3010T.....C.....A..... [4800]
 UNC3011T..A..... [4800]
 UNC3013A..... [4800]

UNC3001 GGTATTGCCGTGGAGCCTGGGAAGAACCCAAAGAACTTTCAAACCATGCCAGGCACTTTTCAGACTACAACAGGGGAAATAGGAGCAATTGCACTGGAT [4900]
 UNC3002C..T..A..... [4900]
 UNC3006 [4900]
 UNC3008A..... [4900]
 UNC3009C..T..A..... [4900]
 UNC3010C.....A.....A.....T..... [4900]
 UNC3011C..T..A..... [4900]
 UNC3013C.....A.....A..... [4900]

UNC3001 TTCAAGCCTGGAACCTCAGGATCTCCTATCATAAACAGAGAGGGAAAGGTAGTGGGACTGTATGGCAATGGAGTGGTTACAAAGAATGGTGGCTACGTCA [5000]
 UNC3002 ..T..... [5000]
 UNC3006C.....C..... [5000]
 UNC3008A..... [5000]
 UNC3009 ..T..... [5000]
 UNC3010T..... [5000]
 UNC3011 ..T..... [5000]
 UNC3013 [5000]

UNC3001 GCGGAATAGCGCAAACAAATGCAGAACCAGATGGACCGACACCAGAGTTGGAAGAAGAGATGTTCAAAAAGCGAAATCTAACATAATGGATCTTCATCC [5100]

| | | |
|---------|-------------------------------------------------------------------------------------------------------|---------|
| UNC3002 |G..... | [5100] |
| UNC3006 |T.....T..... | [5100] |
| UNC3008 | | [5100] |
| UNC3009 |G.....G.....T..... | [5100] |
| UNC3010 |G.....C..... | [5100] |
| UNC3011 |G..... | [5100] |
| UNC3013 |G..... | [5100] |
| UNC3001 | TGGGTCAGGAAAGACACGGAAATACCTTCCAGCTATTGTTAGAGAGGCAATCAAGAGACGTTTAAAGAACTCTAATTTTGGCACCGACAAGGGTGGTTGCA | [5200] |
| UNC3002 | | [5200] |
| UNC3006 |A..... | [5200] |
| UNC3008 | | [5200] |
| UNC3009 |C..... | [5200] |
| UNC3010 |C..C.....T..... | [5200] |
| UNC3011 | | [5200] |
| UNC3013 |C..... | [5200] |
| UNC3001 | GCTGAGATGGAAGAAGCATTGAAAGGGCTCCAATAAGGTACCAAACAACAGCAACAAAATCTGAACACACAGGAAGAGAGATTGTTGATCTAATGTGCC | [5300] |
| UNC3002 | | [5300] |
| UNC3006 | | [5300] |
| UNC3008 | | [5300] |
| UNC3009 | | [5300] |
| UNC3010 |C..... | [5300] |
| UNC3011 | | [5300] |
| UNC3013 | | [5300] |
| UNC3001 | ACGCAACGTTACAATGCGCTTGCTGTCACCAGTTAGGGTCCAAATTATAACTTGATAATAATGGATGAGGCCCATTTACAGACCCAGCCAGCATAGC | [5400] |
| UNC3002 |C.....C.....T..... | [5400] |
| UNC3006 |T..... | [5400] |
| UNC3008 |T..... | [5400] |
| UNC3009 |C.....C.....T..... | [5400] |
| UNC3010 |C.....C.....T..... | [5400] |
| UNC3011 |C.....C.....T..... | [5400] |
| UNC3013 |C.....C.....T..... | [5400] |
| UNC3001 | GGCTAGAGGGTACATATCAACTCGTGTGGGAATGGGAGAGGCAGCCGCAATTTTCATGACAGCAACGCCCTGGAACAGCTGATGCCTTTCCTCAGAGC | [5500] |
| UNC3002 |A.....A..T..... | [5500] |
| UNC3006 |C.....C..... | [5500] |
| UNC3008 | | [5500] |
| UNC3009 |A.....A..T..... | [5500] |
| UNC3010 |A..... | [5500] |
| UNC3011 |A.....A..T..... | [5500] |
| UNC3013 |A.....A..T..... | [5500] |
| UNC3001 | AACGCTCCAATTCAGATGAAGAAAGGGACATACCAGAACGCTCATGGAATTCAGGCAATGAATGGATTACCGACTTCGCTGGGAAAACGGTGTGGTTTG | [5600] |
| UNC3002 |C..... | [5600] |

UNC3006 [5600]
 UNC3008 [5600]
 UNC3009 ..T.....G.....C.....C..... [5600]
 UNC3010 [5600]
 UNC3011C..... [5600]
 UNC3013C..... [5600]

UNC3001 TCCCAGCATTAAAGCCGAAATGACATAGCAAACCTGCTTGCAGAAAAACGGGAAAAAGGTCATTCAACTTAGTAGGAAGACTTTTGACACAGAATATCA [5700]
 UNC3002 ...T...A.....A.....A.....C..... [5700]
 UNC3006 [5700]
 UNC3008 ...T..... [5700]
 UNC3009 ...T...A.....G.....A.....A.....C..... [5700]
 UNC3010 ...T..... [5700]
 UNC3011 ...T...A.....A.....A.....C..... [5700]
 UNC3013 ...T...A.....A.....A.....C..... [5700]

UNC3001 GAAGACTAACTGAATGATTGGGACTTCGTGGTGACAACCTGACATTTAGAAATGGGGCCAATTTCAAAGCAGATAGAGTGATCGACCAAGAAGATGT [5800]
 UNC3002 [5800]
 UNC3006 ..A..... [5800]
 UNC3008 [5800]
 UNC3009 [5800]
 UNC3010 ..A.....T..... [5800]
 UNC3011 [5800]
 UNC3013 [5800]

UNC3001 CTCAAACAGTGATCCTGACAGATGGACCAGAGCGGGTGATCCTGGCTGGACCAATGCCAGTCACCGCGGAGTGCTGCGCAAAGGAGAGGAAGAGTTG [5900]
 UNC3002T.....C.....G..... [5900]
 UNC3006A..... [5900]
 UNC3008A.....G..... [5900]
 UNC3009T.....C.....G..... [5900]
 UNC3010T.....C.....G..... [5900]
 UNC3011T.....C.....G..... [5900]
 UNC3013T.....C.....G..... [5900]

UNC3001 GCAGGAACCCACAAAAAGAAAATGACCAGTACATATTACGGGCCAGCCTCTCAACAATGATGAAGACCATGCTCACTGGACAGAAGCAAAAATGCTGCT [6000]
 UNC3002T..... [6000]
 UNC3006 ..T..... [6000]
 UNC3008 [6000]
 UNC3009T.....T..... [6000]
 UNC3010T..... [6000]
 UNC3011T..... [6000]
 UNC3013T..... [6000]

UNC3001 GGACAACATTAACACACCAGAAGGGATTATACCAGCTCTCTTTGAACCAGAAAGGAGAAGTCAGCCGCATAGACGGTGAGTATCGCCTGAAGGGTGAG [6100]
 UNC3002C.....A..C..... [6100]

UNC3006G.....C..... [6100]
 UNC3008T..... [6100]
 UNC3009C.....A..C..... [6100]
 UNC3010C..... [6100]
 UNC3011C.....A..C..... [6100]
 UNC3013C.....A..C..... [6100]

UNC3001 TCCAGGAAGACTTTCGTGGAACATCATGAGGAGGGGTGACCTTCCAGTTTGGTTAGCCCATAAAGTAGCATCAGAAGGGATCAAATATACAGATAGAAAAT [6200]
 UNC3002T..... [6200]
 UNC3006A..... [6200]
 UNC3008 [6200]
 UNC3009T..... [6200]
 UNC3010 [6200]
 UNC3011T..... [6200]
 UNC3013T..... [6200]

UNC3001 GGTGCTTTGATGGACAACGCAATAATCAAATTTTAGAGGAGAACATGGATGTGGAATCTGGACAAAGGAAGGAGAAAAGAAAAAATTGAGACCTAGGTG [6300]
 UNC3002T..... [6300]
 UNC3006 [6300]
 UNC3008 [6300]
 UNC3009T..... [6300]
 UNC3010G..... [6300]
 UNC3011T..... [6300]
 UNC3013T..... [6300]

UNC3001 GCTTGATGCCCGCACTTATTCAGATCCCTTAGCACTCAAGGAATTTAAGGACTTTGCGGCTGGCAGAAAAGTCAATCGCCCTTGATCTTGTGACAGAAATA [6400]
 UNC3002T.....C.....T..... [6400]
 UNC3006C..... [6400]
 UNC3008 [6400]
 UNC3009T.....C.....T..... [6400]
 UNC3010A.....C..... [6400]
 UNC3011T.....C.....T..... [6400]
 UNC3013T.....C.....T..... [6400]

UNC3001 GGAAGAGTGCCTTCACACCTAGCCACAGAACGAGAAACGCTCTGGACAATCTGGTGATGCTGCATACGTGAGCAATGGCGGTAGGGCCTACAGGCATG [6500]
 UNC3002T..... [6500]
 UNC3006A.....T..... [6500]
 UNC3008T.....C..... [6500]
 UNC3009T.....T..... [6500]
 UNC3010A.....T..... [6500]
 UNC3011T..... [6500]
 UNC3013T..... [6500]

UNC3001 CGGTGGAGGAACTACCAGAAACAATGGAACACTCTTACTTTGGGACTCATGATCTTGTGACAGGTGGAGCAATGCTTTTCTTGATATCAGGAAAAGG [6600]
 UNC3002C.....C.....T.....T..... [6600]
 UNC3006T..... [6600]
 UNC3008A..... [6600]

UNC3009C.....C.....T..... [6600]
 UNC3010C.....T..... [6600]
 UNC3011C.....C.....T.....T..... [6600]
 UNC3013C.....C.....T..... [6600]

UNC3001 GATTGGAAAGACTTCAATAGGACTCATTGTGTGAATTGCCTCCAGCGGCATGTTGTGGATGGCCGAAATCCCACTCCAGTGGATCGCGTCGGCTATAGTC [6700]
 UNC3002T.....TG.....A..... [6700]
 UNC3006A..... [6700]
 UNC3008 [6700]
 UNC3009T.....TG.....A..... [6700]
 UNC3010T.....C.....G.....A..... [6700]
 UNC3011T.....TG.....A..... [6700]
 UNC3013T.....TG.....A..... [6700]

UNC3001 CTGGAGTTTTTATGATGGTGTGCTTATACCAGAACCAGAAAAGCAGAGAACCCCAAGACAACCAACTCGCATATGTCGTGATAGGCATACTTACAT [6800]
 UNC3002 [6800]
 UNC3006T..... [6800]
 UNC3008 [6800]
 UNC3009G..... [6800]
 UNC3010 [6800]
 UNC3011 [6800]
 UNC3013 [6800]

UNC3001 TGGCTGCAATAATAGCAGCCAATGAAATGGGACTGTTGGAAACTACAAGAGAGATTTAGGAATGTCTAAGGAGCCAGGTGTTGTTTCTCCAACCAGCTA [6900]
 UNC3002A..... [6900]
 UNC3006 [6900]
 UNC3008 [6900]
 UNC3009C.....A.....T..... [6900]
 UNC3010G.....C..... [6900]
 UNC3011A..... [6900]
 UNC3013A..... [6900]

UNC3001 TTTGGATGTGGACTTGCACCCAGCATCAGCCTGGACATTGTACGCCGTGGCCACTACAGTAATAACACCAATGTTAAGACATACCATAGAGAATTCTACA [7000]
 UNC3002G..... [7000]
 UNC3006T.....C..... [7000]
 UNC3008C..... [7000]
 UNC3009G..... [7000]
 UNC3010C..... [7000]
 UNC3011G..... [7000]
 UNC3013G..... [7000]

UNC3001 GCAAAATGTGTCCTGGCAGCTATAGCCAACCAGGCAGTGGTCTGATGGGTTTGGACAAAGGATGGCCAATATCAAAAATGGACTTAGGCGTACCACTAC [7100]
 UNC3002A..... [7100]
 UNC3006 [7100]
 UNC3008G..... [7100]
 UNC3009A..... [7100]

UNC3010G.....T [7100]
 UNC3011A..... [7100]
 UNC3013A..... [7100]

UNC3001 TGGCATTGGGTTGCTATTACAAGTGAACCCACTGACTCTAACAGCGGCAGTACTTTTGCTAATCACACATTATGCTATTATAGGTCCAGGATTGCAGGC [7200]
 UNC3002A.. [7200]
 UNC3006 [7200]
 UNC3008 [7200]
 UNC3009C.....C.....C.....A.. [7200]
 UNC3010C.....T.....C.....C.....A.. [7200]
 UNC3011A.. [7200]
 UNC3013A.. [7200]

UNC3001 AAAAGCCACTCGTGAAGCTCAGAAAAGGACAGCTGCTGGAATAATGAAGAATCCAACGGTGGATGGGATAATGACAATAGACCTAGATCCTGTAATATAT [7300]
 UNC3002 [7300]
 UNC3006 [7300]
 UNC3008 [7300]
 UNC3009 [7300]
 UNC3010C..... [7300]
 UNC3011 [7300]
 UNC3013 [7300]

UNC3001 GATTCAAAATTTGAAAAGCAACTGGGACAGGTTATGCTCCTGGTTTTGTGTGCAGTTCAACTTTTGTTAATGAGAACATCATGGGCCTTGTGTGAAGCTT [7400]
 UNC3002A.....C [7400]
 UNC3006 [7400]
 UNC3008 [7400]
 UNC3009C.A.....C [7400]
 UNC3010G.A..... [7400]
 UNC3011A.....C [7400]
 UNC3013A.....C [7400]

UNC3001 TAACTCTAGCTACAGGACCAATAACAACACTCTGGGAAGGATCACCTGGAAAAGTTTTGGAACACCACGATAGCTGTTTCCATGGCGAACATTTTATAGAGG [7500]
 UNC3002C.....G.....T..... [7500]
 UNC3006C..... [7500]
 UNC3008G..... [7500]
 UNC3009C.....G.....T..... [7500]
 UNC3010 .G.C.....G..... [7500]
 UNC3011C.....G.....T..... [7500]
 UNC3013C.....G.....T..... [7500]

UNC3001 GAGCTATTTAGCAGGAGCTGGGCTTGCTTTTCTATTATGAAATCAGTTGGAACAGGAAAAAGAGGAACAGGTTTACAAGGCAAAACCTTAGGAGAAAAA [7600]
 UNC3002C.....T..... [7600]
 UNC3006 [7600]
 UNC3008T..... [7600]
 UNC3009C.....T..... [7600]
 UNC3010C.....T..... [7600]

UNC3011C.....T..... [7600]
 UNC3013C.....T..... [7600]

UNC3001 TGGAAAAAGAAATTAATCAATTATCCCGAAAGAGTTTGACCTTTACAAGAAATCTGGAATCACTGAAAGTGGATAGAACAGAAGCCAAAGAAGGGTTGA [7700]
 UNC3002C..... [7700]
 UNC3006C.....A..... [7700]
 UNC3008 [7700]
 UNC3009C.....C..... [7700]
 UNC3010 [7700]
 UNC3011C.....C..... [7700]
 UNC3013C.....C..... [7700]

UNC3001 AAAGAGGAGAAATAACACATCATGCCGTGTCAGAGGTAGCGCAAACCTTCAATGGTTTGTGGAGAGAAACATGGTCATTCCCGAAGGAAGAGTCATAGA [7800]
 UNC3002C..... [7800]
 UNC3006 .G..... [7800]
 UNC3008 [7800]
 UNC3009C..... [7800]
 UNC3010G.....G..... [7800]
 UNC3011C..... [7800]
 UNC3013C..... [7800]

UNC3001 CTTGGGCTGTGGAAGAGGAGGCTGGTCATACTGTGCAGGACTGAAAAAGTCACAGAAGTGCGAGGATACACAAAAGCGGTCCAGGACACGAAGAA [7900]
 UNC3002T.....T..... [7900]
 UNC3006A..... [7900]
 UNC3008 [7900]
 UNC3009T.....G.....T..... [7900]
 UNC3010T.....A..... [7900]
 UNC3011T.....T..... [7900]
 UNC3013T.....T..... [7900]

UNC3001 CCAGTACCTATGTCTACATATGGATGGAACATAGTTAAGTTAATGAGTGGAAAGGATGTGTTTTATCTTCCACCTGAAAAGTGTGATACCCTGTTGTGTG [8000]
 UNC3002 [8000]
 UNC3006C..... [8000]
 UNC3008T..... [8000]
 UNC3009 [8000]
 UNC3010C..... [8000]
 UNC3011 [8000]
 UNC3013 [8000]

UNC3001 ACATCGGAGAATCTTACCAAGCCCAACAGTGGAAAGAAAGCAGAACTATAAGAGTTTTGAAGATGGTTGAACCATGGCTAAAAACAACCAGTTTGCAT [8100]
 UNC3002T..... [8100]
 UNC3006T.....C..... [8100]
 UNC3008T..... [8100]
 UNC3009T..... [8100]
 UNC3010T.....G..... [8100]
 UNC3011T..... [8100]

| | | |
|---------|------------------------------------------------------------------------------------------------------|---------|
| UNC3013 |T..... | [8100] |
| UNC3001 | TAAAGTATTGAACCTTACATGCCAACTGTGATTGAGCACCTAGAAAGACTACAAAGGAAACATGGAGGAATGCTTGTGAGAAATCCACTTTCACGAAAC | [8200] |
| UNC3002 | | [8200] |
| UNC3006 |A.....T..... | [8200] |
| UNC3008 | | [8200] |
| UNC3009 |T..... | [8200] |
| UNC3010 |T..... | [8200] |
| UNC3011 | | [8200] |
| UNC3013 | | [8200] |
| UNC3001 | TCCACGCACGAAATGACTGGATATCTAATGGCACAGGTAACATTGTCTCTTCAGTCAACATGGTATCTAGATTGCTACTGAACAGTTCCAGATGACAC | [8300] |
| UNC3002 |C.....T.....C.....A..... | [8300] |
| UNC3006 | | [8300] |
| UNC3008 | | [8300] |
| UNC3009 |C.....C.....A..... | [8300] |
| UNC3010 |T.....G.....T..... | [8300] |
| UNC3011 |C.....T.....C.....A..... | [8300] |
| UNC3013 |C.....T.....C.....A..... | [8300] |
| UNC3001 | ACAGGAGACCTACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACTCGACATGTTAATGCGGAACCCAGAAACCCCAACATGGATGTCATTGGGGAAAG | [8400] |
| UNC3002 |G..C.....G.....T..... | [8400] |
| UNC3006 | | [8400] |
| UNC3008 | | [8400] |
| UNC3009 |G..C.....G.....T..... | [8400] |
| UNC3010 |C..... | [8400] |
| UNC3011 |G..C.....G.....T..... | [8400] |
| UNC3013 |G..C.....G.....T..... | [8400] |
| UNC3001 | AATAAAAAGGATCAAGGAGGAGCACAAATCAACATGGCACTATGATGACGAAAACCCCTACAAAACGTGGGCTTACCATGGATCCTATGAAGTCAAAGCC | [8500] |
| UNC3002 |A..T..... | [8500] |
| UNC3006 |T..... | [8500] |
| UNC3008 |T..... | [8500] |
| UNC3009 |A..T..... | [8500] |
| UNC3010 | .G.....T..... | [8500] |
| UNC3011 |A..T..... | [8500] |
| UNC3013 |A..T..... | [8500] |
| UNC3001 | ACAGGCTCAGCCTCCTCCATGATAAATGGAGTCGTGAAACTCCTCACTAAACCATGGGATGTGGTGCCCATGGTGACACAGATGGCAATGACAGATACAA | [8600] |
| UNC3002 | | [8600] |
| UNC3006 |T..... | [8600] |
| UNC3008 | | [8600] |
| UNC3009 |T..... | [8600] |
| UNC3010 |T.....C..... | [8600] |
| UNC3011 | | [8600] |
| UNC3013 | | [8600] |

UNC3001 CTCCATTTGGCCAGCAGAGAGTCTTTAAAGAGAAAGTGGACACCAGGACACCCAGGCCATGCCAGGGACAAGAAAGTTATGGGGATCACAGCGGAGTG [8700]
 UNC3002 .C..T.....T.....A..... [8700]
 UNC3006T.....A..... [8700]
 UNC3008C..... [8700]
 UNC3009 .C..T.....T.....A..... [8700]
 UNC3010A..... [8700]
 UNC3011 .C..T.....T.....A..... [8700]
 UNC3013 .C..T.....T.....A..... [8700]

UNC3001 GCTCTGGAGAACCCTGGGAAGGAACAAAAGACCCAGGTTATGCACAAGGGAAGAGTTTACAAAAAGGTCCAGAACTAACGCAGCCATGGGCGCCTTTTC [8800]
 UNC3002C..... [8800]
 UNC3006 [8800]
 UNC3008 [8800]
 UNC3009A.....C..... [8800]
 UNC3010 [8800]
 UNC3011C..... [8800]
 UNC3013C..... [8800]

UNC3001 ACAGAGGAGAACCAATGGGACAGTGCAGAAAGCTGCTGTTGAGGATGAAGAATTTGGAAACTTGTGGACAGAGAACGTGAACCTCCACAAATTGGGCAAGT [8900]
 UNC3002A..... [8900]
 UNC3006 [8900]
 UNC3008G..... [8900]
 UNC3009A..... [8900]
 UNC3010A..... [8900]
 UNC3011A..... [8900]
 UNC3013A..... [8900]

UNC3001 GTGGAAGCTGTGTTTACAACATGATGGGCAAGAGAGAGAAGAACTTGGGAGAGTTTGGCAAAGCAAAGGCAGTAGAGCTATATGGTACATGTGGTTGGG [9000]
 UNC3002C..... [9000]
 UNC3006 [9000]
 UNC3008 [9000]
 UNC3009C..... [9000]
 UNC3010C.....T.....C..... [9000]
 UNC3011C..... [9000]
 UNC3013C..... [9000]

UNC3001 AGCCAGGTACCTTGAGTTCGAAGCCCTTGGATTCTTAAATGAAGACCACTGGTTCCTCGCGTGACAACCTTACAGTGGAGTAGAAGGAGAAGGACTGCAC [9100]
 UNC3002T.....A..... [9100]
 UNC3006 [9100]
 UNC3008 [9100]
 UNC3009T.....A..... [9100]
 UNC3010T.....A..... [9100]
 UNC3011T.....A..... [9100]
 UNC3013T.....A..... [9100]

UNC3001 AAGCTAGGCTACATATTAAGGGACATTTCCAAGATACCCGGAGGAGCTATGTATGCTGATGACACAGCTGGTTGGGACACAAGAATAACAGAAGATGACC [9200]
 UNC3002A.....A.....G..... [9200]
 UNC3006C..... [9200]
 UNC3008 [9200]
 UNC3009A.....A.....G..... [9200]
 UNC3010C..... [9200]
 UNC3011A.....A.....G..... [9200]
 UNC3013A.....A.....G..... [9200]

UNC3001 TGCACAATGAGGAAAAGATCACACAGCAAATGGACCCTGAACACAGGCAGTTAGCGAACGCTATATTTAAGCTCACATACCAAAACAAAGTGGTCAAAGT [9300]
 UNC3002C..... [9300]
 UNC3006 [9300]
 UNC3008 [9300]
 UNC3009 [9300]
 UNC3010T.....C..... [9300]
 UNC3011C..... [9300]
 UNC3013C..... [9300]

UNC3001 TCAACGACCGACTCCAACGGGCACGGTAATGGACATCATATCTAGGAAAGACCAAAGAGGCAGTGGACAGTGGGAACCTTATGGTCTGAATACATTACC [9400]
 UNC3002 [9400]
 UNC3006T..... [9400]
 UNC3008C..... [9400]
 UNC3009C.....T..... [9400]
 UNC3010T.....A.....A.....C..... [9400]
 UNC3011 [9400]
 UNC3013 [9400]

UNC3001 AACATGGAAGTCCAGTTAGTCAGACAAATGGAAGGAGAAGGTGTGCTGTCAAAGGCAGACCTCGAGAACCCTCATCTGCCAGAGAAGAAAATTACACAAT [9500]
 UNC3002C.....T..... [9500]
 UNC3006C..... [9500]
 UNC3008 [9500]
 UNC3009C.....T..... [9500]
 UNC3010C.....A.....T..... [9500]
 UNC3011C.....T..... [9500]
 UNC3013C.....T..... [9500]

UNC3001 GGTGGAACCAAAGGAGTGGAGAGGTTAAAAAGAAATGGCCATTAGCGGGGATGATTGTGTAGTGAAACCAATCGATGACAGGTTGCTAATGCCCTGCT [9600]
 UNC3002C.....C..... [9600]
 UNC3006 [9600]
 UNC3008C.....C..... [9600]
 UNC3009C.....T.....C..... [9600]
 UNC3010G.....G..... [9600]
 UNC3011C.....C..... [9600]
 UNC3013C.....C..... [9600]

UNC3001 TGCTCTGAACGATATGGGAAAGGTTTCGAAAGACATACCTCAATGGCAGCCATCAAAGGGATGGCATGATTGGCAACAGGTTCTTTCTGCTCCCACCAC [9700]

UNC3002A.....G.....C..... [9700]
 UNC3006 [9700]
 UNC3008C.....AT..... [9700]
 UNC3009A.....G.....A.....C..... [9700]
 UNC3010C..... [9700]
 UNC3011A.....G.....C..... [9700]
 UNC3013A.....G.....C..... [9700]

UNC3001 TTTTCATGAATTGATCATGAAAGATGGAAGAAAGTTAGTGGTTCCTGTAGACCCAGGACGAACTAATAGGAAGCAAGAATCTCTCAAGGAGCGGGAT [9800]
 UNC3002G.....C.....A..T..T..... [9800]
 UNC3006G..... [9800]
 UNC3008 [9800]
 UNC3009G.....C.....A..T..T..... [9800]
 UNC3010G.....C..... [9800]
 UNC3011G.....C.....A..T..T..... [9800]
 UNC3013G.....C.....A..T..T..... [9800]

UNC3001 GGAGCCTTAGAGAGACCGCATGTCTGGGAAAGCCTACGCTCAAATGTGGAGTCTCATGTACTTTCACAGAAGAGATCTCAGACTAGCATCCAACGCCAT [9900]
 UNC3002A..T.....T..C.....T..... [9900]
 UNC3006 [9900]
 UNC3008A.....T.....T..... [9900]
 UNC3009A..T.....T..C.....T.....T..... [9900]
 UNC3010A..T.....C.....T.....T..... [9900]
 UNC3011A..T.....T..C.....T..... [9900]
 UNC3013A..T.....T..C.....T..... [9900]

UNC3001 ATGTTTCAGCAGTACCAGTCCACTGGGTCACAAAGTAGAACGACATGGTCTATTTCATGCTCACCATCAGTGGATGACTACAGAAGACATGCTTACTGTCT [10000]
 UNC3002T..... [10000]
 UNC3006 [10000]
 UNC3008G..... [10000]
 UNC3009T..... [10000]
 UNC3010T.....C.....T..... [10000]
 UNC3011T..... [10000]
 UNC3013T..... [10000]

UNC3001 TGGAACAGGGTGTGGATCGAGGACAATCCATGGATGGAAGACAAAACCTCCAGTTACAACCTGGGAAAATGTTCCATATCTAGGGAAGAGAGAAGACCAAT [10100]
 UNC3002A.....T.....T..... [10100]
 UNC3006A..... [10100]
 UNC3008C.....T.....G..... [10100]
 UNC3009A.....T.....T..... [10100]
 UNC3010A.....A.....T..... [10100]
 UNC3011A.....T.....T..... [10100]
 UNC3013A.....T.....T..... [10100]

UNC3001 GGTGCGGATCACTTATTGGTCTCACCTCCAGAGCAACCTGGGCCAGAACATACCCACAGCAATTCAACAGGTGAGAAGCCTTATAGGCAATGAAGAGTT [10200]
 UNC3002T..... [10200]

UNC3006C..... [10200]
 UNC3008C.C.....T.....T..... [10200]
 UNC3009T..... [10200]
 UNC3010T..... [10200]
 UNC3011T..... [10200]
 UNC3013T..... [10200]

UNC3001 TCTGGATTACATGCCTTCAATGAAGAGATTGAGGAGGAGGAGTGGAAAGGAGCCATTTGGTAACGTAGGAAGTAAAAAGAGGCAAACTGTCAGG [10300]
 UNC3002C.....G.....A.G.....A.....T..... [10300]
 UNC3006T..... [10300]
 UNC3008 [10300]
 UNC3009C.....G.....A.G.....A.....T..... [10300]
 UNC3010 C.....C.....A.....G.....T..... [10300]
 UNC3011C.....G.....A.G.....A.....T..... [10300]
 UNC3013C.....G.....A.G.....A.....T..... [10300]

UNC3001 CCACCTTAAGCCACAGTACGGAAGAAGCTGTGCTGCCTGTGAGCCCCGTTCAAAGGACGTTAAAAGAAGAAGTACGGCCCCAAAGCCACGGTTTGAGCAAA [10400]
 UNC3002C..... [10400]
 UNC3006 [10400]
 UNC3008 [10400]
 UNC3009C..... [10400]
 UNC3010 [10400]
 UNC3011C..... [10400]
 UNC3013C..... [10400]

UNC3001 CCGTGCTGCCTGTAGCTCCGTCGTGGGGACGTAAAACCTGGGAGGCTGCAAACCTGTGGAAGCTGTACGCACGGTGTAGCAGACTAGCGTTAGAGGAGAC [10500]
 UNC3002G..... [10500]
 UNC3006 [10500]
 UNC3008 [10500]
 UNC3009G..... [10500]
 UNC3010G..... [10500]
 UNC3011G..... [10500]
 UNC3013G..... [10500]

UNC3001 CCCTCCCATGACACAACGCAGCAGCGGGGCCGAGCACTGAGGGAAGCTGTACCTCCTTGCAAAGGACTAGAGGTTAGAGGAGACCCCCGCAAAACAAA [10600]
 UNC3002T..... [10600]
 UNC3006 [10600]
 UNC3008 [10600]
 UNC3009T..... [10600]
 UNC3010G.....T..... [10600]
 UNC3011T..... [10600]
 UNC3013 [10600]

UNC3001 ACAGCATATTGACGCTGGGAGAGACCAGAGATCCTGCTGTCTCCTCAGCATATTCCAGGCACAGAACGCCAGAAAATGGAATGGTGTGTTGAATCAAC [10700]
 UNC3002 [10700]
 UNC3006 [10700]

UNC3008 [10700]
UNC3009 [10700]
UNC3010 [10700]
UNC3011 [10700]
UNC3013 ----- [10700]

UNC3001 AGGTTCT [10707]
UNC3002 [10707]
UNC3006 [10707]
UNC3008 ----- [10707]
UNC3009 [10707]
UNC3010 [10707]
UNC3011 [10707]
UNC3013 ----- [10707]

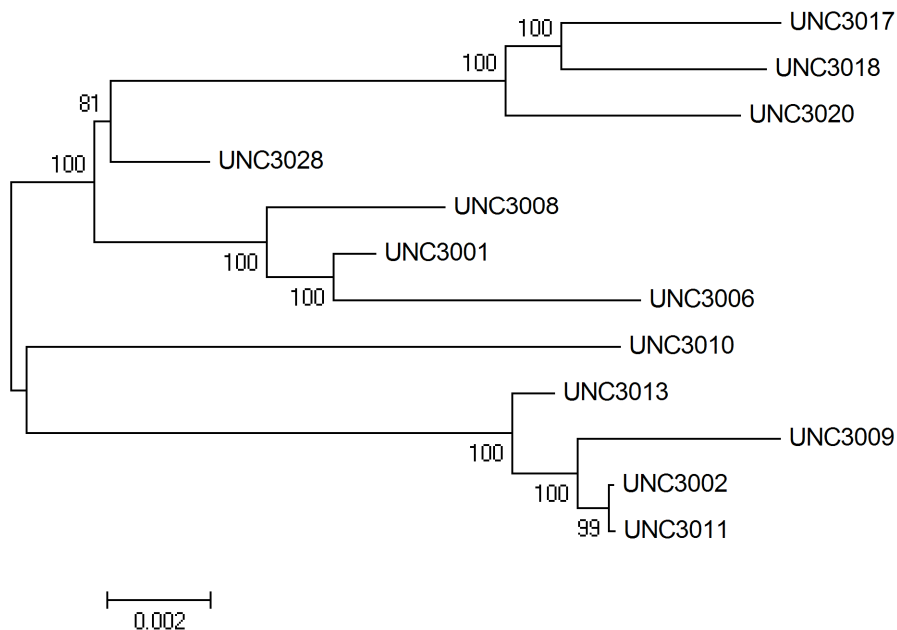


Figure 3.3 Phylogenetic tree depicting relationships between Sri Lankan, Latin American and African DENV3 clinical isolates.

In this tree, the pre-DHF viruses (UNC3002, UNC3009, UNC3011, and UNC3013) group together in a separate clade than the viruses associated with the emergence of DHF in Sri Lanka (UNC3001, UNC3006, UNC3008, UNC3010). The post-DHF viruses are more closely related to viruses found in Africa and Latin American than they are to the pre-DHF viruses. This tree was created in Mega4 using a minimal evolution method. The numbers at each node are bootstrap values and represent the probability that these viruses would be placed on the same branch upon resampling.

| | 27 C(27) | 286 E(6) | 404 E(124) | 499 E(219) | 600 E(320) | 752 E(472) | 775 NS1(2) | 867 NS1(94) | 901 NS1(128) | 946 NS1(173) |
|---------|-------------|-------------|---------------|---------------|---------------|---------------|---------------|----------------|-----------------|-----------------|
| | S | V | S | T | V | S | M | I | I | E |
| UNC3002 | - | - | - | - | - | - | - | - | - | - |
| UNC3009 | - | - | - | - | I | - | T | - | - | - |
| UNC3011 | - | - | - | - | - | - | - | - | - | - |
| UNC3013 | - | - | - | - | - | - | - | - | - | - |
| UNC3001 | P | I | P | - | I | - | - | - | T | D |
| UNC3006 | - | I | P | - | I | - | - | - | T | D |
| UNC3008 | - | - | P | - | I | - | - | - | T | D |
| UNC3010 | - | - | P | A | I | P | - | T | T | - |

| | 990 NS1(217) | 1123 NS1(350) | 1162 NS2A(37) | 1275 NS2A(150) | 1283 NS2A(158) | 1284 NS2A(159) | 1380 NS2B(37) | 1395 NS2B(52) | 1504 NS3(31) | 1642 NS3(169) |
|---------|-----------------|------------------|------------------|-------------------|-------------------|-------------------|------------------|------------------|-----------------|------------------|
| | F | A | F | V | M | F | L | T | L | N |
| UNC3002 | - | - | - | - | - | - | - | I | - | - |
| UNC3009 | - | - | - | I | - | - | M | - | - | S |
| UNC3011 | - | - | - | - | - | - | - | - | - | - |
| UNC3013 | - | - | - | - | I | L | - | - | - | - |
| UNC3001 | L | V | L | - | I | L | - | - | - | - |
| UNC3006 | L | V | L | - | I | L | - | - | F | - |
| UNC3008 | L | V | L | - | I | L | - | - | - | - |
| UNC3010 | L | V | - | - | I | - | - | - | - | - |

Figure 3.4 Amino acid differences in pre- and post-DHF clinical isolates.

This table represents all the amino acid changes in the eight Sri Lankan clinical isolates sequenced. The polyprotein top number is the polyprotein residue and under that is the gene in which the amino acid change is located. The consensus residue is the listed in the top row. Viruses with the consensus residue are represented with a dash and one-letter amino acid codes are given at each position that a virus differs from the consensus. The top four viruses are pre-DHF isolates and the bottom four are associated with the emergence of DHF. Positions at which changes are conserved between pre- and post-DHF DENV are highlighted in blue. All eight viruses are identical at positions not included in the chart above.

| | 1658 NS3(185) | 1921 NS3(448) | 2160 NS4A(68) | 2191 NS4A(99) | 2193 NS4A(100) | 2239 NS4A(147) | 2540 NS5(50) | 2562 NS5(72) | 2743 NS5(253) | 2760 NS5(270) |
|---------|------------------|------------------|------------------|------------------|-------------------|-------------------|-----------------|-----------------|------------------|------------------|
| | <i>R</i> | <i>M</i> | <i>A</i> | <i>D</i> | <i>V</i> | <i>I</i> | <i>T</i> | <i>I</i> | <i>R</i> | <i>I</i> |
| UNC3002 | - | - | <i>V</i> | - | - | - | - | - | - | - |
| UNC3009 | - | - | - | - | - | - | - | - | - | - |
| UNC3011 | - | - | <i>V</i> | - | - | - | - | - | - | - |
| UNC3013 | - | - | - | - | - | - | - | - | - | - |
| UNC3001 | <i>K</i> | - | - | <i>E</i> | <i>I</i> | - | <i>I</i> | - | <i>K</i> | <i>T</i> |
| UNC3006 | <i>K</i> | <i>I</i> | - | <i>E</i> | <i>I</i> | - | <i>I</i> | - | <i>K</i> | <i>T</i> |
| UNC3008 | <i>K</i> | - | - | <i>E</i> | <i>I</i> | - | <i>I</i> | - | <i>K</i> | <i>T</i> |
| UNC3010 | <i>K</i> | - | - | <i>E</i> | - | <i>M</i> | <i>I</i> | <i>V</i> | <i>K</i> | <i>T</i> |

| | 2770 NS5(280) | 2855 NS5(365) | 2864 NS5(374) | 2990 NS5(500) | 3043 NS5(553) | 3106 NS5(616) | 3119 NS5(629) | 3129 NS5(639) | 3139 NS5(649) | 3190 NS5(700) |
|---------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | <i>I</i> | <i>P</i> | <i>E</i> | <i>E</i> | <i>T</i> | <i>A</i> | <i>S</i> | <i>L</i> | <i>T</i> | <i>W</i> |
| UNC3002 | - | - | - | - | - | - | - | - | - | - |
| UNC3009 | - | - | - | - | - | - | - | - | - | - |
| UNC3011 | - | - | - | - | - | - | - | - | - | - |
| UNC3013 | - | - | - | - | - | - | - | - | - | - |
| UNC3001 | - | - | <i>G</i> | <i>D</i> | - | <i>V</i> | - | <i>P</i> | - | - |
| UNC3006 | - | <i>S</i> | <i>G</i> | <i>D</i> | - | <i>V</i> | - | <i>P</i> | - | - |
| UNC3008 | - | - | <i>G</i> | <i>D</i> | - | <i>V</i> | - | <i>P</i> | - | <i>M</i> |
| UNC3010 | <i>V</i> | - | - | - | <i>I</i> | - | <i>T</i> | - | <i>A</i> | - |

| | 3301 NS5(811) | 3310 NS5(820) | 3316 NS5(826) | 3325 NS5(835) | 3354 NS5(864) |
|---------|------------------|------------------|------------------|------------------|------------------|
| | T | D | D | N | P |
| UNC3002 | - | - | - | - | - |
| UNC3009 | - | - | - | - | - |
| UNC3011 | - | - | - | - | - |
| UNC3013 | - | - | - | - | - |
| UNC3001 | - | - | - | - | - |
| UNC3006 | - | - | N | - | - |
| UNC3008 | A | - | - | D | L |
| UNC3010 | - | E | - | - | - |

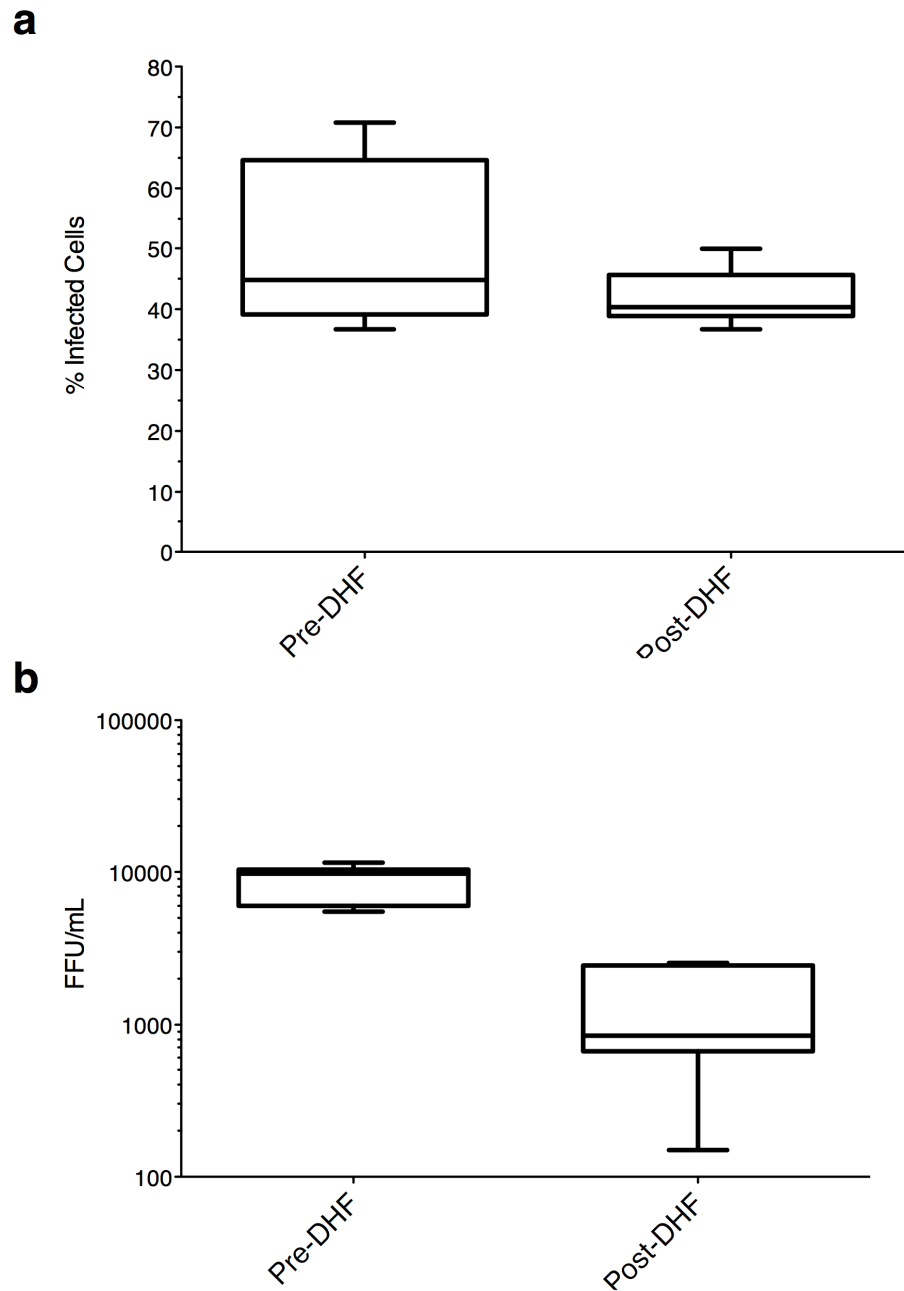


Figure 3.5 Infection of DC-SIGN-expressing U937 cells with Sri Lankan viruses isolated before and after the emergence of DHF in 1989.

(a) U937 cells expressing DC-SIGN were infected with all eight Sri Lankan clinical isolates at a multiplicity of infection of 1.0. The percentage of infected cells was determined at twenty-four hours post infection. (b) The supernatants from infected cells were harvested at twenty-four hours post infection and titered on Vero cells in an immunofocus assay. Supernatant titers are expressed as focus-forming unit per mL. Pre-DHF viruses included UNC3002, UNC3009, UNC3011 and UNC3013. Post-DHF viruses included UNC3001,

UNC3006, UNC3008 and UNC3010. The graph depicts one representative experiment of three performed.

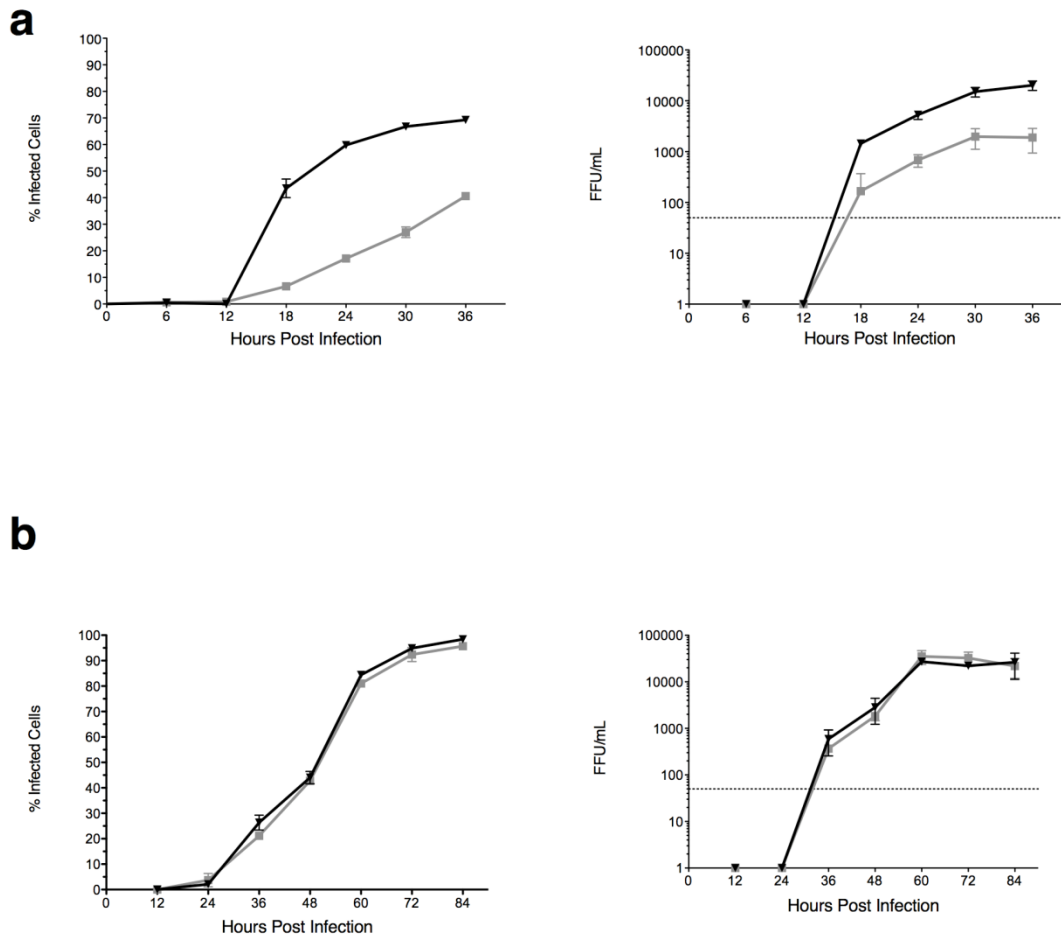


Figure 3.6 Infection of DC-SIGN-expressing U937 human monocytic cells and Aedes C6/36 mosquito cells with one pre-DHF clinical isolate and one post-DHF clinical isolate.

DC-SIGN expressing U937 and C6/36 cells were infected with UNC3006 (gray squares) and UNC3009 (black triangles) at a multiplicity of infection of 0.5. The percentage of cells infected and the amount of virus released into the supernatant were determined at various timepoints post infection. (a) DC-SIGN expressing U937 cells were infected with UNC3006 and UNC3009 and cells and supernatants were harvested every six hours for thirty-six hours post infection. (b) C6/36 cells were infected with both UNC3006 and UNC3009. Cells and supernatants were harvested every twelve hours for eighty-four hours post infection. Each data point represents the mean of three values and error bars represent the standard deviation. Each graph represents one of three or more experiments.

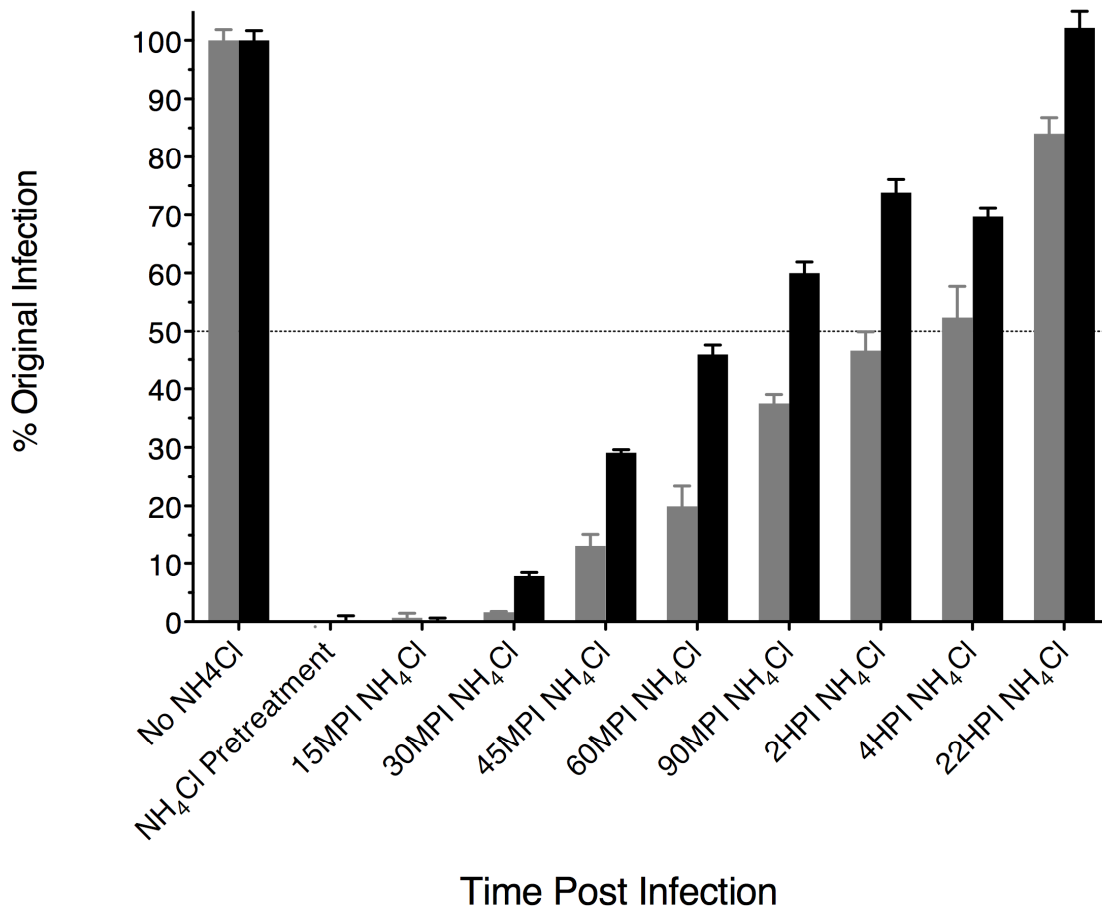


Figure 3.7 UNC3009 may enter DC-SIGN expressing U937 cells quicker than UNC3006.

DC-SIGN expressing U937 cells were infected with UNC3006 (gray bars) and UNC3009 (black bars) at a multiplicity of infection of 1.0. A subset of cells was pretreated with NH₄Cl to raise the pH of endocytic compartments and eliminate DENV infection. Ammonium chloride was added to other cells at various points post infection and the percentage of infection was determined compared to that of untreated cells. The dotted line represents fifty percent of the original infection. The data depicted is representative of three different experiments.

**CHAPTER 4. THE CONSTRUCTION AND CHARACTERIZATION OF A
DENGUE VIRUS TYPE 3 INFECTIOUS CLONE AND GENERATION OF
CHIMERIC VIRUSES**

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ABSTRACT

Following the in vitro characterization and full genome sequencing of two Sri Lankan clinical isolates that behave differently in vitro, we were interested in determining which genetic differences are responsible for the delayed growth of UNC3006. Since there are twenty-five coding differences between UNC3006 and UNC3009, we wanted to identify the specific change or combination of changes responsible for the cell culture growth difference between UNC3009 and UNC3006. To map the relevant dengue mutations, we first needed to develop a DENV3 reverse genetic system. We fixed the viral RNA genome in a cDNA copy on a plasmid that could be propagated in *E. coli* and manipulated genetically. In this chapter, we describe the development and characterization of a DENV3 infectious clone. We also describe the creation of recombinant chimeric viruses containing segments of UNC3006 or UNC 3009 required for genetic mapping studies.

INTRODUCTION

To identify the region(s) of the DENV3 genome responsible for *in vitro* growth differences observed between UNC3006 and UNC3009 and described in chapter 3, we developed a technique to manipulate the viral genome. Because flaviviruses are positive strand RNA viruses, the genetic material is mRNA and therefore, infectious. Thus, we can fix the DENV RNA genome as a cDNA under the control of a promoter, produce viral RNA *in vitro* and introduce this infectious RNA into cells that will translate viral proteins and replicate the viral RNA, resulting in progeny virion production. Once the viral genome is fixed as cDNA, we can manipulate the genetic material through overlap PCR, site-directed mutagenesis, or other cloning techniques. Currently, there is one DENV3 infectious clone that was constructed using the Sleman/78 virus genetic material (14). This virus differs from our virus by 530 nucleotides and 100 amino acids. In our case, we constructed an infectious clone from the genetic material of a DENV3 Sri Lankan clinical isolate, UNC3001 (89SriLan2). UNC3001 was isolated in 1989 and is closely related to other viruses isolated between 1989 and 1997 in Sri Lanka following the emergence of epidemic DHF (142). Next, I will review the literature on DENV infectious clones and then describe our strategy for producing a DENV3 infectious clone.

The first DENV infectious clone was constructed in 1991 using the genetic material of the DENV4 isolate 814669 (123). This infectious clone consisted of the full-length cDNA in pCR322, a low copy number *E. coli* plasmid, under the control of an SP6 RNA promoter. During the construction of this full-length clone the authors learned that the *E. coli* strain had an effect of plasmid stability and were able to isolate full-length cDNA clones more efficiently from low copy number plasmids. Since this initial creation in 1991, infectious

clones have been constructed for all four DENV serotypes. Currently, there are infectious clones for the DENV1 Brazilian prototype (216) and Western Pacific (165) strains, the DENV2 Jamaica (159), 16681 (109, 212), 43 (239), and New Guinea C (59, 107) strains, the DENV3 Sleman78 (14) strain, and the DEN4 814669 (123) strain. In the construction of these various infectious clones, DENV has proven difficult to manipulate and stably maintain in *E. coli*. A number of full-length viral genomes are unstable in *E. coli* and cDNA clones could not be isolated (165, 216). Groups have overcome this problem by using a bacterial artificial chromosome (165) or a yeast shuttle vector (159), by breaking the interrupting the viral coding region and performing in vitro ligation (14, 107), using low copy number plasmids (59, 123), and growing the *E. coli* under low selection pressure at room temperature (212).

The infectious clones of dengue have been used to identify components of the viral genome contributing to virulence. Most of this work has been done with two different DENV2 infectious clones representing 16681 and New Guinea C. DENV2 16681 is a Southeast Asian isolate that efficiently infects MDM and MDDC (168) and is believed to be more virulent than its American DENV2 relatives (172, 173, 229). Using a 16681 infectious clone, an Asn mutation present at position 390 of E in Southeast Asian DENV2 isolates was shown to be an important contributor to increased viral replication efficiency (39, 164). Additionally, structures in the noncoding regions of the Southeast Asian strains absent in the American isolates augment viral replication when paired with the above envelope mutation (39). A reverse genetics system developed with New Guinea C was used to identify mouse neurovirulence determinants (59). By swapping the structural genes of the mouse neurovirulent New Guinea C with those of the non-virulent PUO-218, the authors were able

to identify a lysine at position 126 of E as an important determinant of viral replication in mouse brain. Finally, positions 124 and 128 of E were shown to be important in the mouse adaptation of D2S10 (163). These mutations responsible for increased mouse virulence resulted in decreased affinity for heparin sulfate and decreased viral clearance from the circulation and potential higher serum viremia.

Systems to produce recombinant dengue viruses have also played an important role in vaccine development (reviewed in 13). The DENV4 Δ 30 vaccine candidate was isolated in a genetic screen searching for non-coding deletion viruses attenuated for growth. Using the strategy to create DENV4 Δ 30, the NIH has generated DENV1 Δ 30 and DENV2 Δ 30 vaccine candidates that appear promising in early human trials (13).

Currently, there is one DENV3 infectious clone developed from the Sleman78 virus (14). This virus was isolated from a mild dengue fever outbreak in Java, Indonesia in 1978 and may be partially attenuated since the epidemic attributed to this virus was characterized by lower viremia, milder illness and less spread than previous DENV3 outbreaks in this area (65). In the construction of this infectious clone, the authors amplified and subcloned the viral cDNA in 6 pieces but were unable to isolate *E. coli* clones containing the full-length cDNA (14). It appeared that when E and NS1 were inframe and adjacent to each other, they were toxic to *E. coli*, resulting in plasmid instability. To overcome this obstacle, Blaney et al interrupted the E-NS1 coding region by placing a SpeI linker at the carboxy terminus of E. To produce infectious virus, they removed the linker *in vitro* and ligated the full-length DNA prior to SP6 transcription and transfection (14). The authors were able to recover infectious virus from the supernatants of transfected Vero and C6/36 cells.

Despite the availability of the DENV3 Sleman78 infectious clone, we constructed our own reverse genetics system for a number of reasons. First, the Sleman78 viral genome differs from our Sri Lankan clinical isolates by more than 500 nucleotides and 100 amino acids and characterized as a DENV3, genotype II virus. Sri Lankan DENV3 clinical isolates group into genotype III. We wanted a system for producing virus that was closely related to our viruses so that identifying regions responsible would be simpler. Secondly, we know our viruses are virulent and cause severe disease in humans, as UNC3006 is associated with the emergence of DHF in Sri Lanka (142). Therefore, we did not want to base our experiments on a potentially attenuated DENV3 isolate. Finally, we wanted a system that we could easily manipulate and produce chimeric viruses. The Sleman78 infectious clone system does not contain convenient restriction sites to swap regions of the viral genome. Based on these reasons, we constructed our own infectious clone from UNC3001, a Sri Lankan clinical isolate associated with the emergence of DHF, using a system previously used to create recombinant Coronaviruses (237).

MATERIALS AND METHODS

Cell lines and virus strains

C6/36 *Aedes albopictus* cells were obtained from the American Type Culture Collection (ATCC No: CRL-1660) and propagated in Minimal Essential Media with Earl's salts (E-MEM) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 10% fetal bovine serum (Gibco/Invitrogen). Cells were

grown at 28°C in 5% CO₂. Vero (African Green Monkey) Clone 81 cells were a gift from Robert Putnak at Walter Reed Army Medical Center, and were propagated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.2% sodium bicarbonate and 10% fetal bovine serum (Gibco/Invitrogen). Vero cells were grown at 37°C, 5% CO₂. The DENVs used in this study were UNC3001, UNC3006, and UNC3009 (89SriLan2, 97SriLan1, and 89SriLan1, respectively) and were provided by Duane Gubler (Centers for Disease Control).

Immunofocus titration assay

All viruses were titered on Vero cells in a 24-well format immunofocus assay modified from that described in AP61 cells (45). Subconfluent Vero monolayers were infected with serial 10-fold dilutions of infected cell supernatants in Opti-MEM I (Gibco/Invitrogen) supplemented with 1% penicillin/streptomycin (Gibco/Invitrogen) and 2% heat-inactivated FBS. Virus was allowed to adsorb for 2 hours at 37°C. Overlay media (Opti-MEM I containing 5% FBS, 1% penicillin/streptomycin and 0.8% methyl cellulose (Electron Microscopy Sciences)) was then added to plates and cells were incubated for five days at 37°C, 5% CO₂. Cells were washed two times with 1X PBS (Cellgro) and fixed and permeabilized by incubating in 80% methanol:PBS (v:v) at room temperature for 20 minutes. Cells were then blocked with 5% milk:PBS (w:v) at room temperature for 10 minutes. Monolayers were stained with 400ng anti-flavivirus envelope antibody 4G2 (ATCC HB-112) followed by a 1:500 dilution of a peroxidase conjugated secondary antibody (Sigma-Aldrich) for 1 hour each at 37°C. Foci were visualized with 125µL of TrueBlue Peroxidase Substrate (KPL, Inc). Titers were expressed as focus forming units per mL and were calculated by

multiplying the average number of foci per well at a given dilution by the inverse dilution factor and dividing by the volume added to each well.

cDNA amplification and TOPO cloning

UNC3001 viral RNA was isolated from clarified infected cell supernatants with the QiaAmp Viral RNA Mini Kit (Qiagen) using the manufacturer's protocol as described by Messer et al (65, 142). Viral RNA was recovered and first strand synthesis was performed using Superscript III reverse transcriptase (Invitrogen) and a reverse primer at the 3' terminus of the viral genome (5'-TACATGCCTTCAATGAAGAGATTCAGG-3'). Three different PCR products were amplified using the EXPAND Long Template PCR System (Roche Applied Science). The A fragment containing the first 2034 nucleotides of UNC3001 was PCR amplified using a sense primer containing the T7 RNA promoter directly upstream of the 5' UTR (5'-NNNNNNGCGGCCGCTAATACGACTCACTATAGAGTTGTTAGTCTACGTGGACC-3') and an antisense primer with an engineered BsmBI site (5'-NNNNNNCGTCTCGCCTC↓AATATTGACAGGCTCC-3'). The B fragment spanning nucleotides 2029 to 5210 was amplified with a sense primer containing an engineered BsmBI site (5'-NNNNNNCGTCTCTGAGG↓CTGAACCTCCTTTTGGG-3') and an antisense primer (5'-CCATCTCAGCTGCAACCACC-3'). The third fragment, C/D, spans 5100 to 10707 and was amplified with a sense primer (5'-CTGGGTCAGGAAAGACACGG-3') and an antisense primer with an EciI site that permits cleavage reproducing the viral 3' UTR (5'-GCGGCCGCGGCGGANNNNNNNNNAGAACCTGTTGATTCAACAGCACCATTCC-3'). All three fragments were cloned into pCRXL-TOPO (Invitrogen) according to the manufacturer's protocol. Transformed TOP10 *E. coli* was grown at 25°C under 50µg/ml

kanamycin selection. Plasmids containing the correct coding sequences were selected for viral assembly or site directed mutagenesis was performed to rectify coding changes.

Production of recombinant infectious virus

Restriction enzymes SpeI, SfiI, BsmBI, and EciI were obtained from New England Biolabs and used according to the manufacturer's protocol. Calf Intestinal Phosphatase and T4 ligase were also obtained from NEB. Plasmids dA01 and dC/D01 were digested with SpeI, treated with CIP, and then digested with BsmBI. Plasmid dB01 was digested with SfiI and BsmBI. Restriction fragments corresponding to the appropriate size were gel purified and ligated *in vitro* by T4 ligase using equimolar quantities of the three fragments. DENV cDNA was then digested with EciI to produce the exact 3' terminus of DENV3 and gel purified. Full-length capped T7 transcripts were produced with the mMessage mMachine T7 Kit (Applied Biosystems) according to the manufacturer's protocol and electroporated into Vero81 cells (25µF, 450V, 5 pulses). Vero cells were incubated at 37°C, 5% CO₂ for up to six days, supernatants were clarified, and virus was titered on Vero81 cells, used to perform growth curves, or passaged onto C6/36 cells.

Construction of chimeric viruses

Viral RNA was isolated from the clarified supernatants of C6/36 cells infected with either UNC3006 or UNC3009 and full-length cDNA was generated as described above. The A fragments encoding the structural proteins of UNC3006 and UNC3009 were generated using site directed mutagenesis on the A01 fragment using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies). The primer to convert dA01 to dA06 was 5'-CCGTGTGTCAACTGGATCACAGTTGGCGAAGAG-3'. This primer along with 5'-TGACAATGAGATGTGTGGGAGTAGGAAACAGAGATTTTGTG-3' and 5'-

CGAAATTTCAATGCCTGGAATCAATAGAGGGAAAAGTGGTG-3' were used to convert dA01 to dA09. Non-coding differences were not considered. Fragments encoding B of UNC3006 and UNC3009 were amplified using the primers listed above for B01 and cloned into pCRXL-TOPO. The cDNA spanning the C/D fragments of UNC3006 and UNC3009 was amplified in three steps using primers listed in Table 4.1. The 3' UTR of UNC3001 was amplified from dC/D01 using primers 013'UTRF and 3'BsmBI. The cDNA encoding the C/D nonstructural genes of UNC3006 and UNC3009 was amplified in two pieces and overlap PCR was performed using these PCR products and the UNC3001 3'UTR PCR product to produce a cDNA encoding the C/D nonstructural genes of UNC3006 or UNC3009 adjacent to the 3' UTR of UNC3001. To produce viruses differing only in the 3' non-coding region, the 3' UTR of UNC3001 was mutated to that of UNC3006 and UNC3009 by site directed mutagenesis on dC/D01 (table 4.2). A panel of chimeric full-length cDNAs was assembled, T7 transcripts were produced and Vero cells were electroporated as described above (Table 4.3).

Isolating recombinant virus and verifying viral sequence

Recombinant virus was plaque purified. Briefly 10-fold serial dilutions of electroporated cell supernatants were performed and added to confluent monolayers of Vero81 cells in 6-well plates. Following a one hour absorption, a 2.0% agarose overlay was added to the cells and plates were incubated for seven days at 37°C, 5% CO₂. Seven days post infection, a second overlay containing 0.000075% Neutral Red was added to the cells and incubated for 24 hours. An agar plug was removed from visualized plaques and placed in 500µL of cold PBS for five minutes. The PBS was then added to C6/36 cells in virus production media (E-MEM supplemented with 1% L-glutamine, 1%

penicillin/streptomycin/fungizone, 1% non-essential amino acids, and 2% fetal bovine serum) and incubated at 28°C, 5% CO₂. On the eighth day, the supernatants were removed and clarified, viral RNA was isolated, and first strand synthesis was performed as described above. The viral cDNA was amplified using the EXPAND High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) in six fragments using the primers listed in table A.1. These PCR fragments were then sequenced using primers on both strands, with 500bp coverage (table A.2).

DENV growth curves on U937 cells expressing DC-SIGN

A human monocytic, cell line (U937) constitutively expressing DC-SIGN was obtained from Mark Heise at the University of North Carolina at Chapel Hill (115). The DC-SIGN transduced cells were maintained at 37°C, 5% CO₂ in RPMI complete media (RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 50mM 2-mercaptoethanol, and 10% fetal bovine serum (Gibco/Invitrogen)). For growth curves, virus was added to cells at an MOI of 0.05 as calculated in the immunofocus assay described above. Cells and virus were incubated for two hours at 37°C, 5% CO₂. After two hours, cells were washed to remove unbound virus and cells were resuspended in RPMI complete media. At six-hour intervals, the cells and supernatants were harvested. Cells were fixed and permeabilized and then stained with Alexa488-conjugated anti-flavivirus antibody 2H2 (gift of Robert Putnak, Walter Reed Army Medical Center). Infection percentages were determined by flow cytometry and the titers were calculated in an immunofocus assay described above.

RESULTS

PRODUCTION OF INFECTIOUS VIRUS FROM CLONED DENV3 cDNA

Because previous work in our own lab and other demonstrated that the full-length DENV cDNA is toxic to *E. coli*, we utilized a technique previously described in Coronaviruses (237). Using BsmBI, a type IIS restriction enzyme that recognizes a palindromic sequence but cuts a set distance outside of this recognition site (CGTCTC↓N) (108), allowed us to amplify the entire viral genome in three adjoining cDNA clones that directionally assemble. This method also allowed us to break the genome into three pieces, potentially limiting viral toxicity to *E. coli*. The smaller fragments also provide manipulability of smaller portions of the viral genome in an effort to make chimeric viruses containing fragments from different clinical isolates and identify regions responsible for the *in vitro* growth difference.

In order to develop a DENV3 infectious clone, we produced three overlapping cDNA clones containing the entire genome of UNC3001. RNA was isolated from the supernatants of virus-infected C6/36 cells and full-length cDNA was produced. Three fragments of the cDNA were amplified and TOPO cloned into pCRXL, producing dA01, dB01 and dC/D01. dA01 contained the first 2034 nucleotides of UNC3001 downstream of a T7 RNA promoter, dB01 contained nucleotides 2029 to 5210, and dC/D01 contained nucleotides 5100-10707. BsmBI sites were engineered at the 3' end of A01 and the 5' end of B01. A native BsmBI site is located at nucleotide position 5155 of UNC3001. To produce the exact 3'UTR of the dengue genome, an EciI site was engineered at the 3' end of C/D01. BsmBI and EciI are class IIS restriction enzymes that recognize palindromic sites and cut outside of this recognition site (108). The use of BsmBI allowed for each fragment to have unique sticky

ends that would only anneal to those of adjacent fragments, permitting directional ligation *in vitro*.

We were able to recover plasmids containing the correct coding sequences. These recovered clones did contain some non-coding mutations. These noncoding mutations were not corrected to allow for differentiation of infectious clone and parental virus upon sequencing. Plasmids dA01, dB01 and dC/D01 were propagated in *E. coli* and digested as shown in Figure 4.1. Fragments of the appropriate size were gel purified and ligated *in vitro* overnight at 4°C. The full-length cDNA was then digested with EciI to produce the correct 3' UTR, and capped T7 transcripts were produced *in vitro*. The T7 transcribed DENV RNA was electroporated into Vero cells and the cell supernatants were harvested at three and six days post electroporation. The three days post electroporation supernatants were added to U937 cells expressing DC-SIGN. These cells were fixed and stained for intracellular DENV antigen at twenty-four hours post infection. Approximately seven percent of these cells were positive for dengue antigen (figure 4.2a), indicating that the electroporated cells produced virus capable of infecting a second set of cells. This virus, named 01IC, was plaque purified and sequenced, confirming that the unmarked mutations in our cDNA clones were present in the IC virus and that the proteins produced were identical to the parental virus (figure 4.2b). The supernatants from electroporated Vero cells were also titered in an immuofocus assay (figure 4.2c). The titer of 01IC in the electroporated supernatants increased exponentially over time.

GROWTH CURVES COMPARING PARENTAL AND IC DENV3 VIRUS

To confirm that our infectious clone virus behaved as the parental UNC3001, we compared the foci of both viruses (figure 4.2b) and performed viral growth curves on U937

cells expressing DC-SIGN (figure 4.4). Infected cells and supernatants were harvested every six hours for seventy-two hours. Cells were fixed and stained for intracellular DENV antigen (figure 4.3a) and the supernatants were titered in an immunofocus assay on Vero cells (figure 4.3b). The percentage of infected cells was the same for UNC3001 and 011C at every time point tested. Additionally, virus released into the supernatant was the same at all time points. These growth curves indicate that the *in vitro* produced infectious clone virus, 011C, infects cells and replicates identically to our parental virus, UNC3001.

CONSTRUCTION OF CHIMERIC VIRUSES

To determine which genome regions of the Sri Lankan clinical isolates are responsible for the growth phenotype described in Chapter 3, we constructed chimeric viruses by substituting either A01, B01 or C/D01 with the corresponding fragment from UNC3006 or UNC3009 (table 4.3). We also mutated the 3' UTR of dC/D01 to the sequence of either UNC3006 or UNC3009 using site directed mutagenesis. The chimeric full-length cDNAs were assembled as shown in figure 4.1, capped RNAs were electroporated into Vero cells and supernatants were harvested at three and six days post electroporation. To verify that we had produced the correct chimeras, virus was plaque purified and amplified on C6/36 cells and viral RNA was purified and sequenced. The sequences confirmed that we had produced viruses containing the structural genes of UNC3006 (named 06AIC) or UNC3009 (names 09AIC) and the non-structural genes of UNC3001. Viral titers from these electroporated cells were determined on Vero cells in an immunofocus assay (figure 4.4). Both viruses, 06AIC and 09AIC, were produced in our electroporation and were able to form foci.

DISCUSSION

In this chapter, we describe the development a new infectious clone from a DENV3 clinical isolate using a technique previously used with Coronaviruses (237). Because of toxicity and stability concerns, we amplified and cloned the DENV3 genome in three overlapping pieces: A containing nucleotides 1 to 2034 downstream of the T7 RNA promoter, B containing nucleotides 2029 to 5210, and C/D containing nucleotides 5100 to 10707. BsmBI sites were engineered in A and B outside of the coding region and a native BsmBI site in the UNC3001 genome was taken advantage of. An EciI site was engineered at the 3' end of C/D so that the exact 3' UTR of the DENV3 genome could be produced *in vitro*. Using type II restriction enzymes, which cut outside their palindromic recognition site and produce unique sticky ends, allowing for manipulation of cutting and directional ligation, we were able to assemble full-length DENV cDNA and produce infectious RNA. Once electroporated into cells, the *in vitro* transcribed RNA was translated and replicated, releasing virus into the cell supernatants. This virus was infectious to various cell types and behaved identically to the parental virus in an immuno-focus assay and in growth curves.

We are now able to use this infectious clone system to identify regions of the DENV3 genome responsible for growth differences between two clinical isolates. We constructed recombinant chimeric viruses containing the structural genes of UNC3006 or UNC3009 and the non-structural genes of UNC3001. These viruses were infectious and behaved similar to UNC3006 and the original recombinant 011C in growth curves. Growth kinetic experiments are currently ongoing to determine if the structural gene differences between UNC3006 and UNC3009 are responsible for the growth advantage of UNC3009.

The growth advantage of the DENV2 Asian isolate compared to the American isolate was shown to be partially due to a difference in envelope protein (39, 59). Additionally, the adaptation of the DENV2 D2S1 strain to mice was due to a unique amino acids at positions 124 and 128 of E, resulting in increased viral affinity for heparin sulfate (39, 59).

The development of an infectious clone from a DENV3 clinical isolate and the ability to produce recombinant chimeric viruses provides us with an important new tool for studying DENV3. It can be used to determine which region of the genome is responsible for specific *in vitro* and *in vivo* phenotypes. Additionally, we can potentially determine the consequence of specific residues in DENV3 pathogenesis.

| <u>Primer Name</u> | <u>Primer Sequence</u> |
|--------------------|-----------------------------------------------|
| 06CD1F | 5' TGGAAGAAGAGATGTTCAAAAAGCG 3' |
| 06CD1R | 5' CATGTTGACTGAAGAGACAATGTTACCTGTGCC 3' |
| 06CD2F | 5' GGCACAGGTAACATTGTCTCTTCAGTCAACATG 3' |
| 06CD2R | 5' CTACGTTTACCAAATGGCTCCTTCCGAC 3' |
| 09CD1F | 5' TGGAAGAAGAGATGTTTCAGAAAGCG 3' |
| 09CD1R | 5' CATGTTGACTGAAGATACTATGTTACCTGTGCC 3' |
| 09CD2F | 5' GGCACAGGTAACATAGTATCTTCAGTCAACATG 3' |
| 09CD2R | 5' CTACGTTTACCAAATGGCTCCCTCTGAC 3' |
| 013'UTRF | 5' GGAGCCATTTGGTAAACGTAGGAAGTGAAAAAGAGGCAA 3' |
| 3'BsmBI | 5' CGTCTCNNNNNAGAACCTGTTGATTCAACAGCACC 3' |

Table 4.1 UNC3006 and UNC3009 CD fragment cloning primers.

| <u>Virus</u> | <u>A Fragment</u> | <u>B Fragment</u> | <u>C Fragment</u> | <u>D Fragment</u> | <u>3' UTR</u> |
|---------------------|--------------------------|--------------------------|--------------------------|--------------------------|----------------------|
| 01IC | UNC3001 | UNC3001 | UNC3001 | UNC3001 | UNC3001 |
| 06AIC | UNC3006 | UNC3001 | UNC3001 | UNC3001 | UNC3001 |
| 09AIC | UNC3009 | UNC3001 | UNC3001 | UNC3001 | UNC3001 |
| 06BIC | UNC3001 | UNC3006 | UNC3001 | UNC3001 | UNC3001 |
| 09BIC | UNC3001 | UNC3009 | UNC3001 | UNC3001 | UNC3001 |
| 06CDIC | UNC3001 | UNC3001 | UNC3006 | UNC3006 | UNC3001 |
| 09CDIC | UNC3001 | UNC3001 | UNC3009 | UNC3009 | UNC3001 |
| 06C09DIC | UNC3001 | UNC3001 | UNC3006 | UNC3009 | UNC3001 |
| 09C06DIC | UNC3001 | UNC3001 | UNC3009 | UNC3006 | UNC3001 |
| 06UIC | UNC3001 | UNC3001 | UNC3001 | UNC3001 | UNC3006 |
| 09UIC | UNC3001 | UNC3001 | UNC3001 | UNC3001 | UNC3009 |

Table 2 Virus panel to determine genome region responsible for growth phenotype

| <u>Position</u> | <u>Protein (Position)</u> | <u>UNC3001</u> | <u>UNC3006</u> | <u>UNC3009</u> |
|-----------------|-------------------------------|----------------|----------------|----------------|
| 27 | C (27) | P | S | S |
| 286 | E (6) | I | I | V |
| 404 | E (124) | P | P | S |
| 775 | NS1 (2) | M | M | T |
| 901 | NS1 (128) | T | T | I |
| 946 | NS1 (173) | D | D | E |
| 990 | NS1 (217) | L | L | F |
| 1123 | NS1 (350) | V | V | A |
| 1162 | NS2A (37) | L | L | F |
| 1275 | NS2A (150) | V | V | I |
| 1283 | NS2A (158) | I | I | M |
| 1284 | NS2A (159) | L | L | F |
| 1380 | NS2B (37) | L | L | M |
| 1504 | NS3 (31) | L | F | L |
| 1642 | NS3 (169) | N | N | S |
| 1658 | NS3 (185) | K | R | R |
| 1921 | NS3 (448) | M | I | M |
| 2191 | NS4A (99) | E | E | I |
| 2192 | NS4A (100) | D | D | V |
| 2540 | NS5 (50) | I | I | T |
| 2743 | NS5 (253) | K | K | R |
| 2760 | NS5 (270) | T | T | I |
| 2855 | NS5 (365) | P | S | P |
| 2864 | NS5 (374) | G | G | E |
| 2990 | NS5 (500) | D | D | E |
| 3106 | NS5 (616) | V | V | A |
| 3129 | NS5 (639) | P | P | L |

Table 3 Amino acid comparison between UNC3001, UNC3006 and UNC3009.

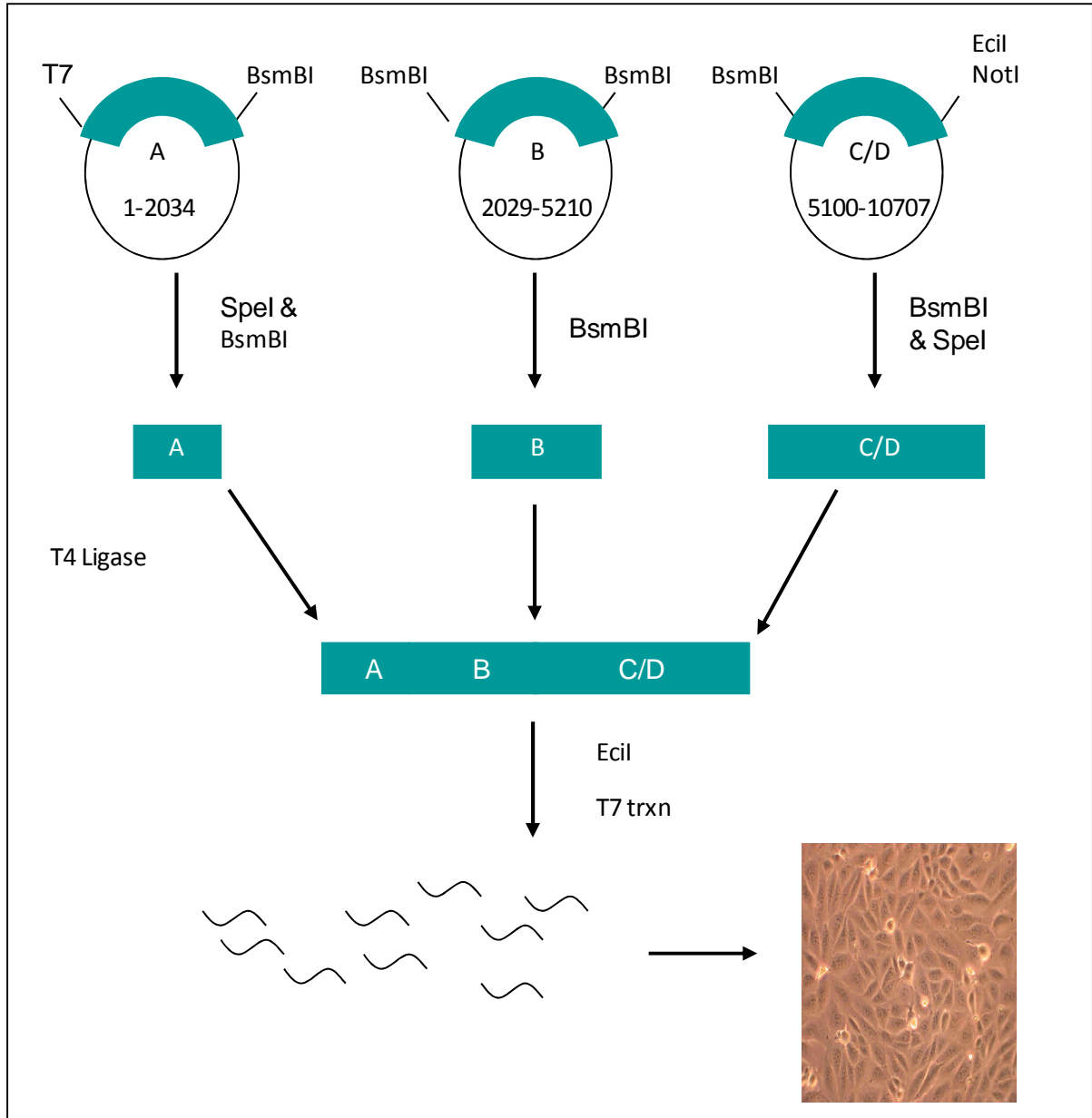
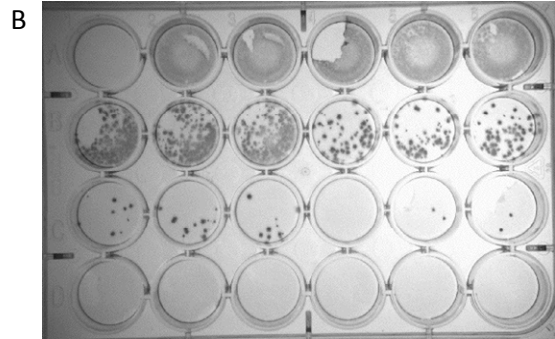
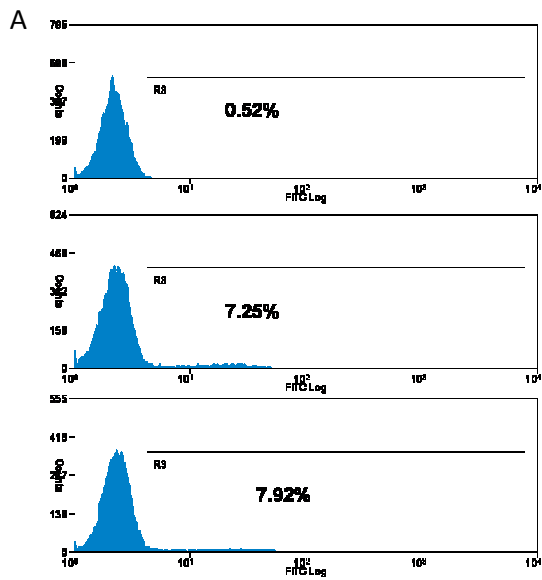
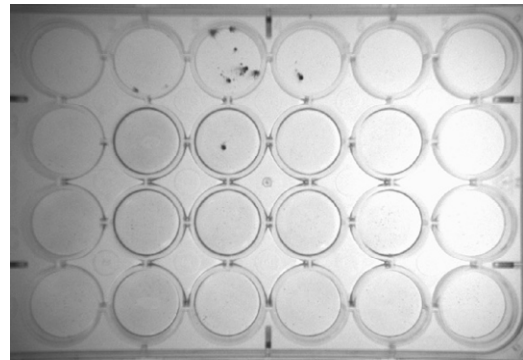


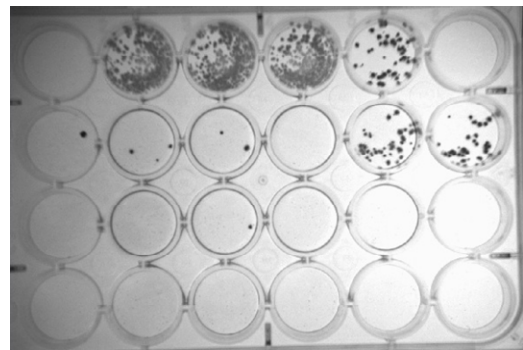
Figure 4.1 Construction of a DENV3 infectious clone.



Parental UNC3001 titration

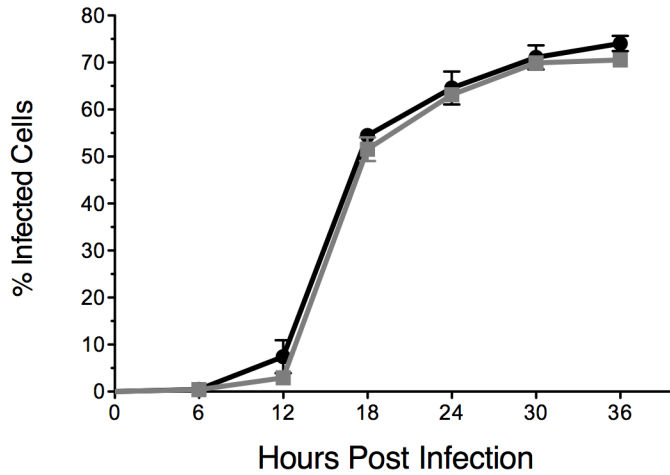


O1 IC 3 days post electroporation
supernatant titration



O1 IC 6 days post electroporation
supernatant titration

Figure 4.2 Virus is released from vero cells electroporated with T7-transcribed DENV RNA. Supernatants were recovered from electroporated Vero cells and add to U93 cells or more Vero cells. The parental and the recombinant viruses were able to infect both cell types.



b

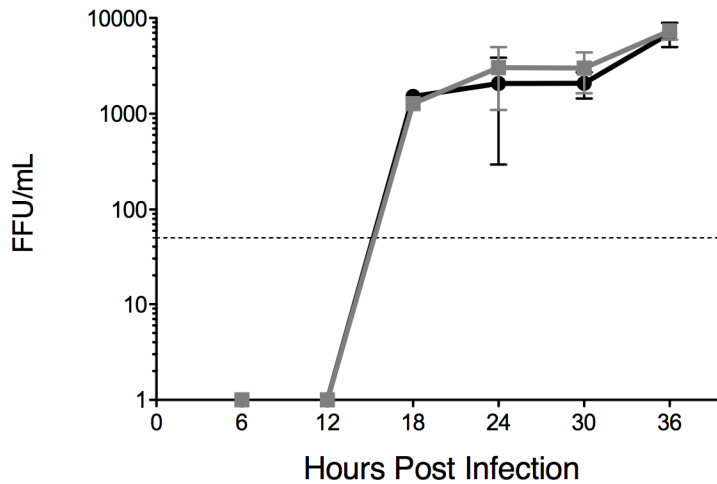


Figure 4.3 Recombinant 01IC grows as well as UNC3001 in DC-SIGN expressing cells. DC-SIGN expressing U937 cells were infected with either the parental or the recombinant in vitro generated virus. At 6-hour intervals, cells and supernatants were harvested and % infected cells and supernatant titers were determined.

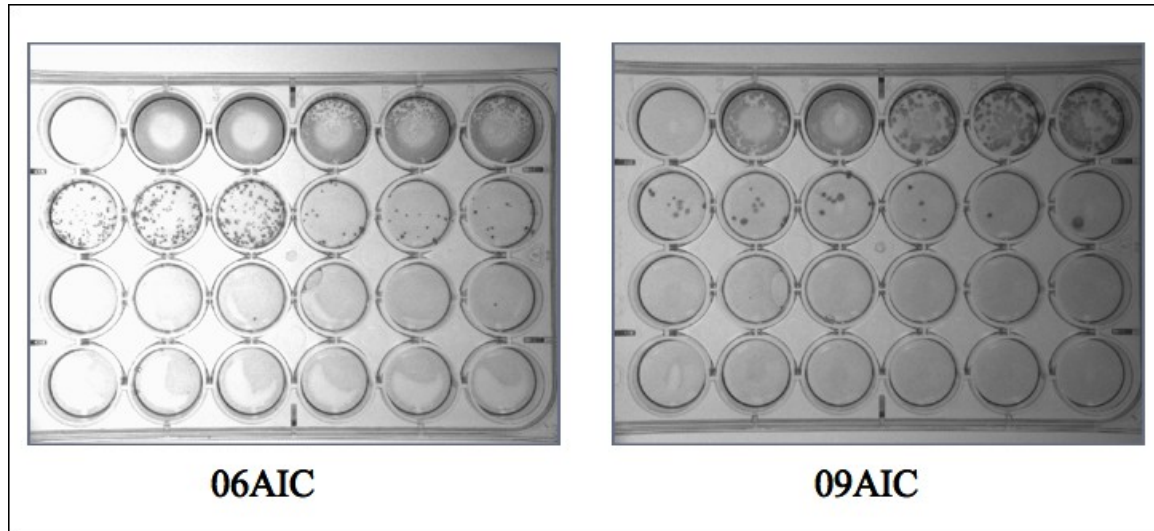


Figure 4.4 Chimeric viruses are viable and generate immunofoci on Vero cells.

Supernatants from 06A and 09AIC electroporated cells were added to Vero cells. Both viruses replicated well in Vero cells.

CHAPTER 5. SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

A number of studies with Arboviruses have examined the role of structural motifs and nonstructural mutations involved in viral pathogenesis and virulence. In this body of work, we have sought to examine how dengue, a mosquito-borne Flavivirus, interacts with host cells and to characterize *in vitro* differences between viruses isolated before and after the emergence of DHF in Sri Lanka. The first goal of this study was to determine if viral glycoprotein structures differ when propagated in arthropod versus mammalian cells. The second part sought to analyze evolutionary relationships and *in vitro* growth characteristics of Sri Lankan clinical isolates. Using a panel of viruses isolated prior to or following the emergence of DHF in Sri Lanka, we performed kinetic growth experiments to determine if the viruses associated with DHF had a fitness advantage in human cells similar to that seen in mosquitoes (85). The third goal was to develop a reverse genetic system for DENV so that various mutant viruses could be constructed and characterized. The work presented in this dissertation will help further the understanding of how DENV interacts with host cells and potentially elucidate viral virulence factors.

N-LINKED GLYCANS ON DENGUE Viruses

Previous work with alphaviruses and West Nile virus has shown that virus produced in the arthropod vector is structurally different than virus produced in the mammalian host (42, 43, 112, 194, 195). The viral glycoproteins of mosquito-derived virus are high mannose, whereas those of mammalian derived virus are more highly processed (43, 194, 195). The high mannose glycans on mosquito-derived virus particle allow the virus to efficiently interact with C-type lectins, rendering the virus highly infectious on monocytic target cells (43, 194, 195). In addition to mediating infection, these high mannose glycans alter the host

immune response to alphaviruses (194). Upon infection, mosquito-derived alphaviruses evade or suppress the host antiviral response, leading to less IFN production compared to mammalian-derived virus.

Since DENV is an arbovirus like alphaviruses, we were keen to characterize potential differences between mosquito- and mammalian-derived DENV. Unlike previously characterized arboviruses, mosquito-derived DENV did not infect a higher percentage of DC-SIGN expressing cells or primary human dendritic cells. Infection of both viruses was mediated by DC-SIGN and the kinetics of infection was similar. Upon characterization of the major viral glycoprotein E, we discerned an explanation for similar infectivity. Both mosquito- and mammalian-derived DENV have a high mannose glycan at position N-67 of E. This glycan can mediate infection of DC-SIGN expressing cells by both viruses. The second E glycan at N-153 is more highly processed in viruses produced by both cell types. The mosquito-derived virus has a low mannose glycan whereas the mammalian-derived virus has large a large complex sugar. Following characterization of glycans on various forms of E, we learned that membrane-anchoring plays an important role in determining whether the glycan at N-67 is processed. When E is tethered to the membrane and arranged in viral or subviral particle, host cell glycans are unable to access the N-67, resulting in minimal glycan trimming during viral egress and high mannose sugars on E. If E is expressed without the transmembrane anchor, the soluble protein is secreted and host enzymes are able to access E N-67 and the resulting glycan is complex.

CHARACTERIZATION OF SRI LANKAN CLINICAL DENV3 ISOLATES

The emergence of DHF in new geographic areas has been associated with the replacement of existing DENV viruses with new viruses associated with DHF in other

geographical regions (64, 142, 173). This finding led to the hypothesis that some viruses within specific serotypes are more fit than others (38). We were fortunate to have access to a panel of DENV3 isolated in Sri Lanka before and after the emergence of DHF in 1989. The growth of these viruses has been characterized in *Aedes aegypti* mosquitoes. The viruses associated with DHF replicate to higher titers and disseminate within mosquitoes better than those isolated prior to the emergence (85). Thus, it appears that post-DHF viruses possess a fitness advantage over pre-DHF viruses in mosquitoes, potentially explaining the clade replacement seen in Sri Lanka (85, 142).

Previous work with DENV2 showed the DHF-associated viruses had a fitness advantage in mosquitos and in primary human DC (38). Therefore, we wanted to determine if the pre- and post DHF viruses behaved differently while infecting human DC-SIGN expressing cells. Unexpectedly, in our experiments, the pre-DHF viruses infected human cells better than the post-DHF viruses. Additionally, more virus was released from cells infected with pre-DHF viruses. To further characterize this potential *in vitro* growth difference, we focused on one representative virus from each group. The viruses clearly replicated with different kinetics: the pre-DHF virus produced viral antigen earlier than the post-DHF virus and replicated up to 100 times higher than the post-DHF virus. Our preliminary studies indicate that the pre- and post-DHF isolates bind to and enter cells with similar efficiency. Thus, a post entry defect is likely to be responsible for the different infectivity of pre- and post-DHF viruses. More studies are needed to confirm and extend this observation.

In order to identify potential viral virulence factors, we compared the entire genome sequences of our Sri Lanka clinical isolates and other DHF-associated viruses. We

confirmed that the post-DHF viruses are more closely related to other DHF-associated viruses isolated in Africa and South American than to the pre-DHF viruses. We were also able to identify coding and 3' UTR mutations that differentiated the Sri Lanka pre-DHF viruses from all DHF-associated viruses. These changes were located in E, NS1, NS3, NS4A, NS5 and the 3' UTR. It is currently unknown which of these mutations are important for the fitness advantage seen in mosquitoes or the growth differences observed in human cells.

THE CONSTRUCTION AND CHARACTERIZATION OF A DENV3 INFECTIOUS CLONE

DENV is a RNA virus, precluding genetic manipulation without a reverse genetics system. In such systems, the viral RNA genome is fixed as DNA and propagated in *E. coli* or yeast. Once fixed, we can manipulate the genetic code of RNA viruses and produce recombinant progeny viruses. These viruses can be characterized in a number of systems to describe phenotypic consequences of genetic changes.

For the final portion of this dissertation, we developed a reverse genetic system for DENV3. We chose a post-DHF isolate as the parental strain and used NoSeeM technology to construct an infectious clone in three pieces. We were able to assemble full-length DNA and produce T7 transcripts. Once electroporated with RNA, host cells produced infectious virus. The recombinant virus replicated with similar growth kinetics to the parental virus. In addition to producing recombinant wildtype virus, we were able to produce chimeric viruses containing genetic material from more than one virus. Thus far, we have isolated four recombinant chimeric viruses.

The development of a DENV3 reverse genetic system is a major contribution that can be used to characterize chimeric and single mutation viruses. We've already produced viruses containing structural and nonstructural mutations and shown we can isolate mutants. Using chimeric viruses containing regions of the genome or specific genes from pre- and post-DHF viruses, we may be able to determine which changes conserved between groups contribute to the *in vivo* and *in vitro* phenotypes described above.

APPENDIX

| Fragment | Forward Primer | Reverse Primer |
|-----------------|-------------------------------|-----------------------------|
| 1-2497 | AGTTGTTAGTCTACGTGGACCGACA | CCAGGTATGGACCTCGTTGGT |
| 2250-5171 | GAAGTGCTTACACAGCCCTATTCAG | GAGTTCTTAAACGTCTCTTGATTGCC |
| 5000-7108 | AGCGGAATAGCGCAAACAAATGC | CAATGCCAGTAGTGGTACGCCTAA |
| 6650-8613 | ATGTTGTGGATGGCCGAAATC | TGGCCAAATGGAGTTGTATCTGTC |
| 8315-10452 | ATAGAGAAAGATGTGGATTTAGGAGCAGG | TTTGCAGCCTCCCAGGTTTTA |
| 9951-10707 | CTATTCATGCTCACCATCAG | TACATGCCTTCAATGAAGAGATTCAGG |

Table A.1 PCR primers to amplify segments of DENV viral isolates for use in sequencing.

| <u>Primer Name</u> | <u>Primer Sequence</u> |
|---------------------------|-------------------------------------|
| 1F | 5' AGTTGTTAGTCTACGTGGACCGACA 3' |
| 276F | 5' CAACAGCAGGAGTCTTGGCTAGA 3' |
| 470F | 5' ATGATTGTGGGAAGAATGAAAGAG 3' |
| 980F | 5' TCAGGAGCTACGTGGGTTGACG 3' |
| 1490F | 5' CGGACAGGTTTGGATTTC AATG 3' |
| 1995F | 5' CAGTGGTGACTAAGAAGGAGGAGC 3' |
| 2500F | 5' AGAGCAATACAAATTCCAAGCAGAC 3' |
| 3000F | 5' CCGACATGGGCTATTGGATAGA 3' |
| 3500F | 5' GGTGTCTTGTGTTTGGCAATCC 3' |
| 4001F | 5' TTACCAGTGTGCCAGTCTTCGAG 3' |
| 4449F | 5' TCTTCCATACTCCATACCCGCA 3' |
| 5000F | 5' AGCGGAATAGCGCAAACAAATGC 3' |
| 5502F | 5' ACGCTCCAATTCAAGATGAAGAAAG 3' |
| 6000F | 5' TGGACAACATTAACACACCAGAAGG 3' |
| 6500F | 5' GCGGTGGAGGA ACTACCAGAA 3' |
| 7001F | 5' GCAAATGTGTCCCTGGCAGC 3' |
| 7503F | 5' GCTATTTAGCAGGAGCTGGGCT 3' |
| 8000F | 5' GACATCGGAGAATCTTCACCAAGC 3' |
| 8495F | 5' AAAGCCACAGGCTCAGCCTC 3' |
| 9000F | 5' GAGCCAGGTACCTTGAGTTCGAA 3' |
| 9500F | 5' TGGTTGGAACCAAAGGAGTGG 3' |
| 10001F | 5' TGGAACAGGGTGTGGATCGA 3' |
| 10251F | 5' AAGGAGCCATTTGGTAAACGTAGG 3' |
| 10501F | 5' CCCTCCCATGACACAACGCA 3' |
| 257R | 5' TGAGGAAAGCTATGAACGCCAT 3' |
| 525R | 5' CCAGAGGCTGTCTTAAAAAGTAGGGATT 3' |
| 942R | 5' CATCTCATTGTCATGGATGGGGT 3' |
| 1233R | 5' CCTCTGTCTACGTATGTATGCTTACACAC 3' |
| 1696R | 5' TTGCGATCCAAGGACGACTACT 3' |
| 2200R | 5' TGATCCAAAGTCCCAAGCTGTG 3' |
| 2712R | 5' CTTGCTCTAAGACCCCAATTGTA 3' |
| 3232R | 5' CAATTTTCCTAAGTGCCAGGGTC 3' |
| 3732R | 5' GCCAAGAATGGCTGGATTTTAAATG 3' |
| 4209R | 5' GCCATGGGCACATCATTCTAAG 3' |
| 4717R | 5' GTTTGGTTCCAGTCTTTTCCCAT 3' |
| 5187R | 5' GTCGGTGCCAAAATTAGAGTTCTTA 3' |
| 5737R | 5' TGTCACCACGAAGTCCCAATCA 3' |
| 6255R | 5' TCCACATCCATGTTCTCCTCTAAAA 3' |
| 6746R | 5' GCTTTTCTGGTTCTGGTATAAGCAACA 3' |
| 7232R | 5' CTGTCCTTTTCTGAGCTTCACGA 3' |
| 7734R | 5' CTGGACACGGCATGATGTGTTA 3' |
| 8255R | 5' CTGAAGAGACAATGTTACCTGTGCC 3' |

| | |
|--------|-----------------------------------|
| 8724R | 5' TTCCTTCCCAGGGTTCTCCA 3' |
| 9240R | 5' TCAGGGTCCATTTGCTGTGTG 3' |
| 9709R | 5' TTCATGAAAGTGGTGGGAGCA 3' |
| 10172R | 5' CCTGTTGAATTGCTGTGGGTATGT 3' |
| 10452R | 5' TTTGCAGCCTCCCAGGTTTT 3' |
| 10707R | 5' TACATGCCTTCAATGAAGAGATTCAGG 3' |

Table A.2 DENV3 whole genome sequencing primers.

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