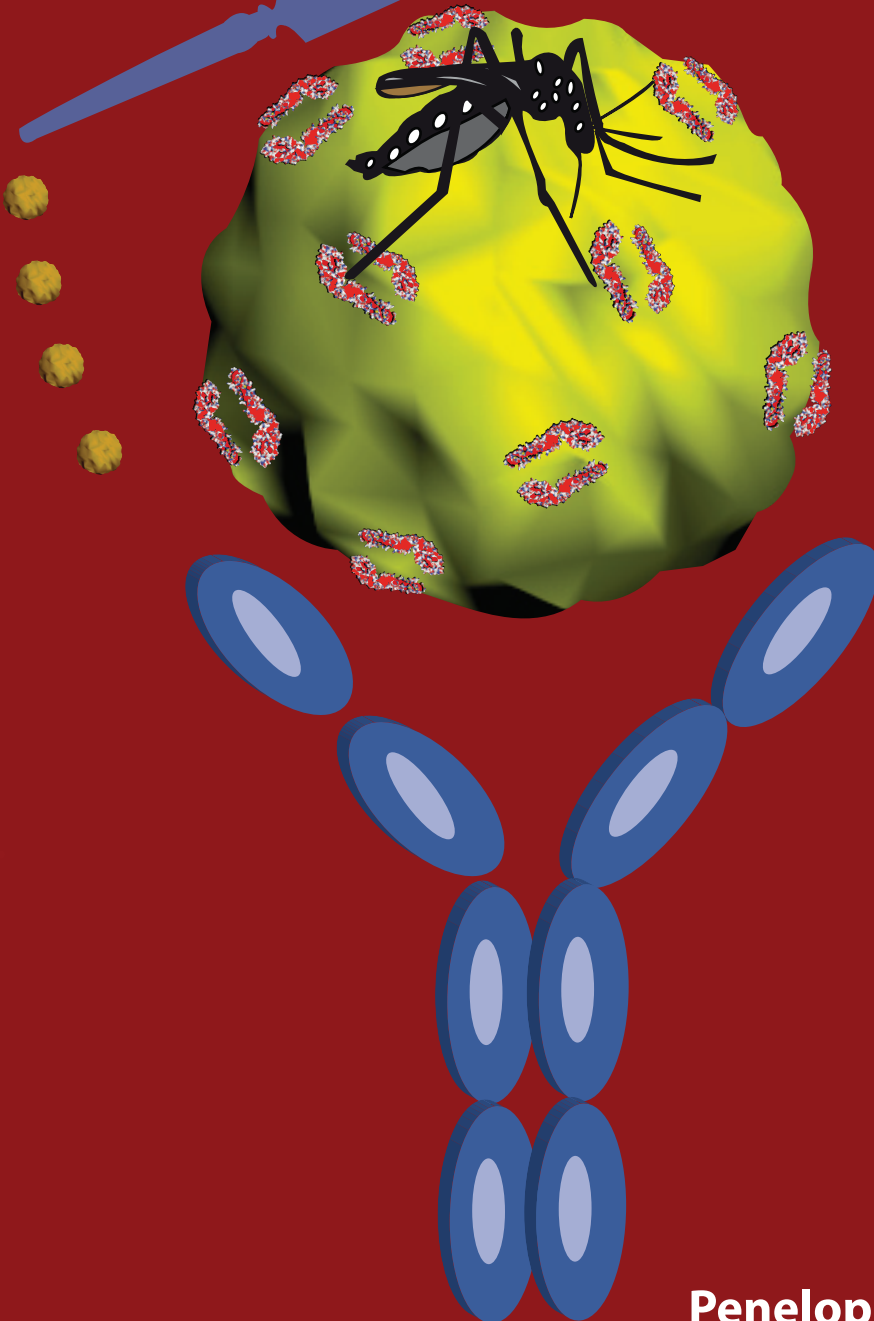# Dengue virus specific immune response:

Implications for laboratory diagnosis and vaccine development

Penelope Koraka

Dengue Virus Specific Immune Response:

Implications for laboratory diagnosis and vaccine development

Dengue virus specifieke immuunrespons:

Implicaties voor laboratorium diagnose en vaccin ontwikkeling

Thesis

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Mairuhu ATA, Setiati TE, **Koraka P**, Leyte A, Soemantri A, Osterhaus ADME, Brandjes DPM, Meijers JCM, ten Cate H and van Gorp ECM. Are dengue virus associated coagulation abnormalities related to disseminated intravascular coagulation? Submitted.

Mairuhu ATA, Joop K, Setiati TE, **Koraka P**, Soemantri A, Osterhaus ADME, Brandjes DPM, Sturk A, Nieuwland R, ten Cate H, Leyte A and van Gorp ECM. Dicreased number of microparticles in severe dengue virus infections: possible involvement in disease pathogenesis. Submitted.

Setiati TE, de Kruif MD, Mairuhu ATA, **Koraka P**, Aberson HA, Spek CA, Osterhaus ADME, Reitsma PH, Brandjes DPM, Soemantri A and van Gorp ECM. Inflammatory gene expression changes in children with severe dengue virus infection. Submitted.

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List of Abbreviations

aa	amino acid
Ab	antibody
ADE	antibody dependent enhancement
Ag	antigen
ANOVA	analysis of variance
BHK	baby hamster kidney
BSL	bio-safety level
C	nucleocapsid
CMV	cytomegalovirus
D	domain
DBI	dot-blot immunoassay
DENV	dengue viruses
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
E	envelope
EBV	Epstein-Barr virus
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
ELIspot	enzyme linked immunospot
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HBV	hepatitis B virus
HCV	hepatitis C virus
HI	haemagglutination-inhibition
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRPO	horse-radish peroxidase
HSV	herpes simplex virus
IFA	immunofluorescence assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
JEV	Japanese encephalitis virus
LAT	live attenuated tetravalent

M	membrane
MHC	major histocompatibility complex
mRNA	messenger RNA
NHS	normal human serum
NS	non-structural
NT	neutralization test
O.D.	optical density
PAF	platelet activation factor
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PRNA	plaque reduction neutralization assay
RIT	rapid immunochromatographic test
RSV	respiratory syncytial virus
RT	reverse transcriptase
SD	standard deviation
SLEV	St. Luis encephalitis virus
TBEV	tick-borne encephalitis virus
TCID	tissue culture infectious dose
TNF	tumor necrosis factor
UTR	un-translated region
UV	ultra-violet
VCAM	vascular cell adhesion molecule
VEEV	Venezuela equine encephalitis virus
VN	virus neutralizing,
VZV	varicella zoster virus
WHO	World Health Organization
WNV	west Nile virus
YFV	yellow fever virus

Chapter 1

Introduction

Dengue viruses (DENV 1-4) belong to the family *Flaviviridae*, genus *Flavivirus*. They are transmitted to humans through the bite of infected mosquitoes of the *Aedes* species. An estimated 100 million people are annually infected with DENV and over two billion people are at risk in acquiring DENV infection in tropical and subtropical regions of the world. Infection with DENV may be asymptomatic or may be characterized by a variety of clinical symptoms including mild dengue fever or more severe forms of disease characterized by haemorrhages which may lead to shock. Treatment of DENV infection is supportive but non-specific. The world-wide distribution of the mosquito vector as well as the high morbidity and mortality rates of DENV infection have led to the emergence of DENV as one of the most important public health problems world-wide.

Laboratory diagnosis of DENV infection is based on virus isolation from cell cultures and/or detection of viral RNA, or on the detection of DENV specific immunoglobulin M (IgM) and IgG serum antibodies. Recent studies have revealed that serological diagnosis can be difficult due to cross-reactions observed with other members of the genus *Flavivirus*.

Despite several decades of research the pathogenesis of DENV infection is poorly understood. Antibody dependent enhancement (ADE) of infection has been associated with severe DENV disease outcome. Although ADE is at the basis of the predominant theory to explain different forms of severe DENV infections, it is now generally accepted that other factors such as virological, immunological and other host factors may play important roles in the pathogenesis of severe DENV disease.

Theoretically, as for other arthropod-borne viral infections, prevention of DENV can be achieved either through vector control or through immunization strategies. Prevention of DENV through vector control is largely ineffective, expensive and with only temporary benefit. Several groups have attempted to develop a vaccine against DENV with limited success so far. A live attenuated tetravalent candidate vaccine against all four DENV serotypes has been developed and evaluated also in phase I and II human trials, but it is not yet licensed for public use. The lack of suitable animal models to test DENV disease has hampered the development of a safe and effective vaccine and extensive studies on the pathogenesis of DENV infections.

STRUCTURE MOLECULAR AND BIOLOGICAL CHARACTERISTICS OF DENGUE VIRUSES

Dengue viruses belong to the family *Flaviviridae*, genus *Flavivirus* together with Yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and many other human pathogenic viruses, mainly causing heamorrhagic fevers or encephalitides in man [1]. Four serologically and genetically distinguishable types, DENV 1-4, can be recognised on the basis of plaque reduction neutralization assays and polymerase chain reaction (PCR) [2;3]. Similar to other flaviviruses, DENV virions consist of an RNA genome, encapsulated in a nucleocapsid, which is surrounded by a lipid envelope (figure 1). The particle size is about 45 to 55 nm [4].

The viral genome is single stranded RNA of approximately 11,000 bases in length. The genomic RNA has a positive polarity, is infectious and encodes three structural and seven non-

structural proteins. The single open reading frame of DENV is flanked by a capped 5' untranslated region (UTR) and a 3' UTR lacking a polyA tail (figure 2) [5;6]. The viral genome is translated as a single polyprotein, which is cleaved by host and viral proteases during or post translation [7;8].
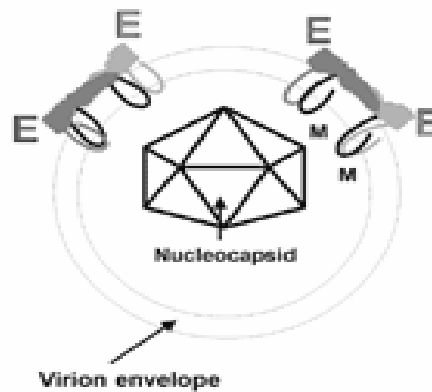

Figure 1: Schematic representation of mature DENV virions. An icosahedral nucleocapsid encloses the viral RNA. E: envelope protein; M: membrane associated protein and C: nucleocapsid protein (www.cdc.gov/ncidod/eid/vol7no4/petersen.htm).

The nucleocapsid (C) protein has a molecular weight of ~ 13,500 Dalton and is rich in arginine and lysine residues. C is the first protein to be synthesised during translation [9]. The membrane (M) protein of ~8,000 Dalton, is a small non-glycosylated protein which is formed after proteolytic cleavage of a glycosylated prM precursor during virus maturation. The formation of M from pre-M appears to be crucial in virus morphogenesis, increasing virus infectivity [10]. The role that the M protein plays in mature virion is not known. The large envelope glycoprotein (E) has a molecular weight varying among the different serotypes from ~51,000 to ~59,000 Dalton. Henchal et al., showed that the E protein harbours at least four antigenic determinants, the flavivirus group reactive, the DENV complex specific, the DENV subcomplex specific and the DENV serotype specific [6]. These heterologous antigenic determinants of the E protein of DENV are related with the cross reactivity among the four DENV serotypes. The E protein mediates several important biological activities as it is the viral hemagglutinin and the receptor binding protein, it induces virus neutralizing (VN) antibodies and participates in membrane fusion and viral assembly. The viral envelope consists of a lipid bilayer, which is derived from the host cell membrane. Three structural domains have been identified in the E: DI, DII (includes the fusion peptide) and D III (includes major antigenic sites involved in VN) [11].
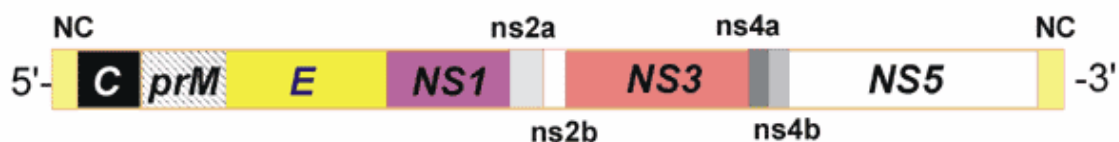

Figure 2: DENV genome organization. 5'UTR is followed by the open reading frame coding for the structural and non-structural proteins. Following the stop codon at the end of NS5 is the 3'UTR (www.cdc.gov/ncidod/eid/vol7no4/petersen1b.gif).

The seven non-structural proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. The NS1 protein exists as cell-associated, cell-surface and extracellular nonvirion forms [12]. Upon infection with DENV, antibodies are formed against the NS1 protein with complement fixing activity [13]. The second largest viral protein, the NS3, has two main functions, protease activity and triphosphate/helicase activity, suggesting of a cytoplasmic localization of the NS3 protein [4;14;15]. The NS5 protein is presumably located in the cytoplasm, although it is membrane associated, and is the viral RNA-dependent RNA polymerase [4]. Part of the NS5 acts as methyltransferase, for capping of the viral genome [16]. The small hydrophobic NS2a, NS2b, NS4a and NS4b proteins are thought to be membrane associated, forming membrane components of the viral replication complexes and may also be involved in membrane localization of NS3 and NS5 proteins through protein-protein interactions [17;18]. Moreover, the NS2a protein has been associated with proteolytic processing of the NS1 protein [19]. The 5' and 3' UTR of DENV have been associated with virus replication, translation and virulence [20-25]. The importance of the 5' and 3' UTRs in the virus cycle has been linked to conserved structural elements that have been shown to form among all flaviviruses irrespective of sequence homology [26].

As defined with the use of monoclonal and polyclonal antibodies, DENV 1 and 3 form a subcomplex [6]. Additionally, some antigenic heterogeneity may be seen among different strains of each serotype. At the amino acid level, nucleotide sequences have revealed a positional homology of 63% to 68% within the DENV group in contrast to 44% to 51% between DENV and YFV and DENV and WNV respectively [27-29].

DENV is stable at pH 8 whereas it is inactivated at acidic pH. Lipid solvents, trypsin, chymotrypsin, papein and pancreatic lipase may chemically inactivate DENV. Photoinactivation of DENV by UV light is also possible.

DENV can replicate in a variety of cell lines including primary and continuous cell lines, however it is difficult to obtain cytopathic effects in these cell lines. The most susceptible cell lines are human monocytes, monkey kidney or Vero cells, baby hamster kidney cells (BHK) and mosquito cells especially of the *Aedes* or *Toxorhynchitis* spp.

REPLICATION OF DENGUE VIRUSES

Dendritic cells and other cells of the mononuclear lineage have been suggested as the main target cells of DENV infection [30-32]. DENV enters the cells through endocytosis and viral replication takes place in perinuclear foci of the cytoplasm with subsequent proliferation of smooth and rough endoplasmic reticulum (Figure 3). After translation of the incoming genomic RNA, which serves directly as messenger RNA (mRNA), the replication of RNA involves synthesis of complementary minus strands that are subsequently used as templates for production of additional plus-stranded molecules. Plus stranded molecules are then used for translation of structural and non-structural viral proteins, or they may be encapsidated into virions [12]. Virion assembly occurs on membranes of the endoplasmic reticulum. Individual viral proteins are the products of cleavage of a large precursor

polyprotein, which is formed after complete translation of the large open reading frame of the mRNA starting from the 5' end.
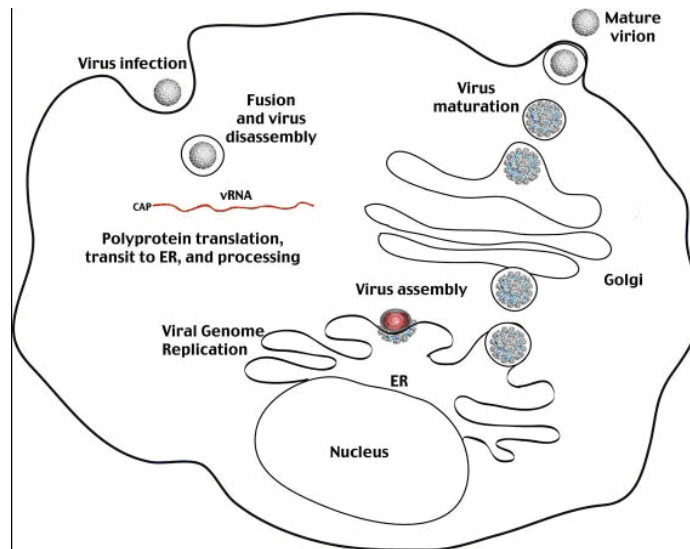

Figure 3: DENV infection of host cells and replication cycle of the virus (translation of the viral polyprotein and replication of the viral genome) (http://chen.bio.purdue.edu/images/flavi/viruslifecycle.jpg).

The latent period is approximately 12 hours or more, whereas 10 to 15 minutes are required for completion of DENV genome-length products. These long periods of latency and translation indicate that some mechanism must exist that regulates synthesis of plus-stranded RNA relative to minus-strand RNA. It has been observed that the ratio of plus- to minus- stranded RNA at the peak of DENV-2 RNA synthesis is as high as 10 to 1 [33]. In contrast to other arboviruses, DENV continuous to replicate after infection for days without shutdown of nucleic acid or protein synthesis [33].

TRANSMISSION OF DENGUE VIRUSES

Dengue viruses are considered to be the most important arboviruses with the greatest impact in public health. They are transmitted to humans with the bite of an infected mosquito of the *Aedes* species (now known as *Stegomya* species), mainly *A. aegypti* or *A. albopictus* (Figure 4). The mosquito vector gets infected after feeding on an infected person. After 10-12 days of viral development in the vector, the mosquito is infectious and remains infectious for life [34]. The principal vector, *A. aegypti*, is a small black-and-white, highly domesticated mosquito. It prefers to lay its eggs in peri-domestic artificial containers, rests indoors and is day active, biting and transmitting the virus to susceptible hosts, during early morning and late afternoon [5]. The female mosquitoes are also very nervous feeders, disrupting their meal after a slight move to continue feed only moments later, possibly to a different host. Therefore it is common to see persons of the same household becoming ill with DENV within the same time frame, suggesting that a single mosquito may have infected all of them [5]. Infected female mosquitoes may pass the virus through trans-ovarian transmission to the

next generation. Infected humans are viraemic, approximately as long as they have fever, whereas after defervescence the virus usually disappears from the blood circulation [35].

In contrast to the highly domesticated *A. aegypti*, *A. albopictus* is originally a forest mosquito, feeding on a variety of animals and breeding in tree holes, plant axils, cut bamboo stumps and opened coconuts [36]. However, larvae also use outdoor artificial water containers, barrels and trash receptacles as their habitats. The diversity of larval habitats resulted in the abundance of this species in rural areas, peri-urban areas and city parks. In the rainy season a much larger number of potential *A. albopictus* larval habitats is available. The adults of *A. albopictus* are both zoophylic and anthropophilic and like *A. aegypti* they feed outdoors during the day.

Figure 4: Aedes aegypti, the black and white "tiger mosquito" the main vector transmitting DENV
(http://www.doctortravel.ca/diseases/dengue/dengue_mosquito.jpg)

The most important transmission cycle of DENV is the urban endemic/epidemic cycle often seen in the urban centres of the tropics and is responsible for periodic epidemics and maintenance of the virus in human populations. In these cases the viruses are maintained in an *A. aegypti*-human-*A. aegypti* cycle causing periodic epidemics (Figure 5). In several parts of the world it is common to see multiple serotypes circulating a phenomenon called hyperendemicity [34].
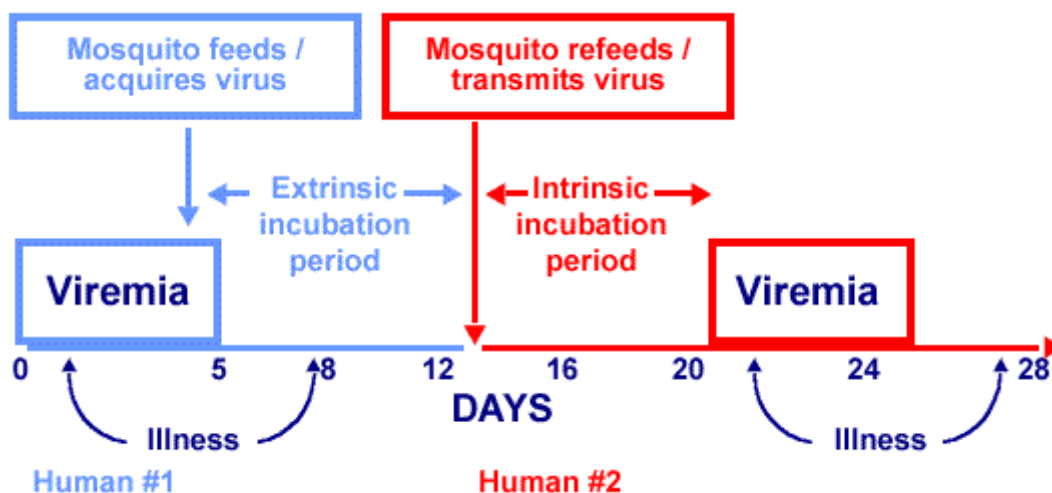

Figure 5: Transmission cycle of DENV involving mosquitoes and susceptible hosts (www.cdc.gov).

CLINICAL ASPECTS OF DENGUE VIRUS INFECTIONS

Infection with DENV may cause a variety of clinical symptoms, from asymptomatic or mild febrile illness to severe haemorrhagic fever with or without shock. After the bite of an infected mosquito an incubation period of 4 to 7 days (range 3 to 14 days) follows before symptoms appear. The following clinical patterns may be encountered: mild atypical febrile illness, classic dengue fever (DF) and dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS) (Figure 6).

Dengue Fever (DF)

Infants and young children may present with an undifferentiated febrile disease usually accompanied by maculopapular rash. In older children and adults symptoms of DF include sudden onset of high fever, severe frontal headache, muscle- bone- or joint-pain, vomiting, nausea and rash. Fever may persist for six to seven days or may be biphasic. The pulse rate may be slow due to the fever and especially children may present respiratory symptoms such as cough, sore throat and rhinitis [37]. The rash may apper on the first or second day of fever and by the time of defervescence or shortly after, a secondary maculopapular or morbilli-like rash may appear. Generalised lymphadenopathy and cutaneous hypersthesia are symptoms that accompany this stage of the disease. The peripheral white blood cells count is decreased with absolute granulocytopenia. Thrombocytopenia is also common with platelet count as low as <100,000/mm^3. Patients with DF may also have elevated levels of liver enzymes as was reported in one epidemic of DENV-4 [38].

DF should also be differentiated from other viral infections with similar clinical symptoms, such as chikungunya or hantavirus infections, especially in areas with overlapping endemicity for these viruses [37]. Neurologic manifestations have also been reported including encephalopathy, peripheral mononeuropathy and polyneuritis. Myocarditis has also been associated with DF [39;40]. DF may have a prolonged convalescence period with weakness, depression and bradycardia.

Dengue Haemorrhagic Fever (DHF)-Dengue Shock Syndrome (DSS)

DHF is characterised by high fever, haemorrhagic phenomena accompanied by increased vascular permeability and hepatomegaly as well as circulatory failure. Patients present with high fever (sometimes higher than 40°C), which persists for 2 to 7 days and other non-specific symptoms similar to DF or other infections seen in tropical areas such as measles, rubella, chikungunya and malaria. During the acute stage of illness it is not possible to differentiate DF from DHF. The hallmark of DHF that differentiates it from DF is plasma leakage and haemoconcentration [41]. Haemorrhagic symptoms include positive tourniquet test, petechiae, epistaxis and mild gastrointestinal bleeding [37]. The time of defervescence is critical since signs of circulatory failure may accompany the fall of the temperature. Depending on the severity of DHF, plasma leakage may be mild with spontaneous recovery after fluid replacement. In the more severe cases that plasma leakage becomes critical, the patient develops shock, which may be fatal if not treated adequately. Early diagnosis is critical in DHF patients since replacement of plasma loss may modify the severity of disease. Usually, thrombocytopenia and haemoconcentration are detectable before the onset of shock [37].

DSS is characterised by the symptoms of DHF and additionally, rapid pulse and fall of blood pressure, acute abdominal pain and hypotension [42]. Patients, who progress to DSS, get into critical condition usually at the time or soon after the fall of temperature following the symptoms described in DHF and the additional abdominal pain. During profound shock the pulse and blood pressure become undetectable. Patients if not treated immediately may die within 12-24 hours [37].

Patients with DHF and DSS have leukopenia, thrombocytopenia and hemoconcentration. The white-blood-cell count may be variable from leukopenia to mild leukocytosis with a marked reduction in the number of neutrophils. Thrombocytopenia is presented with a platelet count of <100,000/mm^3 with simultaneous changes in the hematocrit. Rise in the hematocrit level indicates plasma leakage and is present in all DHF cases with or without shock. Hemoconcentration is the result of increased vascular permeability, leading to plasma leakage into the extravascular compartment and is found in almost all patients with DHF and DSS. In severe cases of DHF plasma volume may be reduced by as much as 20%. Hepatomegaly and elevated liver enzymes are common in patients with DHF and DSS [43]. Vascular changes, thrombocytopenia and coagulation disorders are the main factors involved in hemostatic changes during DHF and DSS.
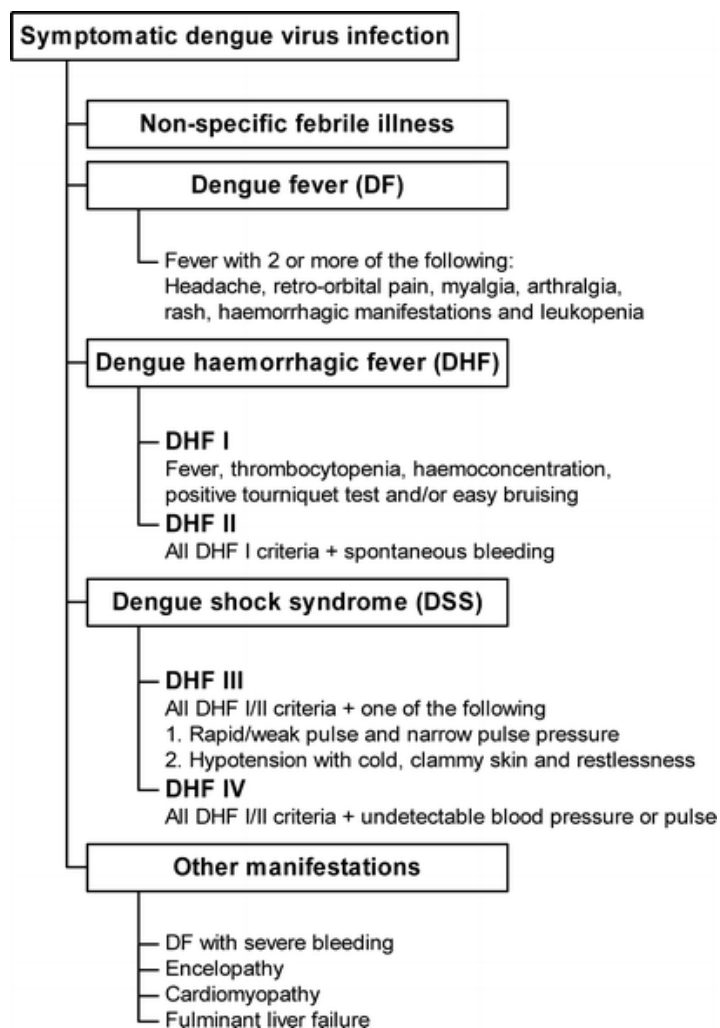

Figure 6: Clinical aspects of DENV infections [44].

Recovery from DHF and DSS is usually rapid and uneventful. Patients that survive, even the ones with undetectable pulse and blood pressure, will recover within two to three days.

The World Health Organization (WHO) has established criteria for the classification and grading of DHF (Figure 6). Recent studies on the clinical aspects of DENV have highlighted the importance of reconsidering the strict criteria of WHO for classification and grading of DHF [45;46].

Treatment of DENV infections

The WHO has formulated special guidelines for the management of DENV infections. DF is a self-limiting infection and no specific therapy is necessary. Treatment is supportive including bed rest, antipyretics and analgesics. In case of dehydration, fluid and electrolyte replacement is also used [37]. The treatment of DHF and DSS patients is based on replacement of plasma volume and close monitoring of the patient for signs of shock. In the acute phase of illness thirst and dehydration result from high fever, therefore fluid and electrolyte replacement are recommended. Antipyretics should also be administrated in the cases of hyperpyrexia. Patients with DSS are considered a medical emergency, however it is not necessary to hospitalise patients with suspected DHF but it is important to closely monitor them and observe for signs of shock especially at the crucial point when fever subsides, usually on day three to five of illness. Replacement of plasma loss with plasma expander or fluid and electrolyte solutions early at the course of DHF may be very effective in recovering from DHF or even from DSS. However, since plasma leakage is not constant, the rate of fluid replacement should be determined according to the plasma loss. The changes of the plasma loss can be monitored by the changes in the haematocrit value. Disseminated intravascular coagulation can be prevented with early and rapid resuscitation from shock and correction of metabolic disturbances with sodium bicarbonate [37]. Patients with unusual manifestations such as hepatic failure or abnormal neurological signs should be treated with extreme caution.

Antiviral therapy in case of DENV infections has not been extensively evaluated [47]. During the 1981 DENV epidemic in Cuba the use of interferon-α gave some indication of decreased mortality. Ribavirin has been demonstrated to show some anti-dengue activity in vitro, however efficacy of this drug in vivo can be achieved only in combination with high toxicity. No antiviral effect was observed, when ribavirin was administrated in experimentally infected monkeys with DENV [48].

LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTIONS

Appropriately collected samples during the acute and convalescent stage of DENV disease are needed for accurate laboratory diagnosis of DENV infections. The reliability of laboratory diagnosis of DENV infections depends on the quality and the timing of the specimens collected, as well as optimal procedures for handling of specimens. Several tests have been developed and are in use for the laboratory diagnosis of DENV.

Virological assays

Virus isolation from the blood of patients with febrile illness has been the method of choice for the laboratory diagnosis of DENV infections for many years [37]. DENV can replicate in a variety of cell lines, such as insect, monkey and baby hamster cells. Insect cells are the most sensitive and widely used cells for isolation of DENV. Since cytopathic effects are rarely seen, cultures must be examined by e.g. immunofluorescence to detect the presence of the virus. However isolation of the virus from cell cultures may be hazardous (DENV is classified as BSL 3 microorganism) and time consuming (it may take up to three weeks to isolate the virus from the blood of infected individuals). In addition, the period that DENV can be recovered from the serum of infected individuals is very brief [49]. Soon after the rise of fever, antibody levels start rising clearing the virus from the circulation. The handling of specimens for virus isolation is also very important for successful virus culture. The virus is heat-labile and serum samples for virus isolation should be stored at -70° C. The lack of appropriate laboratory facilities for virus culture and the difficulties in isolating the virus from serum samples have made virus isolation of limited use for diagnosis of DENV infections.

Detection of DENV RNA in blood samples of infected patients has also been described [2]. Using specific primers in a (real time) reverse transcriptase polymerase chain reaction (RT-PCR) amplification assay, it is possible to detect virus RNA from serum, plasma or cells of a DENV infected individual. However this method is as labour intensive as virus isolation but less time consuming. Detection of viral RNA has the same limitiations as virus isolation since viremia is short-lived after onset of symptoms and sample collection and handling is critical.

Serological assays

Plaque reduction neutralization assays (PRNA) have also been used for the laboratory diagnosis of DENV infections [50]. With PRNA one can diagnose DENV infections on paired samples from the acute and convalescent phase of illness, but also identify the serotype with which the individual is infected. The disadvantage of PRNA is that is labour-intensive and time consuming for routine diagnosis of DENV infections and in addition is hazardous since the use of live virus is essential. In addition, although PRNA is DENV specific (even serotype specific), some cross-reactions with antibodies against other flaviviruses have been observed.

Haemagglutination-inhibition (HI) tests have been extensively used for measurement of DENV specific antibodies. The HI test was developed by Casals in 1954 and has become the WHO standard test for the serological conformation and classification of DENV infections [51]. Interpretation of the HI test is based on titers and time after onset of illness. Fourfold or greater rises in HI titers between paired sera are indicative of recent infections. In primary infections detectable HI antibodies generally appear on day six or later after onset of symptoms, whereas in secondary infections an anamnestic IgG response may occur, which results in rapid elevation of the HI titers just a few days after onset of symptoms (i.e. in acute phase specimens). HI titers of >2560 are considered indicative for secondary DENV infections. This test is relatively simple and sensitive to perform, although it has rather low specificity in discriminating among closely related flaviviruses such as JEV and WNV. Another disadvantage of the HI test is that for a definite diagnosis of DENV infections acute and convalescent paired serum samples of at least seven days interval are required.

Alternatively to HI and PRNA, a wide variety of other serological tests have been developed based on classical ELISA systems. A four-fold rise on DENV specific IgG serum antibodies is usually diagnostic of recent DENV infection. The presence of DENV specific IgM antibodies is also diagnostic of recent DENV infection [37]. Measurement of IgM and IgG antibodies is based on enzyme linked immunosorbent assays (ELISA), which are relatively easy assays to develop and perform, therefore widely applicable for routine diagnosis of DENV infections. Several commercial ELISA kits are available for the measurement of DENV specific IgM and/or IgG serum antibodies with varying sensitivity and specificity [52]. In addition to traditional capture and indirect ELISA tests for the detection of DENV specific IgM and IgG serum antibodies other approaches such as dot blot assay and dipstick ELISA have been developed for the detection of DENV specific IgM and IgG serum antibodies [53;54]. These tests are simple and rapid to perform, do not require the equipment needed for conventional serology and can be performed in a small laboratory or field station.

Recent studies have revealed the importance of IgA serum antibodies in the diagnosis of DENV infections and its potential as diagnostic tool in DENV infections [55;56]. The presence of IgA antibodies is indicative of recent infection. IgM antibodies may persist up to eight months after infection. It has been demonstrated that patients with DHF and/or DSS have elevated levels of total and DENV specific IgE antibodies in the acute phase of disease. However, the diagnostic value of these antibodies in the serum samples of DHF and DSS patients, needs to be further evaluated [57]. DENV infections should be differentiated from a wide range of other viral, bacterial or parasitic infections, early in the acute phase of febrile illness [37]. Infection with other flaviviruses, chikungunya virus, malaria and several other pathogens may be presented with a similar clinical picture early in the course of disease. Serological tests may often give cross-reactions especially with other flaviviruses and in the presence of pre-existing antibodies due to prior infection or immunisation with a flavivirus [58;59]. Usually by day three to four of illness, laboratory findings can establish the diagnosis before shock may occur.

PATHOGENESIS OF DENGUE VIRUS INFECTIONS

Despite several years of research, the pathogenesis of DENV infections is poorly understood (Table 1). The most widely accepted theory to explain the development of DHF and DSS is antibody dependent enhancement (ADE) of infection [60;61]. According to this hypothesis, patients, who experience a secondary DENV infection with a different DENV serotype than the one of the primary infection, are more likely to develop DHF and/or DSS due to pre-existing antibodies to DENV. For ADE to occur two prerequisites should be fulfilled. First, cross reactive but non-neutralizing antibodies elicited during the primary infection bind to the heterologous DENV serotype during the secondary infection, and second, non-neutralized antibody-virus complexes bind to Fc receptors on macrophages and are subsequently internalised into the cell. Since these heterologous antibodies are non-neutralizing, the virus is free to replicate in the infected cell. These pre-existing antibodies may enhance infection with a heterologous DENV serotype. Cases of DHF/DSS in children less than a year

old of age and with no pre-exposure to DENV have been explained with this hypothesis as the effect of residual, sub-neutralizing maternal DENV specific antibodies.

Table 1: Existing hypotheses to explain DENV pathogenesis. For references see text.

Hypothesis	Mechanism	Result
ADE	Immune-complexes	Enhancement of infection of target cells
T-cell mediated	Cytokine production	Increased vascular permeability
Viral virulence	Highly virulent strains	Increased infection of target cells
Molecular mimicry	Autoimmune reactions	Hemorrhagic manifestations

Although ADE as an explanation for severe DENV associated pathogenesis has predominated DENV research for many years, recent studies have suggested that the pathogenesis of severe DENV infections is more likely to be multi-factorial, with virological, immunological and host factors contributing to the development of DHF/DSS.

Increased viral virulence has been associated with DHF/DSS. The most convincing evidence came from epidemiological studies of DENV-2 outbreaks. South East Asia has experienced severe outbreaks of DHF/DSS caused by virulent strains of DENV-2 (SE Asian genotypes) whereas DENV-2 epidemics in Latin America are mild with most cases presented as DF. The American genotypes of DENV-2 seem to be less virulent [62] whereas introduction of the virulent SE Asian genotypes into the Americas resulted in the introduction of DHF/DSS in this region [63;64]. Experimental studies have demonstrated that less virulent strains of DENV replicate less well in ADE assays [65], suggesting that those strains are less fit to sustain a DHF outbreak. In contrast to viral virulence, the implication of viral load during DENV infection has been contradictory. Some studies have found association with increased viral load and disease severity whereas others did not [66;67]. The role of magnitude of viremia in the pathogenesis of DENV remains to be clarified.

Increasing experimental evidence implicates DENV-specific T cells as mediators of immune-mediated plasma leakage resulting in DHF. Figure 7 illustrates a model for plasma leakage directly caused by the effects of cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) on vascular endothelial cells. According to this model, during secondary infection, memory DENV specific T cells are able to recognize DENV infected cells more rapidly than naïve cells, become activated in shorter time and secrete high amounts of the cytokines leading to increased vascular permeability and plasma leakage. In addition, ADE during secondary DENV infection results in higher amounts of viral antigens, readily available for T cell recognition, activation and subsequent cytokine production.

Molecular mimicry resulting in autoimmune reactions is an alternative to explain the pathogenesis of DHF and DSS. It has been demonstrated that a part of the DENV envelope protein, a 20-amino acid sequence, shares a sequence similarity with a family of clotting factors, including plasminogen and in addition, cross-reactive antibodies to plasminogen appeared during DENV infections [68]. These findings indicate that a relation between these cross-reactive antibodies and development of haemorrhagic manifestations may exist. In addition, antibodies to the NS 1 protein

have been found to cross-react with epitopes on human blood clotting factors and integrins and bind to endothelial cells [69].
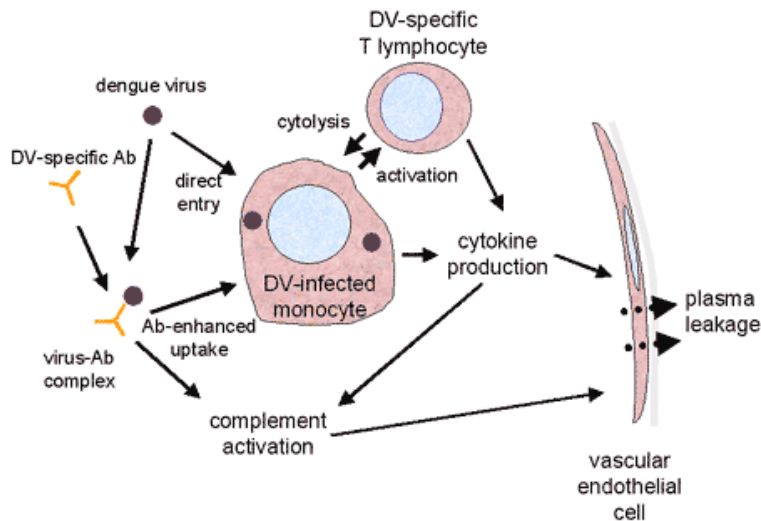

Figure 7: Model of T cell mediated immunopathogenesis in DHF leading to plasma leakage (http://www.umassmed.edu/cidvr/faculty/rothman.cfm).

These hypotheses are all supported by varying degrees of laboratory evidence. An interesting feature of DENV infections is the rare incidence of DHF and DSS when compared to the high incidence of DF. Moreover the identification of primary DHF and DSS during DENV epidemics as well as the fact that several individuals experience infections with more than one DENV serotypes during their lives without developing DHF, has underlined the fact that ADE is not always responsible for the development of DHF and DSS. In addition to the ADE hypothesis and the viral virulence factors, it is also reasonable to speculate that viral load in the infected individual may play an important role in the development of DHF and/or DSS. Also the genetic background of the infected individual may be crucial to determine the disease outcome of DENV infection. The lack of a suitable animal model for testing the immunological and viral virulence factors aspects *in vivo* has posed a major stumble block in the understanding of the pathogenesis of DENV infections.

In response to infection with DENV, macrophages, dendritic cells and other infected mononuclear cells, secrete vasoactive mediators, which cause vascular permeability and may lead to shock, the most important feature of severe DENV infection. It is hypothesised that a complicated network of cytokines and other mediators, involving TNF, interleukin-1 (IL-1), IL-2, IL-6 and platelet activation factor (PAF) as well as complement activation products such as C3a and C5a and histamine may be responsible for vascular permeability. During DENV infection, viral antigens are presented by infected cells, in the context of MHC antigens resulting in priming and stimulation of CD4+ and CD8+ T cells. As a consequence of T cell activation, is the production of several cytokines including IL-2, IL-4, IL-5 and IL-6, whereas infected macrophages produce TNF, PAF and IL-1 and IL-6. This complicated network acts synergistically, resulting in increased vascular permeability observed during DHF and

DSS [70]. In addition, the production of IFN-γ during DENV infection up-regulates the expression of Fc receptors and MHC expression, which results in increased numbers of DENV infected cells. These chain reactions and production of the cytokine cascade results in immunopathology seen in DHF and DSS (Figure 8). A shift has been demonstrated in the cytokine production from Th1 responses during DF towards Th2 responses during severe DSS [70]. The association of Th2 cytokine profiles and exacerbation of disease has been demonstrated in other viral infections such as RSV, HSV and HIV [71]. In a study that was conducted to determine the cytokine profiles during DENV infections, it was demonstrated that 71% of the cases with DSS had an immune response shifted towards the Th2 type [72]. Immunoglobulin class and subclass responses are in agreement with these observations. It has been demonstrated that patients that develop DHF and/or DSS have higher levels of IgA, IgE and IgG 4 serum antibodies than patients with DF or non-dengue patients [57;73]. IL-4, a potent Th2 cytokine, found elevated in DHF/DSS patients is also a cytokine associated with immunoglobulin class and subclass switch to the IgA, IgE and IgG 4 types [74].
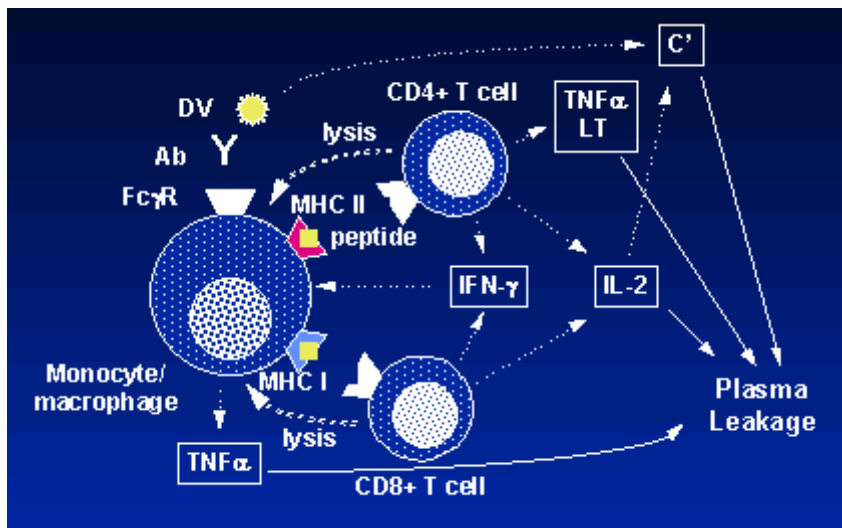
Figure 8: Cytokine production after DENV infection resulting in plasma leakage (www.nlv.ch)

Upon infection, DENV specific antibodies are rising a few days after onset of symptoms. In the absence of any previous infection or vaccination against a flavivirus, the infected individual will mount a high IgM response, whereas later in the course of DENV infection IgG antibodies will appear. On the contrary, secondary infection with DENV or after previous infection or vaccination with other flavivirus, the individual will mount an immune response of mainly the IgG isotype, with relatively low or even undetectable levels of IgM in 20-30% of the cases [37]. Upon infection with DENV, specific IgM serum antibodies may persist for up to eight months after infection, whereas IgG antibodies may be detectable for several years after infection. Independent of primary or secondary DENV infection, DENV specific IgM and IgG antibodies have neutralizing effect on the virus that circulates in the blood, therefore these antibodies play a role in virus elimination from the blood. DENV specific IgA antibodies are also present during acute phase DENV infection, however they disappear early in convalescence, approximately three to four weeks after infection [55;56]. Low levels of IgE serum antibodies have also

been measured during acute DENV infections. Patients that developed DHF and/or DSS were found to have significantly higher DENV specific IgE serum antibodies early in the acute phase of their disease that declined soon after the development of haemorrhagic complications [57].

EPIDEMIOLOGY OF DENGUE VIRUS INFECTIONS

The first DENV reports in medical literature date from the 18th century, although in some Chinese medical encyclopaedia there are reports of a DENV-like illness already several centuries before. DENV or DENV-like epidemics were reported throughout the 19th and early 20th century in the Americas, South East Asia, South Europe and several other parts of the world [5]. However, the prevalence of DENV-associated illness was minor and DENV was not considered a public health problem until the global prevalence of DENV increased dramatically in recent decades. It wasn't until 1953 when the first epidemic of DHF occurred in Manila, the Philippines [5]. In the following two decades DENV re-emerged and today is the most prevalent vector-borne disease worldwide [37]. Prior to 1970 only nine countries had experienced DHF epidemics, whereas today DF, DHF and DSS occur in more than 100 countries and territories (Figure 9) and threaten the health of more than 2.5 billion people living in urban, peri-urban and rural areas of the tropics and sub-tropics. Although there are over one million DENV infections reported every year, it is estimated that over 100 million cases actually occur annually [37]. DENV is endemic in Africa and Eastern Mediterranean, the Americas and the Caribbean, South East Asia and the Western Pacific. The major disease burden of DENV infections is in South East Asia and the Western Pacific, although the increased reporting of DENV from the Americas has raised concerns in the recent years.
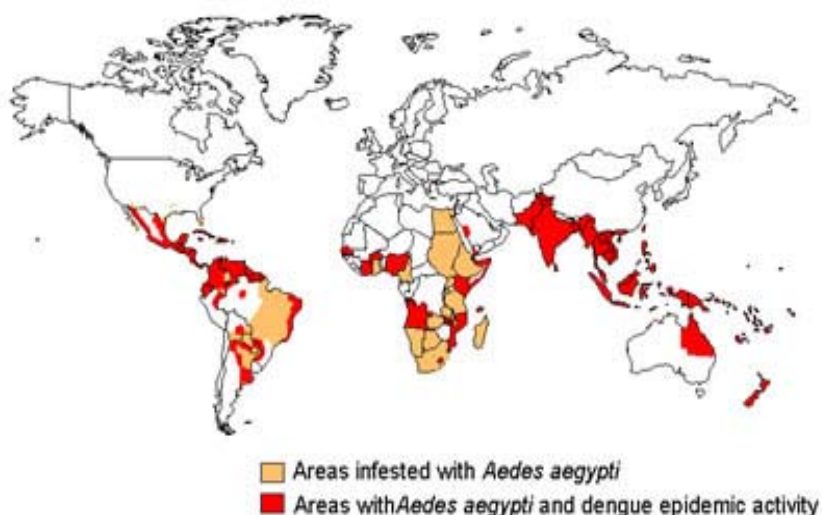

Figure 9: World distribution of DENV in the year 2005, highlighting the areas at risk for transmission of DENV (source: www.cdc.gov).

DENV is transmitted all year around in the tropics, although in most countries there is a seasonal increase in transmission during the hot rainy season. The possible explanation could be that mosquito survival increases with the increase of humidity. In addition, during heavy rainfalls mosquitoes fly less, remaining indoors, therefore increasing the chances for contacts with humans [75].

Introduction of new viral strains into a receptive population may be the most important factor for initiation of a DENV epidemic. When a new virus is introduced into areas free from DENV, transmission could be easily initiated causing a subsequent epidemic. However, once a DENV epidemic has started, several factors may influence the individual's risk in getting infected. Poor house screening, limited use of insecticides or slum housing and poverty have been associated with high infection rates during a DENV epidemic.

Molecular epidemiology of DENV infections

Nucleotide sequencing of the entire gene of the envelope protein has allowed the classification of DENV serotypes into a number of E genotypes, which are usually correlated with geographic distribution as seen in Table 2 [76-78]. Genetic changes in the virus population are seen within the same geographic region and are often results of mutation and selection or introduction of a new variant from a different region. Differences in the sequence homology between two virus strains circulating in the same region may be explained by the introduction of a new strain by a viraemic host into the region. Therefore, genotyping of viral strains is useful to determine the origin and spread of epidemics.

Table 2: Classification of DENV into genotypes according to nucleotide sequences of the E protein.

Virus	Genotype	Geographical distribution
DENV-1	Genotype I	Japan, SE Asia, S Pacific, Hawaii
	Genotype II	SE Asia, W Africa, the Americas
DENV-2	Genotype I	New Guinea, SE Asia, SE China, the Americas
	Genotype II	Asia, Africa, Saudi Arabia
	Genotype III	India, S Pacific, the Americas
	Genotype IV	W Africa
DENV-3	Genotype I	SE Asia, S Pacific
	Genotype II	Thailand
	Genotype III	Sri Lanka, India, Africa
	Genotype IV	S Pacific, the Americas
DENV-4	Genotype I	Indonesia, S Pacific, the Americas
	Genotype II	Thailand, Philippines

PREVENTION AND CONTROL OF DENGUE VIRUS INFECTIONS

Limited options are available for prevention and control of DENV infections. No vaccine is yet available for human use, therefore the only way in preventing DENV infection is to control contact with the mosquito vectors. On the other hand, prevention of DENV infections has become urgent with the wide geographic distribution and high incidence of the disease.

Mosquito control

Prevention and control of DENV currently relies on controlling the mosquito vector, however this option has several limitations. The most effective means for control of the *A. aegypti* is environmental management. This should include planning, organization, carrying out and monitoring activities for the modification or manipulation of environmental factors and prevention or reduction of vector propagation and contact between humans and vectors [37]. Improvement of water supply and storage, solid waste management and modification of artificial larval habitats are methods to be used for environmental management. An important point of focus for controlling the vector is destruction, alteration and disposal or recycling of artificial and natural larval habitats in each community.

Elimination of *A. aegypti* can be achieved by elimination of breeding sites, use of larvicides and perifocal spraying with insecticides. Spraying insecticides is not effective unless used indoors [79]. The most effective way to control mosquitoes is elimination or cleaning of water-holding containers that serve as the larval habitats for *A. aegypti* in the domestic environment [79].

It is very important that all programmes applied for mosquito control, to retain sustainability. Once the mosquito has been eliminated and transmission of DENV has been controlled it is important that the vector remains under control. If sustainability is not applied, the vector will rapidly re-emerge and so will the transmission of the disease, which could then reach epidemic levels. The "gold standard" in controlling *A. aegypti* would include a solid regional programme for reduction of larval source as well as participation on the community level for sustaining such programmes [37].

Vaccine development

The great impact that DENV infections have on public health and the fact that prevention is difficult to achieve through vector control have made the development of a DENV vaccine essential for long-term control and elimination of DENV infections. Vaccines for other closely related flaviviruses, such as yellow fever, Japanese encephalitis and tick borne encephalitis viruses, are available but no safe and effective vaccine has yet been developed for DENV. The unique features of DENV, namely the existence of four closely related but still antigenically distinct serotypes and the observation that subsequent infection with a different serotype may be of increased severity, have made the development of a safe and effective vaccine against DENV infection a difficult task.

Epidemiological studies have suggested that antibodies are the main protective mechanism against DENV infection; therefore antibody responses are used as the "gold standard" to assess the validity of any candidate DENV vaccine[80;81]. On the other hand, the most widely accepted theory for the pathogenesis of severe DENV infections, the "ADE hypothesis", has made the development of DENV vaccines a challenge. To be in agreement with this theory a candidate DENV vaccine should be able to induce high-titre protective antibodies against all four DENV serotypes, in order to protect from

DENV infection and avoiding the risk of DHF/DSS induction. Nevertheless, the borderline between protective immunity and disease enhancing immunity is not well defined. In addition, the lack of a suitable animal model of DENV-associated disease to test candidate vaccines makes the development and evaluation of DENV vaccine even more difficult [82].

The current approaches in DENV vaccine development include live attenuated vaccines, killed vaccines, subunit and DNA candidate vaccines (Table 3 and for review [83-85]). The Mahidol University group in Thailand and the Walter Reed Army Institute of Research in the USA have developed live attenuated tetravalent DENV candidate vaccines [86;87]. Attenuation of the virus was achieved through serial passages of the virus in primary dog kidney cells (DENV 1, 2 and 4), or in primary African green monkey kidney cells (DENV-3) following the hypothesis of Sabin and Schlesinger [88]. The passage history of each monovalent vaccine differs between the preparations of the different institutes. Monovalent, bivalent and tetravalent vaccine formulations of the vaccine produced at the Mahidol University have been tested and evaluated in adult Thai volunteers whereas a tetravalent formulation was tested in Thai children. Clinical trials of these vaccine formulations revealed that the vaccines are immunogenic with acceptable levels of adverse reactions [89;90]. The vaccine formulation prepared at the Mahidol University, although proved to be immunogenic in clinical trials in adult American volunteers, was associated with increased reactogenicity [91]. Ongoing investigations will reveal the causes of those adverse reaction to vaccination.

Table 3: Summary of DENV candidate vaccines that were shown to be immunogenic in the respective animals that were tested.

Type of vaccine	Virus/Antigen	Tested in animals	Clinical status	Reference
Whole virus inactivated	DENV-2	Mice, non-human primates	Phase I	[92]
Subunit	DENV-2 Domain III part of NS1	Mice		[93]
Subunit	DENV-2 E protein	Mice		[94]
Subunit	DENV-2 E protein	Non-human primates		[95]
Subunit	DENV-4 C-M-E-NS1	Mice, non-human primates		[96;97]
DNA	DENV-2 NS1	Mice		[98]
Live attenuated	DENV-2	Non-human primates		[95]
Live attenuated	tetravalent	Non-human primates	Phase I	[87];[86]

Attempts were made to develop subunit vaccines against DENV infections, despite the fact that subunit vaccines are considered to have poor immunogenicity, inducing low-level antibody and T cell responses. Recombinant DENV E and NS-1 proteins have been generated using a baculovirus expression system and have been tested in mice with varying success [97;99-101]. The purity of

recombinant proteins to be used in subunit vaccines is a problem that needs to be solved. It has been observed that contaminated recombinant DENV proteins can suppress or even divert the DENV specific T cell responses (Bielefeldt-Ohmann, unpublished data).

The DNA vaccine approach has also been considered from some investigators for DENV. DNA vaccines have the advantage of a long-term antigen source in the vaccinee, however in some instances they induce low levels of antibody responses [102].

Aim of the thesis

DENV infections are endemic in essentially all tropical parts of the world sometimes co-endemic with other (closely-related) flaviviruses. A key issue in studying DENV infections in humans is the availability of a diagnostic test with high degree of sensitivity and specificity. In Chapter 2 we aimed to compare several commercially available diagnostic tests to accurately diagnose DENV infections and define the sensitivity and specificity of several currently available assays. Since the limitations of these tests became obvious an alternative test was developed for accurate, timely and easy diagnosis of DENV infections.

Characterization of humoral immune responses of acute infections such as DENV infections is crucial in understanding the pathogenesis of DENV infections and the mechanisms of the humoral arm of the immune system associated with disease severity. Little is known about the nature of antibody responses during DENV infections further than classical IgM and IgG responses and neutralizing antibodies. In Chapter 3 we studied humoral immune responses in patients with DENV infections of varying disease severity. In addition to adaptive humoral immune responses, the effect of soluble VCAM-1 a molecule associated with plasma leakage, was measured in patients with varying disease severity.

Finally, for elucidating the pathogenesis of DENV infections, the need of an appropriate animal model is obvious. In the absence of such a model, neither existing nor newly proposed hypotheses can be tested to explain the pathogenesis of DENV-associated disease. In addition, candidate vaccines could not be tested and may reach clinical trials too early. Chapter 4 describes the evaluation of an animal model to study DENV infections and the application of such a model in evaluation of a candidate live attenuated tetravalent DENV vaccine.

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Chapter 2

Laboratory diagnosis
of
dengue virus infections

Chapter 2.1

Evaluation of Six Immunoassay Systems for the Detection of Dengue Virus Specific IgM and IgG Antibodies

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ABSTRACT

The performance of six commercially available immunoassay systems for the detection of dengue virus specific IgM and IgG antibodies in serum was evaluated. These included two IgM and IgG enzyme immunoassays (EIA) from MRL Laboratories and PanBio, a rapid immunchromatographic test (RIT) from PanBio, immunofluorescence assays (IFA) from Progen, a Dot-blot assay from Genelabs and a dipstick EIA from Integrated Diagnostics (INDX). For this study a panel of 132 serum samples were used including 90 serum samples from patients suspected of a dengue virus infection and 42 serum samples from patients with other viral infections. In addition, serial serum samples from two monkeys experimentally immunized and challenged with dengue virus type 2 were used. Results were considered conclusive when a concordant result was obtained with four out of the six (4/6) antibody specific assays. Based on this definition the calculated overall agreement for the human serum samples for the respective IgM immunoassays was 97% (128/132) with 34% (45/132) positive serum samples, 63% (83/132) negative and 3% (4/132) samples with a discordant result. The calculated overall agreement for the IgG assays was 94% (124/132) with 49% (65/132) positive, 45% (59/132) negative and 6% (8/132) discordant results, respectively. The sensitivities of the evaluated dengue virus specific assays varied between 71%-100% for IgM and 52%-100% for IgG with specificities of 86%-96% and 81%-100%, respectively. The relative sensitivities of the respective IgM assays measured with the monkey serum samples were comparable with those obtained with twelve serial serum samples from humans. The overall performance was based on the sum of the agreement, sensitivity, specificity and *Kappa* statistics of the IgM and IgG immunoassays and showed that the antibody detection systems from INDX, Genelabs, MRL EIA and the PanBio EIA are useful and reliable assays for dengue virus serodiagonsis.

INTRODUCTION

Dengue virus (DENV) infections are among the most common arthropod borne infections in tropical and subtropical areas. The four serotypes DENV 1, DENV 2, DENV 3 and DENV 4 are transmitted by several mosquito's species including *Aedes aegypti* and *Aedes albopictus*. At least 50 million people are infected with any of the four serotypes annually (3). The majority of the DENV infections are asymptomatic or cause mild dengue fever (DF), characterized by 'flu-like' symptoms including fever, chills, headache and myalgia. Rash, lymphadenopathy, arthralgia or myalgia usually follows these initial symptoms. In some cases the infection may lead to the more severe dengue hemorrhagic fever (DHF) with plasma leakage. Usually also conjunctival suffusion, facial flushing and truncal erythema are then present. The usually lethal dengue shock syndrome (DSS) may follow DHF after circulatory collapse (6,8).

Differential diagnosis may be important in order to distinguish DF from influenza, measles, rubella, other arthropod-borne viral infections, malaria and other hemorrhagic fevers (15). Therefore, a good laboratory diagnosis is important. The classic hemagglutination inhibition (HAI) assay and virus neutralization assay are still widely used, despite their tedious nature (1,4,9). Recently other immuno systems for the diagnosis of DENV infection have become commercially available. Among these are enzyme immunoassays (EIA), immunochromatographic assays, and a dot-blot assay (2, 10, 11, 12, 13, 16). Differences in assay format, usage of antigen and detection system makes it difficult to estimate the value of each individual assay without proper comparison. This prompted us to evaluate six commercially available immuno assay systems for the detection of DENV specific IgM and IgG antibodies. Ninety serum samples, both single and serially collected from European and Asian patients suspected of acute DENV infections and 42 serum samples from Dutch patients with confirmed viral infections other than DENV infections were used to evaluated eleven different assays from five companies. In addition serial serum samples from experimentally vaccinated monkeys and subsequently challenged with DENV 2 were used to study their antibody kinetics in the respective assays.

MATERIALS AND METHODS

Human serum samples. A panel of 132 human serum samples from DENV suspected patients and patients with other viral infections were included in this study. Serial serum samples were collected from patients suspected of an acute DENV infection, living in DENV endemic areas. Thirteen patients from Curaçao, seven paired samples and six single serum samples (n=20), six patients with paired samples (n=12) and 12 patients with serial samples (n=36) from Indonesia were included. Serum samples from patients with suspected primary DENV infections (n=22) were collected from Dutch travelers: 16 single serum samples and three paired samples (n=6). As controls, serum samples from patients with other viral infections confirmed by the detection of specific IgM antibodies were used. These included sera with specific IgM antibodies to Epstein-Barr virus (EBV) (n=5), cytomegalovirus (CMV) (n=8), Yellow Fever virus (YFV) (n=4), varicella zoster virus (VZV) (n=8), herpes simplex virus (HSV) (n=6) and tick-borne encephalitis virus (TBEV) (n=2). Eight samples from chronically infected patients with hepatitis B virus (HBV) (n=8) were also included. All samples had been collected between 1993 and 1998 and stored at -20 °C until use.

Monkey serum samples. Serum samples from two cynomologous monkeys (*Macaca fascicularis)* experimentally immunized with live attenuated DENV 2 vaccine and subsequently challenged with homologous wild DENV 2 virus, as previously described, were included in this study (14). Serum samples were collected at different times after immunization and challenge and stored at -20 °C until use.

IgG and IgM assays. The characteristics of the respective immuno assays are depicted in table 1. Included in this evaluation are two Enzyme Immuno based Assay (EIA), an Immuno Fluorescence Assay (IFA), a Rapid Immunochromatographic Test (RIT), a DipStick EIA and an Immunoblot Assay (blot). The MRL EIA (MRL Diagnostics, Cypress, CA, USA) and the PanBio EIA (PanBio, Brisbane, Australia) are both based on indirect systems for the detection of IgG serum antibodies using microwell plates coated with the DENV 1-4 antigens. The detection of IgM serum antibodies for both these EIAs is based on an IgM capture system followed by an incubation with DENV 1-4 antigens and virus specific monoclonal antibodies conjugated with horseradish peroxidase. The assay times for these EIAs are two hours for the detection of IgG serum antibodies with MRL EIA and one hour for the detection of IgG serum antibodies with the PanBio EIA and for the detection of IgM serum antibodies the assay time is four hours with the MRL IgM and two and half hours for the PanBio IgM. The Pan Bio RIT is a rapid (seven minutes) assay based on a capture principle for the detection of IgM and IgG serum antibodies followed by an incubation with a mixture of DENV 1-4 antigens and a gold-labelled DENV-specific monoclonal antibody. The IFA from Progen (Progen Biotechnik, Heidelberg, Germany) is based on an indirect system for the detection of both IgM and IgG serum antibodies, using IFA slides coated with DENV 2 antigen. To detect DENV specific IgG antibodies a Goat-anti-Human IgG-FITC conjugate (DAKO, Glostrup, Denmark) was used. To detect DENV specific IgM antibodies, the IgG-FITC conjugate was replaced by a Rabbit-anti-Human IgM-FITC conjugate (DAKO, Glostrup, Denmark). Prior to the detection of DENV specific IgM antibodies by IFA, serum samples were pre-treated with Gull-sorb (Gull Laboratories, Salt Lake City, USA) to remove IgG antibodies. The total assay time for detection of IgG serum antibodies is 90 minutes and for IgM detection is two hours. The INDX DipStick EIA (Integraded Diagnostics, Baltimore, MD, USA) is based on an indirect system for the detection of both IgM and IgG serum antibodies. In this assay nitrocellulose membrane is coated with DENV 2 antigen and binding antibodies are detected with anti human IgM or IgG conjugate labelled with alkaline phosphatase. The assay times for detection of IgM and IgG serum antibodies are 90 and 45 minutes respectively. Finally the Genelabs blot (Genelabs Diagnostics, Singapore) is based on nitrocellulose membranes coated with DENV 1-4 antigens and binding IgG antibodies are detected using a peroxidase labelled protein A conjugate. IgG results were obtained within two and a half hours. For the detection of IgM serum antibodies the nitrocellulose membranes are coated with anti-human IgM. After the binding of IgM antibodies the membranes are incubated with a mixture of DENV 1-4 antigens followed by incubation with a DENV-specific monoclonal antibody and a rabbit anti-mouse Ig peroxidase labelled conjugate and developed with chloro-naphthol as substrate. The minimum assay time is eight hours although the manufacturer recommends an overnight incubation with the antigen. Monkey serum samples were analyzed for both IgM and IgG in the appropriate assays according to the procedures described by the manufacturer, with modifications described below. In the Progen IgM respectively IgG IFA an anti monkey IgM or IgG FITC conjugate was used (DAKO, Glostrup, Denmark). In the Genelabs IgG blot the anti-human IgG conjugate was replaced by an anti-monkey IgG HRPO (Sigma Chemical, St. Louis, USA). It was not possible to detect IgG antibodies in the monkey sera with the PanBio RIT.

Calculation, statistics and ranking. The overall agreement, sensitivities and specificities of the respective assays were determined in relation to the consensus values as the "gold standard" (17). Results were considered to be "true values" (consensus values) when a concordant result was obtained from at least four out of the six assays. When three of the six assays were positive the result was defined as discordant. *Kappa* statistics were used to evaluate the measure of agreement between the consensus value above the results expected by change. If the agreement reach a κ-value of 1, indicate a good agreement while a κ-value of 0 indicate no agreement (7).

Table 1: Characteristics of IgG and IgM assays for the detection of DENV antibodies

Company	Type of Assay	Antigen	Principle	Serum dilution	Total assay Time (minutes)
MRL Diagnostics Cypress, CA, US	IgM EIA IgG EIA	DENV 1-4 DENV 1-4	Capture Indirect	1:101 1:101	240 120
PanBio Brisbane, Australia	IgM EIA IgG EIA IgM/IgG RIT	DENV 1-4 DENV 1-4 DENV 1-4	Capture Indirect Capture	1:100 1:100 Undiluted	150 60 7
Progen Biotechnik, Heidelberg, Germany	IgM IFA IgG IFA	DENV-2 DENV-2	Indirect Indirect	1:16 1:16	120 90
Integrated Diagnostics (INDX), Baltimore, MD, USA	IgM Dipstick EIA IgG Dipstick EIA	DENV-2 DENV-2	Indirect Indirect	1:200 1:200	90 45
Genelabs Diagnostics, Singapore	IgM Blot IgG Blot	DENV 1-4 DENV 1-4	Capture Indirect	1:100 1:100	480 210

Ranking of each assay group (IgM and IgG) is determined by the sum of the calculated κ-value, overall agreement, sensitivity, and specificity. The overall ranking of each diagnostic system is determined by calculating the sum of the IgM and IgG ranking.

RESULTS

The overall agreement between all the six immuno-assays for the detection of specific IgM and IgG antibodies against DENV in the human sera are summarized in table 2. Of the 132 samples tested the overall agreement resulted in 45 (34%) positive, 83 (63%) negative and 4 (3%) discordant for DENV IgM serum antibodies as well as 65 (49%) positive serum samples, 59 (45%) negative serum samples and 8 (6%) discordant samples for DENV IgG serum antibodies

Table 2: Overall agreement of all the respective DENV specific IgM and IgG immunoassays according to the consensus model.

Result	IgM		IgG	
	Numbers	%	Numbers	%
Positive[a]	45	34.1	65	49.2
Negative[b]	83	62.9	59	44.7
Discordant[c]	4	3.0	8	6.1

[a] Positive is defined as at least 4 out of six assays positive

[b] Negative is defined as at least 4 out of six assays negative

[c] Discordant is defined as 3 out of six assay positive and 3 out of six assays negative

The results of the individual assays with respect to the agreement, sensitivity, specificity and κ-value using the consensus value as golden standard are summarized in table 3. In general the measure of

agreement between the six IgM assays was good (κ = 0.884 to κ = 0.966). The overall agreements of the respective IgM assays varied between 88% to 98% for the PanBio EIA (κ = 0.912) and PanBio RIT (κ = 0.966). Calculation of the sensitivities resulted in 100% sensitivity for both the PanBio RIT IgM and Genelabs blot IgM to 71% for the Progen IgM IFA. Calculation of the specificity's resulted in values varying from 86% for the INDX EIA and 96% for the PanBio EIA.

The overall agreement between the DENV specific IgG assays varied between 75%-98% for the INDX EIA (κ = 0.913) and the PanBio RIT (κ = 0.803), respectively. The measure of agreement between the respective DENV IgG immunoassays was relatively good (κ = 0.785 to κ = 0.917). High sensitivities of 100% and 97% were obtained with the MRL IgG EIA, PanBio IgG EIA and INDX IgG EIA, whereas the sensitivities of the Genelabs IgG blot, Progen IgG IFA and PanBio IgG RIT were respectively, 85%, 77% and 52% compare to the consensus value. Calculations of the specificities of these IgG assays resulted in values between 86%-100%.

Figure 1 presents the results of the performance of the six different immunoassays for the detection of DENV IgM antibodies measured in serial serum samples from 12 patients suspected of an acute DENV infections at different times after onset of clinical symptoms. During the acute phase in 7 patients DENV–specific IgM antibodies could be detected with the Genelabs blot, whereas in the MRL EIA, PanBio EIA and the INDX 5, in the PanBio RIT 6 and in the Progen IFA 4 patients were positive. During the early convalescent phase IgM was detected in the Genelabs blot in 9 patients out of 12, in the MRL EIA and PanBio RIT 8, in the PanBio EIA and the INDX 7 and in the Progen IFA 4. In the convalescent phase in 9 patients DENV specific IgM antibodies were detected with the MRL EIA and the Genelabs blot. Eight patients were positive for the presence of DENV-specific IgM antibodies in the PanBio EIA, PanBio RIT and the INDX. In the Progen IFA seven patients were positive.
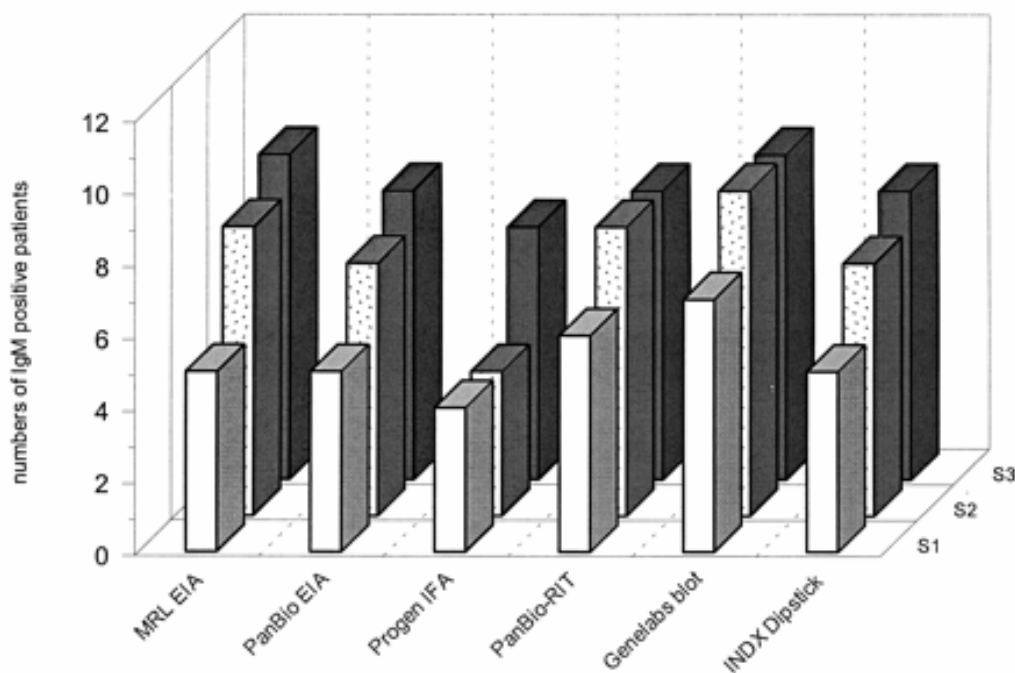

Figure 1: Results of DENV-specific IgM detection of six different assays in sera from 12 patients suspected of an acute DENV infection at different time points after onset of clinical manifestation. S1: serum sample in the acute phase; S2: Serum sample in the early convalescent phase; S3: serum sample in the convalescent phase.

Detection of DENV virus specific IgM antibodies in the non-DENV group varied between 0 to 6 positive samples out of 42 tested serum samples. DENV specific IgM serum antibodies were detected in two samples (VZV) using the PanBio RIT, in three samples (2 HBV and 1 VZV) using the MRL IgM EIA, in four samples (2 HBV and 2 VZV) using the PanBio IgM EIA and in six samples (1 CMV, 1 HBV, 2 HSV, 1 VZV and 1 TBEV) using the INDX IgM EIA. DENV-specific IgM serum antibodies were not detected using the Progen IgM IFA and Genelabs IgM blot. Serum antibody reactivities of the 42 non-DENV serum samples measured with the DENV specific IgG assays show four positive samples with the MRL IgG EIA (1 CMV, 1 TBEV and 2 VZV), three positive serum samples using the PanBio IgG EIA (1 TBEV and 2 VZV), five positive samples in the Progen IgG IFA (2 CMV, 2 TBEV and 1 VZV), two positive VZV in the INDX IgG EIA and three positive reactions in the Genelabs IgG blot (1 CMV, 1 EBV and 1 VZV). Al the non-DENV serum samples were negative with the PanBio IgG RIT.

Figure 2 presents the IgG and IgM antibody kinetics to DENV 2 in monkeys experimentally vaccinated and subsequently challenged with DENV 2 measured with different assays. In the monkeys E1 and E2 the IgM antibody kinetics measured with the quantitative IgM EIAs from MRL and PanBio showed identical patterns after immunization and challenge, although the ratios measured with the IgM MRL EIA were slightly lower. In sera from monkey E1 the MRL EIA, the PanBio EIA and the PanBio RIT IgM antibodies were detected on day 14, whereas the INDX Blot and the Progen IFA became positive on day 21. The PanBio RIT, PanBio EIA IgM and the MRL IgM EIA remained positive for IgM antibodies during the whole period. The EIAs showed a gradual decrease until 3 to 6 days after challenge with homologous DENV 2 virus. In sera from monkey E2 lower IgM antibody response were detected as shown by the EIAs. The Progen IFA remained negative for IgM antibodies during the whole period.

DENV specific IgG antibodies were detected only after challenge with homologous DENV-2 virus in all assays except the Progen IFA, which was already positive on day 21 after immunization and remained positive during the whole period.

DISCUSSION

Recently, a number of DENV specific immunoassays have become available for the detection of IgM and IgG antibodies in serum varying from dipstick based assays to more sophisticated enzyme linked immunoassays. Some of these immunoassays have been evaluated in different studies (16, 10, 2). We have evaluated eleven DENV immunoassays comprising 6 different systems for the detection of IgM and IgG. Based on a consensus model using serum sample from DENV suspected patients and non-DENV patients the performance of each assay was validated. In addition, the relative sensitivity of the respective assays was studied with serial serum samples from monkeys experimentally infected with DENV-2 virus followed by homologous challenge with wild-type 2 DENV virus (14).

In general, all assays were easy to perform, but the most simple and fastest assay to perform is by far the PanBio RIT. The results of PanBio RIT are available in less than 10 minutes and both IgM and IgG antibodies are detected simultaneously.

Table 3: Agreement, specificity, sensitivity and ranking of DEN virus antibody assays based on the consensus value

Assay	IgM				IgG				Ranking
	Agreement	Sensitivity	Specificity	κ-value	Agreement	Sensitivity	Specificity	κ-value	
MRL EIA	91	96	91	0.930	92	100	88	0.830	4
PanBio EIA	88	87	96	0.912	96	100	98	0.917	2
PanBio RIT	98	100	92	0.966	75	52	100	0.803	5
Progen IFA	83	71	89	0.884	82	77	86	0.785	6
INDX EIA	92	96	86	0.904	98	97	98	0.913	3
GeneLabs blot	95	100	92	0.950	88	85	95	0.847	1

Except the Genelabs blot and the Progen IFA IgM assays and the PanBio RIT for detection of IgG antibodies all the other DENV immunoassays detected non-specific DENV IgM and IgG serum antibodies. These reactions were mainly found in-patients with a CMV, EBV and VZV infection. In contrast to a previous study showing no DENV IgM reactivity in serum samples from VZV, CMV and EBV patients using the INDX dipstick EIA and a home-made DENV specific IgM capture EIA (14). Although the non-DENV serum panel was carefully selected, using serum samples from patients in The Netherlands where no flaviviruses are circulating, the presence of flavivirus specific IgG antibodies can not completely be ruled out. The TBEV and YFV vaccination status of the patients as well as their history of possible flavivirus infections was not available. DENV virus IgM and IgG reactivity was detected with several assays in serum samples from patients with a TBEV infection. These flavivirus cross-reactivities are in agreement with several other studies clearly showing DENV virus antibody reactivity in patients with YFV and Japanese encephalitis virus infections (16, 2, 5).

Serial serum samples from patients were used to evaluate the respective DENV IgM assays in the acute, early convalescent and convalescent phase of disease. The results with these serum samples clearly showed the best performance of the Genelabs IgM blot assay and a strong under performance of the Progen IgM IFA in the acute and early convalescent phase. Using serial serum samples from humans with a DENV infection the estimated time-point after infection is variable. Therefore, we used serial serum samples from monkeys experimentally vaccinated and subsequently challenged with wild-type DENV 2 to study the relative sensitivity of the IgM assays. Despite the fact that the monkeys were vaccinated with DENV 2, which is the only virus present in the Vero cells coated on the Progen slides IgM antibodies could not be detected after vaccination of the monkeys. The Progen IgG IFA on the other hand seems to be more sensitive, compared to the other assays (figure 2). Therefore, it seems not clear yet why the Progen IgM is performing less well compared to the other assays. In general, the results obtained with the serial samples from humans, in particular for the Progen IgM, are in agreement with the relative sensitivity measured in the serial samples from the monkeys. Well-defined serial serum samples from experimentally infected animals, like the monkey samples in this study, clearly show the value of these samples. Since the results of these well-defined monkey samples are not influenced by pervious infections or vaccinations and geographical background. These samples may also contribute to the composition of quality control panels for flavivirus serology.

The consensus model resulted in an overall agreement of 45 IgM and 65 IgG DENV positive serum samples, 83 IgM and 59 IgG DENV negative serum samples and 4 IgM and 8 IgG DENV discordant serum samples. On basis of this consensus model the calculated sensitivities of the evaluated DENV immunoassays varied between 71 to 100% for the respective IgM assays, 52% to 100% for the respective IgG assays with specificities of 86% to 92% for IgM assay and 86% to 100% for IgG assays.

The variation in sensitivity and specificity are comparable with previous published data (2, 5) and might be caused by the different principles of the assays, different antigens, conjugates (table 1) and the selection on the respective serum panels. In a multi-center evaluation using a commercial DENV IgM dot assay it was shown that the sensitivities varied between 80% to 98% depending on the serum samples of the respective collaborating centers (10).
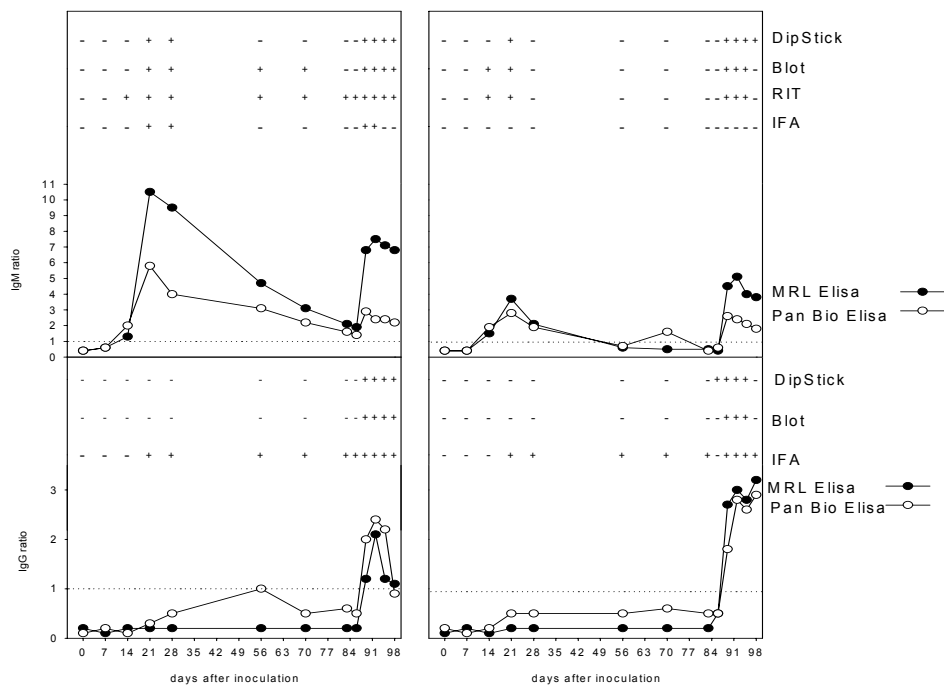
Figure 2

Figure 2: Results of DENV-specific IgM and IgG detection of several assays in sera from two monkeys at different time point after experimental immunization (day 0) and subsequent challenge (day 84) with homologous DENV-2 virus. Left graphs Monkey E2; Right graphs: Monkey E1; Lower panel: IgG results; Upper panel: IgM results. The cut-off value (ratio of 1) is indicated by a dotted line. The respective assays are indicated on the right.

Taken together we conclude that the best complete DENV IgM and IgG detection systems are the INDX Dipstick EIA and PanBio EIA followed by the MRL EIA and Genelabs blot, whereas the PanBio RIT and Progen IFA perform less well. If separated assays are selected to perform the DENV diagnostic in the laboratory a combination of the PanBio RIT for IgM detection and PanBio IgG EIA would be the most sensitive and specific combination. The PanBio RIT assay seems to be in favour for bedside diagnostic and fieldwork, because of its high sensitivity combined with a relative high specificity for IgM, ignoring the simultaneously obtained IgG results. In our view, for laboratories with a relative high workload of DENV samples a combination the of MRL IgM EIA and the PanBio IgG EIA could be a good choice, since both assays can easily be automated. The commercially available DENV immunoassay systems offer a good alternative for homemade DENV assays including HAI and EIA based systems. These commercial assays make the sero-diagnosis of DENV infection available to general and peripheral laboratories. However, for the isolation, molecular diagnosis and determination of DENV specific neutralizing antibodies, reference laboratories will continue to play an important role, which will also be the case for immunopathogenic, epidemiological and vaccine studies.

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Chapter 2.2

Reactivity of serum samples from patients with a Flavivirus infection measured by IFA and ELISA

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ABSTRACT

Flavivirus infections are a significant public health problem, since several members of the *Flaviviridae* family are highly pathogenic to humans. Accurate diagnosis and differentiation of the infecting virus is important especially in areas where more Flaviviruses are circulating. In this study we evaluated a newly developed commercially available immunofluorescence assay (IFA) (INDX, Baltimore, USA) for the detection of IgM and IgG antibodies against dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV). The IFA was compared with standard diagnostic enzyme immunoassays (EIAs) specific for the detection of IgM and IgG antibodies against these viruses. Forty-seven serum samples from patients with a defined Flavivirus infection were tested. As controls, serum samples from individuals with antibodies against tick-borne encephalitis virus, Hepatitis C virus and healthy individuals were included. The results obtained from this study indicated that the IFA showed a significant better discrimination for Flavivirus specific IgM antibodies compared to the standard IgM specific EIAs (the overall cross reactivity varied between 4-10% by IFA and 30-44% by EIA for the respective viruses). In contrast, the detection of Flavivirus specific IgG antibodies showed high cross-reactions in both the IFA and the EIAs (overall cross reactivity 16-71% and 62-84% respectively). This study clearly stated the complexity of Flavivirus diagnosis, showing that one cannot rely on one assay or search for one virus only. The Flavivirus IFA is a useful tool for the identification of Flavivirus infections during the acute stage of disease. In particular, IFA can be an important diagnostic tool for testing samples from travellers that have accidentally been exposed to these viruses.

INTRODUCTION

Arboviral infections have gained considerable importance in international public health during the last decades, mainly due to their worldwide distribution and their merit in morbidity and mortality among human populations [1]. Today over 70 Flaviviruses have been identified, belonging to the Arbovirus group, including four serotypes of dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) [2]. Flaviviruses and their mosquito vectors are prevalent globally and in many regions several Flaviviruses are endemic, i.e. DENV and JEV are both endemic in Southeast Asia [3], DENV and YFV are endemic in South America [4], whereas DENV, YFV and WNV are present in Africa [5].

Laboratory diagnosis of Flavivirus infections is based on isolation of the virus, detection of viral RNA using reverse transcription polymerase chain reaction (RT-PCR), neutralization test (NT) and antibody detection. Detection of viral RNA with RT-PCR has been described for several Flaviviruses [6, 7], however these assays can only be performed in reference laboratories. NT although the assay of choice for many Flaviviruses, since it is type specific [8, 9], is time consuming and like RT-PCR, can only be performed in reference laboratories. Detection of IgM and IgG serum antibodies based on EIA is widely used for routine serodiagnosis of Flavivirus infections. Today several commercial immunoassays are available for Flavivirus diagnosis, in particular for DENV, JEV and tick-borne encephalitis virus (TBEV) [10, 11, 12]. The majority of the Flavivirus diagnosis is based on in-house IFA and EIA systems [13, 14]. Flaviviruses contain cross-reactive epitopes, which poses a problem in serological diagnosis of these viruses [3], especially in regions where several Flaviviruses are present. This also implicates that specimens should be tested against different Flaviviruses to ensure accurate diagnosis. Therefore, there is a strong need for routine laboratories, for rapid and easy to perform assays, with high specificity and sensitivity against the respective Flaviviruses for routine and epidemiological studies.

Recently there has been a rapid growth of commercially available assays for Flavivirus diagnosis. The aim of this study was to evaluate the complexity of Flavivirus serology using commercial as well as in-house developed immunoassays.

MATERIALS AND METHODS

Serum samples: A panel of 75 human serum samples from patients with Flavivirus infections and healthy individuals was used in this study. The samples obtained from the Flavivirus infected individuals, were previously diagnosed in various laboratories, described in various publications and characterised as DENV, YFV, JEV WNV or TBEV cases. The characteristics of these samples are summarised in table 1. Briefly, the panel included serum samples from 13 patients with DENV infection (six patients had a primary DENV infection and seven patients had secondary DENV infection according to the criteria set by WHO [15]), 10 patients with YFV virus infection, 12 patients with WNV infection, 12 patients with JEV infection, five patients with TBEV infection and 10 individuals that had received TBEV vaccine. As controls, serum samples from patients with Hepatitis C virus (HCV) infection (n=8) and from healthy individuals (n=5) were used. All samples had been tested positive by means of EIA and/or NT and/or PCR for the above mentioned viruses for diagnostic purposes prior to inclusion in the study. All samples had been collected between 1997 and 2001 and stored at –20°C until use.

Table 1: Characteristics of the serum panel used in the study.

Flavivirus infection	No of samples	Confirmation	Origin of samples (n)	Patient history [Ref]
DENV	13	EIA	Dutch (9)	travellers[11]
			Malaysian (4)	unknown
YFV	10	EIA	Dutch (5)	vaccinees
		NT	African (5)	patients [16]
WNV	12	EIA	African	unknown [17, 5]
JEV	12	EIA	Dutch (4)	travellers
			Asian (8)	unknown
TBEV	15	EIA	German (10)	vaccinees
			Lithuanian (5)	patients [12]
HCV	8	blot	Dutch	chronic patients

Serology

IgM EIAs: For the detection of DENV IgM serum antibodies a commercially available kit was used (Focus Technologies, Cypress CA, USA) and performed according to the procedures described by the manufacturer. For the detection of WNV, YFV and JEV IgM serum antibodies, in-house capture EIAs were used for each virus [5]. Briefly, commercially available 96-well plates coated with rabbit anti-human IgM antibodies (Meddens Diagnostics, Vorden, The Netherlands) were blocked with 5% (w/v) skim milk (ELK Campina, Eindhoven, The Netherlands) in PBS for 30 min at 37° C. Serum samples were diluted 1:100 in PBS containing 0.2% (w/v) milk, 0.1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo, USA), 1% (v/v) fetal bovine serum (FBS) (ICN Biochemicals Inc, Ohio, USA), 5% (v/v) normal rabbit serum (DAKO, Glostrup, Denmark) and 5% (v/v) normal goat serum (ICN Biochemicals Inc) (EIA-buffer) and added to the plates. After one hour incubation at 37° C, unbound antibodies were washed away and the respective viral antigen was added to the plates. WNV antigen was prepared after cultivation of the virus on Vero cells and treatment of the infected cells with 1% Triton X-100. YFV antigen was obtained from CDC, Fort Collins, USA and JEV antigen is commercially available (Denka Seiken Co, Tokyo Japan). Viral antigens were diluted in EIA buffer supplemented with 5% (v/v) normal human serum (NHS) and incubated overnight at 4^oC. After washing the plates to remove excess of antigen commercially available affinity purified monoclonal antibodies against WNV YFV and JEV (PanBio INDX Inc, Baltimore, USA, Chemicon International, CA, USA and Trop Bio, Queensland, Australia, respectively) were diluted in EIA+NHS buffer and added to the plates. After one hour incubation at 37^oC plates were washed and incubated for an additional hour with a goat anti-mouse peroxidase labelled conjugate (DAKO). Plates were washed to remove excess of conjugate before they were developed with 2,2,4,4 tetramethylbenzidine (TMB) for 15 minutes at room temperature. The reaction was stopped by adding 100µl of 1N H_2SO_4 and the extinction of the samples was read at 450 nm (with 620 nm reference filter).

IgG EIAs: For the detection of DENV IgG serum antibodies a commercially available kit was used (Focus Technologies, Cypress CA, USA) and performed according to the procedures described by the manufacturer. For the detection of WNV, YFV and JEV IgG serum antibodies, in-house indirect EIAs were developed for each virus. Briefly, medium binding plates were coated with WNV, or YFV or JEV antigens overnight at room temperature. Unbound antigens were washed away and serum samples diluted 1:100 in EIA buffer were added on the plates. After incubation for one hour at 37^oC, unbound antibodies were washed away and a goat anti-human IgG peroxidase labelled conjugate (Biosource, Camarillo, CA, USA) was added to the plate. The plates were returned

for an additional hour at 37°C and after a washing step were developed with TMB as described in the procedure for IgM EIA.

IFA: Commercially available IFA slides (PanBio INDX Inc, Baltimore, USA) coated with WNV, Venezuela equine encephalitis (VEE), JEV, YFV, DENV and control antigens, were used for the detection of IgM and IgG serum antibodies against the respective viruses. Serum samples were diluted 1:32 and applied on the IFA slides for an overnight incubation when tested for the presence of IgM or for 30 minutes when tested for the presence of IgG antibodies. To detect virus specific IgM or IgG antibodies, a rabbit anti-human IgM or IgG respectively, fluorescein isothiocyanate (FITC) conjugate (DAKO) was applied on the slides for 30 minutes and the results were read under a fluorescence microscope. Prior to the detection of virus specific IgM antibodies by IFA, serum samples were pre-treated with anti-human IgG (Gullsorb, Meridian Salt Lake City, USA).

Calculations: DENV specific antibody ratios were calculated according to the instructions of the manufacturer. For the calculation of the ratios of WNV, YFV, and JEV IgM and IgG serum antibodies the following formula was used:

IgM/G ratio = (O.D. sample – O.D. blank)/ (mean of negative controls + 3X S.D).

Where O.D.: Optical density (extinction) of each sample and S.D.: Standard deviation.

RESULTS

Comparison of IFA with EIA for the detection of IgM serum antibodies. Figure 1 shows the results of the IgM antibody detection measured by the IFA and by Flavivirus specific EIAs using different groups of patients with various Flavivirus infections. Six out of the 12 serum samples that were obtained from patients with JEV infection were positive in the IFA whereas, 10 out of these 12 serum samples were positive in the JEV specific EIA (Table 2). When these 12 samples were tested for other Flaviviruses by IFA the highest cross-reactivity was observed with DENV (9/13), followed by WNV (3/12) and YFV (2/10). Using the respective EIAs, the cross-reactivity observed was 8/13 for DENV, 4/12 for WNV and 6/10 for YFV (Figure 1a).

Twelve out of the 13 samples obtained from patients with a DENV infection were positive in the IFA, whereas all 13 were positive in the DENV specific EIA (table 2). Only one serum sample tested positive by IFA against WNV, in contrast to the EIA where cross-reactions were detected against WNV (n=6), JEV (n=7) and YFV (n=10) (figure 1b).

Table 2: Overall comparison of the IFA vs. the EIA

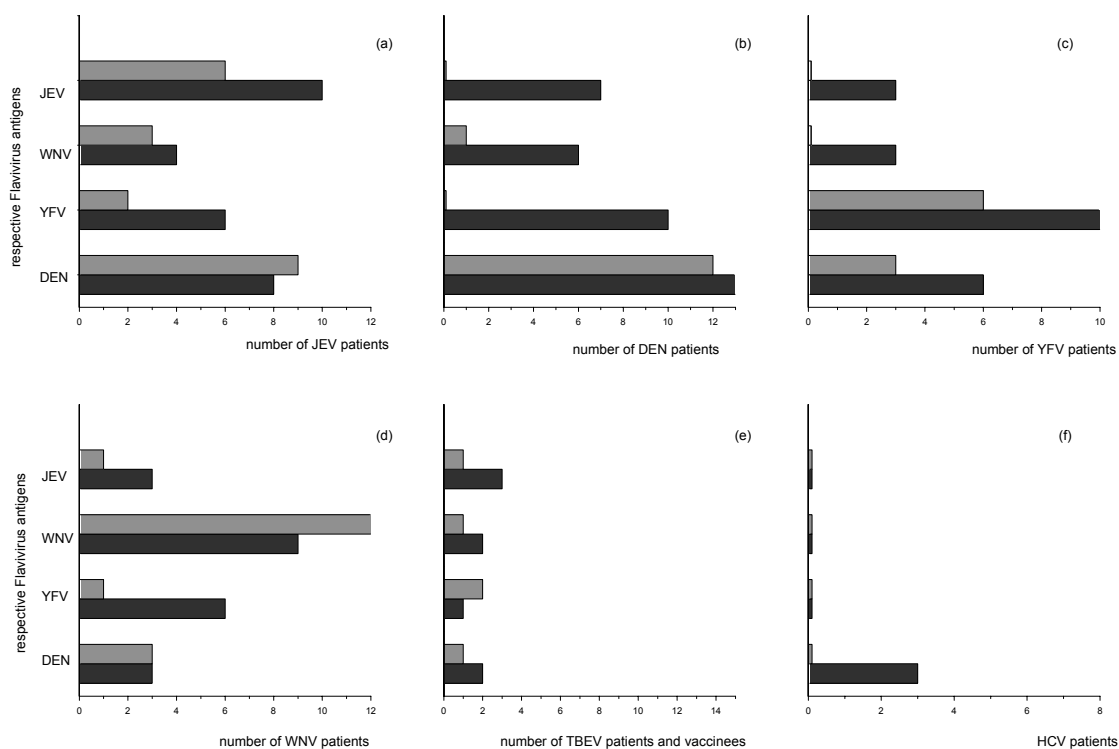| Flavivirus infection	No of samples	No of positive samples			
		IgM		IgG	
		IFA	EIA	IFA	EIA
JEV	12	6	10	10	11
DENV	13	12	13	11	10
YFV	10	6	10	10	9
WNV	12	12	9	11	11

Figure 1: Detection of IgM serum antibodies in patients with Flavivirus infections measured with the IFA (grey bars) and the specific EIAs (black bars).

Six out of the 10 serum samples obtained from patients with YFV infection were positive in the IFA and all 10 samples were positive in the YFV specific EIA (table 2). Only three of the YFV samples tested positive for DENV by IFA and none against WNV and JEV. In the WNV, JEV and DENV EIAs, cross-reactions were detected in three, three and six samples respectively (figure 1c).

All 12 serum samples obtained from patients with WNV infection were positive by the IFA and nine out of the 12 samples were positive in the WNV specific EIA (table 2). When these 12 samples were tested for other Flaviviruses by IFA the highest cross-reactivity was observed with DENV followed by JEV and YFV (n=3, n=1 and n=1 respectively). Using the DENV, JEV and YFV EIA, the cross-reactivity observed in these samples was n=3, n=3 and n=6 respectively (figure 1d).

In total five out of 15 serum samples obtained from patients suspected of TBEV infection or TBEV vaccinees cross reacted in the IFA, one serum sample tested positive for WNV, one serum sample for JEV, two for YFV and one for DENV. The reactivity of the TBEV serum samples measured by EIA resulted in two serum samples positive for WNV, three for JEV, one for YFV and two for DENV (figure 1e).

Among the eight samples obtained from HCV patients, three tested positive for DENV specific IgM antibodies measured by EIA, whereas none of these serum samples tested positive by IFA against JEV, YFV, DENV or WNV (figure 1f). None of the samples obtained from healthy blood donors tested positive for any of the Flaviviruses by the IFA or the EIAs.

Comparison of IFA with EIA for the detection of IgG serum antibodies. In figure 2 are depicted the results of the IgG detection measured in the respective assays using different groups of patients with Flavivirus infections. Ten out of the 12 serum samples obtained from patients with JEV infection were positive by IFA and 11 out of 12 serum samples were positive in the JEV specific IgG EIA (table 2). When these 12 samples were tested for other Flaviviruses by IFA the highest cross-reactivity was observed with DENV, followed by WNV and YFV (n=10, n=7 and n=7 respectively). Using the DENV, WNV and YFV EIA, the cross-reactivity observed in these samples was n=10, n=9 and n=9 respectively (figure 2a).

Eleven out of the 13 serum samples obtained from patients with a DENV infection were positive in the IFA, whereas ten out of the 13 serum samples were positive in the DENV specific IgG EIA (table 2). When these serum samples were tested for other Flaviviruses by IFA the cross reactivity observed was n=4 for JEV, n=9 for WNV and n=8 for YFV. The cross reactivity of these serum samples by EIA against JEV, WNV and YFV was n=10, n=8 and n=8, respectively (figure 2b).

All ten serum samples obtained from patients with a YFV infection were positive by IFA, whereas nine out of the 10 serum samples were positive in the YFV specific IgG EIA (table 2). The cross reactivity of these samples by IFA was n=6 for DENV, n=4 for WNV and n=3 for JEV. In contrast, the cross reactivity of these serum samples by DENV, WNV and JEV specific IgG EIA was n=7, n=6 and n=7, respectively (figure 2c).

Eleven out of the 12 serum samples obtained from patients with a WNV infection were positive for IgG antibodies by both the IFA and the EIA (table 2). When these 12 samples were tested for other Flaviviruses by IFA the highest cross-reactivity was observed with DENV, followed by YFV and JEV (n=8, n=7 and n=1 respectively). Using the DENV, YFV and JEV EIA, the cross-reactivity observed in these samples was n=11, n=9 and n=10 respectively (figure 2d).

Of the 15 serum samples obtained from TBEV infected patients or TBEV vaccinees, none cross reacted in the JEV IFA, five cross reacted in the WNV IFA, one in the YFV IFA and 11 in the DENV IFA. In contrast, the cross reactivity of these samples in the JEV, WNV, YFV and DENV IgG EIAs was ten, seven, eight and 13, respectively (figure 2e).

Among the eight serum samples obtained from HCV patients, three tested positive for DENV specific IgG antibodies measured by IFA, whereas none of these serum samples tested positive by IFA against JEV, WNV or YFV. One of these serum samples tested positive for IgG antibodies against JEV, WNV and YFV and two serum samples tested positive for DENV by EIA (figure 1f). None of the samples obtained from healthy blood donors tested positive for IgG antibodies against any of the Flaviviruses by the IFA or the EIAs.

Overall cross reactivity of the IFA among the Flavivirus infected individuals. The overall cross reactivity using the IFA for the detection of Flavivirus specific IgM and IgG antibodies was calculated among the Flavivirus infected individuals (table 3). For the detection of IgM antibodies the highest cross reactivity was 33% and was observed with the DENV antigen (16 out of the 49 serum samples cross reacted with DENV), followed by WNV antigen (10% or five out of 50 serum samples), YFV antigen (10%, or five out of the 52 serum samples) and finally JEV antigen (4% or two out of the 50 serum samples).

For the detection of Flavivirus IgG antibodies by IFA the highest cross reactivity was 71% and was observed with the DENV antigen (35 out of the 49 serum samples cross reacted with DENV), followed by WNV (50% or 25 out of the 50 serum samples), YFV (44% or 23 out of the 52 serum samples) and JEV (16% or 8 out of the 50 serum samples).

Table 3: Overall cross reactivity of the IFA and the EIA among the Flavivirus infected patients.

Flavivirus Ag	% (No of cross reactive samples/total No of samples tested)			
	IFA		EIA	
	IgM	IgG	IgM	IgG
DENV	33 (16/49)	71 (35/49)	39 (19/49)	84 (41/49)
YFV	10 (5/52)	44 (23/52)	44 (23/52)	65 (34/52)
WNV	10 (5/50)	50 (25/50)	30 (15/50)	60 (30/50)
JEV	4 (2/50)	16 (8/50)	32 (16/50)	74 (37/50)

Overall cross reactivity of the EIA among the Flavivirus infected individuals. The overall cross reactivity using the respective EIA system for the detection of Flavivirus specific IgM and IgG antibodies was calculated among the Flavivirus infected individuals (table 3). For the detection of Flavivirus specific IgM antibodies, the highest cross reactivity was observed with the YFV EIA (44% or 23 out of the 52 serum samples cross reacted with the YFV EIA), followed by the DENV EIA (39% or 19 out of the 49 serum samples), JEV EIA (32% or 16 out of 50 serum samples) and WNV (30% or 15 out of the 50 serum samples).

For the detection of Flavivirus specific IgG antibodies by the EIA system, the highest cross reactivity was 84% and was observed with the DENV EIA where 41 out of the 49 serum samples cross reacted with the DENV EIA. The JEV EIA showed an overall cross reaction of 74% (37 out of the 50 serum samples), YFV showed 65% (34 out of the 52 serum samples) and finally WNV EIA showed an overall cross reaction of 60% (30 out of the 50 serum samples).

DISCUSSION

IgM and IgG serology is the method of choice for routine diagnosis of Flavivirus infections. Today many commercial and in-house immunoassays are used world-wide [11, 10, 12, 13, 14]. Although the NT for Flaviviruses would be the method of choice for type specific diagnosis, it is not often used since it is time consuming and paired sera are required. Also virus isolation and/or detection of viral RNA by PCR could be the method of choice during the acute phase of infection. However, this is hardly ever possible, since in most of the cases patients show clinical symptoms after they have passed the viremic phase. In addition, Flavivirus infections are often presented with similar clinical features and that in several regions of the world, more than one of these viruses are endemic. Taken into consideration these epidemiological and clinical features of Flavivirus infection, it is obvious that one should rely on appropriate serological assays for the diagnosis of Flavivirus infections. A rapid screening for differential diagnosis would be beneficial for the serological diagnosis of Flaviviruses.
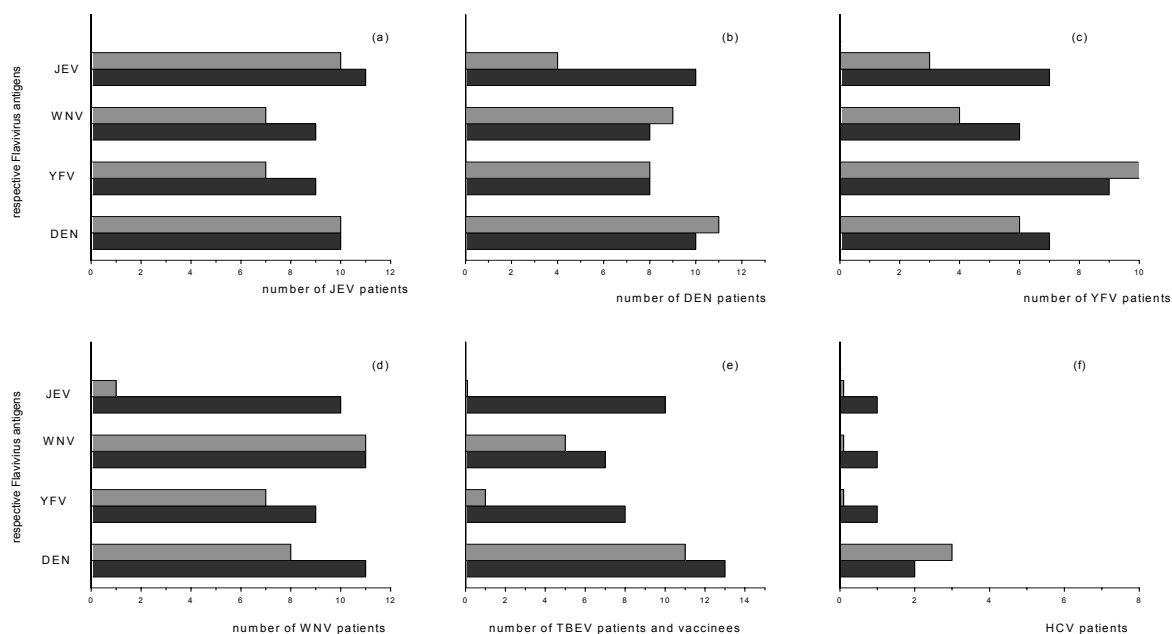
Figure 2: Detection of IgG serum antibodies in patients with Flavivirus infections measured with the IFA (grey bars) and the specific EIAs (black bars).

Here, we studied the reactivity pattern of serum samples obtained from patients with a Flavivirus infection, measured by IFA and ELISA.

In general, the IFA for the detection of IgM serum antibodies was more type specific than the in-house and commercial EIAs used for the respective viruses. The overall cross-reactivity for the detection of IgM antibodies was generally lower by the IFA than by the EIA when patients with suspected Flavivirus infections were screened for several different Flaviviruses. With the exception of DENV antigen of which the cross-reactivity was high by both IFA and EIA, the other Flaviviruses tested gave lower cross-reactions by IFA (between 4-10%), compared to the EIAs (overall cross reactivity between 30-44%). In contrast, the EIAs were more sensitive for the detection of IgM serum antibodies, with the exception of WNV IgM serum antibodies measured in WNV suspected patients, all had detectable IgM serum antibodies by the IFA but not by the EIA.

For the detection of Flavivirus IgG antibodies, both the IFA and the EIAs showed very poor specificity, with the IFA being slightly better when compared to the EIA. The overall cross-reactivity of the IFA for the detection of IgG antibodies was lower when compared with the EIA (overall cross-reactivity between 16-71% for IFA and 62-84% for EIA). DENV antigen was again the antigen with the highest overall cross-reactivity. Previous reports have demonstrated cross-reactions observed among Flaviviruses belonging to the same antigenic subgroup of viruses i.e. JEV and WNV belong to the same group whereas DENV and YFV belong to independent antigenic subgroups [18]. The preparation of the antigens and the method of fixation and inactivation also contribute to the differences in sensitivity and specificity for both the IgM and IgG serum antibody detection. The origin of the samples may also play a role for the cross-reactions observed. Several of the samples from patients with suspected DENV and JEV infections were from Southeast Asia where both viruses are endemic and it might be possible that patients have been exposed to both viruses. Therefore, these samples may show reactivity to both antigens not only due to cross-reactions but also due to the presence of specific antibodies to both viruses. Other serum samples were obtained from patients that

had travelled to the tropics and had possibly been vaccinated against YFV, therefore having cross-reactive antibodies with this virus.

To further asses the specificity of the IFA, serum samples obtained from patients with detectable antibodies to other members of the *Flaviviridae* family as well as samples from healthy individuals, were tested. Although both the IFA and the EIAs gave cross-reactions in the detection of both IgM and IgG antibodies to the respective Flaviviruses when TBEV suspected patients and vaccinees were tested the IFA resulted in higher specificity than the EIA (figures 1 and 2). When samples from HCV patients were tested the IFA only gave three positive results in the detection of IgG antibodies against DENV, whereas the EIAs gave more positive results in the detection of both IgM and IgG antibodies against the Flaviviruses tested, resulting in lower specificity than the IFA. In the contrary, both assays did not show any cross-reactions when samples from healthy individuals were tested. The vaccination and travel history of the HCV patients is not known and the observed cross-reaction against a Flavivirus could be due to previous vaccination or due to a trip to a Flavivirus endemic area in the past.

The results of this study are in agreement with previous studies in which it was demonstrated that cross reactions among viruses belonging to the same antigenic subgroup are observed [19, 20] and these cross reactions are more frequently observed among IgG antibodies detection and in lower extent for the detection of IgM antibodies against Flaviviruses [21]. The use of DENV recombinant antigens has recently proved to be a useful tool for specific diagnosis of DENV infections showing low cross-reactivity in serum samples from patients with other Flavivirus infections [22].

In conclusion, the results of this study showed that the commercially available IFA coated with antigens from several Flaviviruses, is a rapid and easy to perform test, which seems to be slightly more specific for the detection of IgM antibodies by means of less cross reactions in comparison to the Flavivirus IgM EIAs. However the Flavivirus IgM EIAs proved to be slightly more sensitive. Our results clearly show that both methods are useful for the detection of Flavivirus serum antibodies. It is also clearly demonstrated that testing a serum sample only for one virus does not lead, in many cases, to a possible diagnosis. To enable appropriate diagnosis of Flavivirus infection, one should consider the country of origin of the sample, the vaccination status and travel history of the patient. Therefore, a combination of the two tests would enable to correctly diagnose and identify the infecting Flavivirus subtype. To improve the serological diagnosis of Flavivirus infections, further studies should be undertaken for the development of more specific serological tests for the detection of Flavivirus specific serum antibodies, for example using recombinant proteins or peptides.

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Chapter 2.3

Detection of Immune-Complex Dissociated Nonstructural-1 (NS-1) Antigen in Patients with Acute Dengue Virus Infections

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ABSTRACT

Accurate and timely diagnosis of dengue virus (DENV) infections is essential for the differential diagnosis of patients with febrile illness and hemorrhagic fever. In the present study, the diagnostic value of a newly developed immune-complex dissociated NS-1 antigen dot-blot immunoassay (DBI) was compared with a commercially available DENV antigen detection kit (denKEY Blue kit, Globio Co., MA, USA) and an RT-PCR. Serial serum/plasma samples (n=181) obtained from 55 acute DENV-infected patients were used. In samples obtained from 32 of these 55 DENV-infected patients, viral RNA could be detected using the RT-PCR. DENV antigen was detected in only 10 of these 55 patients' samples using the denKEY kit. When these samples were treated with acid to release the immune complex-associated NS-1 antigen for detection using the DBI, 43 of these 55 patients were found to be positive for DENV NS-1 antigen. In non-dissociated samples, 22 of these patients were found to be positive using the DBI. In the presence of DENV specific IgM antibodies, both viral RNA and DENV (NS-1) antigen could be detected. The number of positive samples identified from these patients with primary DENV infections using the RT-PCR and DBI varied between 28 and 78%. In secondary DENV infections, the number of samples tested positive using the DBI after immune-complex dissociation (DIS-DBI) was 25% higher than those tested positive using the RT-PCR and 35% higher compared to non-dissociated antigen (NDIS-DBI) detection. We conclude that the denKEY kit has limited diagnostic value for acute DENV infections when compared to the RT-PCR and the NDIS-DBI and DIS-DBI. We clearly demonstrated that in secondary DENV infections the dissociation of NS-1 immune-complexes is essential for early diagnosis of DENV infections.

INTRODUCTION

Dengue virus (DENV) is one of the most widespread mosquito-borne human pathogens worldwide accounting for more than 50 million infections per year [2]. Mosquitoes of the *Aedes* species are responsible for transmitting the four serotypes of DENV (DENV 1-4) to humans. Infection with DENV may be asymptomatic or may cause a variety of symptoms ranging from mild dengue fever (DF) to the more severe form of dengue hemorrhagic fever (DHF) with or without shock (dengue shock syndrome [DSS]) [3]. In DENV endemic areas DHF has become an increasingly important cause of pediatric morbidity and mortality since it was described half a century ago [3]. Accurate diagnosis of DENV infections is therefore essential.

The diagnostic methods of choice for the identification of DENV infections have been the plaque reduction neutralization assay and/or virus isolation from patients serum samples using mosquito cell lines [3,4]. However, both of these assays are laborious to perform and a period of at least 7 days is required to obtain accurate diagnostic results using them. Recently, several enzyme-linked immunosorbent assays (EIAs) have become commercially available for the detection of DENV specific antibodies of different isotypes [5,1]. However, DENV serology is not virus specific, but shows a high amount of cross-reactivity with other Flaviviruses [6]. Detection of viral RNA in serum samples from acute-phase DENV-infected patients using a reverse transcriptase PCR (RT-PCR) has been described and is a valuable tool for both diagnosis of DENV infections and the identification of the viral serotype [7]. RT-PCR provides an accurate diagnosis for DENV infections during the early stages of DENV illness, even in the presence of DENV specific IgM antibodies [8]. The RT-PCR is however relatively expensive to use as a routine diagnostic test and requires specialized laboratory equipment and trained personnel. In addition, the storage of the serum samples at -70°C that is essential for RT-PCR in order to maintain viral RNA in optimal conditions, is not feasible in many DENV endemic areas. As an alternative, the detection of viral antigens has been proposed [9] and a suitable EIA [10] can be performed using patients serum samples that have been stored at 4°C. A simplified immunoassay for the detection of DENV antigen in patients samples with a sensitivity and specificity comparable to the RT-PCR would therefore be highly desirable. The DENV nonstructural-1 (NS-1) protein has been identified as either an intracellular membrane-associated protein or a soluble extracellular protein [11]. Since high concentrations of the NS-1 protein were found in blood samples of patients obtained during the early acute phase of both primary and secondary DENV infections, and for up to nine days after the onset of symptoms (1), DENV NS-1 detection assays are likely to be valuable diagnostic tools. Anti-NS-1 antibodies were rarely detected in samples from patients with primary DENV infections using immunoblot assays, but these antibodies were detected much more frequently in patients with secondary DENV infections, in particular among patients from areas where DHF/DSS is more frequent (such as Indonesia), when compared with patients from areas that DHF/DSS is not that common (such as the Caribbean) (10).

Dissociation of antibody-antigen (Ab-Ag) immune-complexes has proven to be important for the early diagnosis of several blood-borne viruses such as HIV and both hepatitis B and C viruses [12,13,14]. This technique has not however been applied in the diagnosis of Flavivirus infections and in particular for DENV infections.

In this study, we assessed the diagnostic value of dissociated and non-dissociated DENV NS-1 Ab-Ag immune complexes in samples from primary and secondary DENV patients using a dot-blot immunoassay (DBI). The results were compared with a commercially available DENV antigen detection kit (denKEY Blue, Globio Co., MA, USA) and DENV specific RT-PCR.

MATERIALS AND METHODS

Serum/Plasma samples. Serial serum/plasma samples were collected from January 2000 to March 2002 from 55 patients with acute DENV infections and stored in aliquots at -80^oC until use. The diagnosis of the patients was based on the clinical and serological criteria set by the WHO [3]. Thirty-nine patients were from Indonesia and plasma samples (n=156) were obtained upon admission to the hospital (D 0) and on days 1, 2 and 7 post admission (D 1, D 2 and D 7 respectively). All of these Indonesian patients were children, 20 of them were suffering from DSS. Twenty-five serum samples were obtained from 16 patients from the Dutch Antilles. From nine of these patients serum samples were obtained upon presentation to their treating physician (D 0) and two weeks later (D 14), whereas from the remaining seven patients, only one serum sample was available obtained upon presentation to the clinic (D 0). The patients from the Antilles were all adults, suffering from DF. In total, 18 patients were suffering from primary DENV infections and 37 patients were suffering from secondary DENV infections based on their serological profile [3]. Sixteen serum samples were obtained from healthy Dutch blood donors with no evidence of current or previous DENV infection (as defined by the absence of DENV specific IgM and/or IgG antibodies) served as the negative controls.

IgM/IgG EIA. A commercially available kit (Focus Technologies, Cypress CA, USA) was used for the detection of DENV specific IgM and IgG antibodies in the samples and performed according to the procedures described by the manufacturer. The sensitivity and specificity of the assays has been described elsewhere [5].

Dot-blot assay. Patients' serum/plasma samples were either diluted 1:3 in PBS (non-dissociated samples) or 1:2 in dissociation buffer (1.5M glycine pH 2.8). Ab-Ag immune complexes were dissociated for one hour at 37^oC and the reaction was stopped by the addition of one volume neutralization buffer (1.5M Tris-HCl pH 9.7) for end dilution of the sample 1:3. The samples were then spotted on nitrocellulose membranes (High Bond-P membranes, Amersham Biosciences, Little Chalfont, UK). After spotting the samples, membranes were air-dried and treated with methanol containing 0.3% H_2O_2, washed with distilled water and PBS and blocked overnight at 4^oC with 5% skimmed milk (ELK Campina, Eindhoven, The Netherlands) in PBS containing 0.1% Tween-20 (PBS-T). The blots were then washed with PBS-T and incubated for 30 min at RT with monoclonal antibody 3D1.4, which defines the LX1 epitope on the NS-1 proteins of each DENV serotype [15] diluted in PBS-T buffer containing 5% skimmed milk, 5% normal goat serum (ICN Biochemicals Inc. Ohio, USA), 5% fetal bovine serum (ICN Biochemicals Inc.) and 20% normal human serum (blot-buffer). The blots were then washed thoroughly for 30 min with PBS-T and incubated for 30 min at RT with goat anti-mouse HRPO conjugate (Dako, Glostrup, Denmark) diluted 1:10,000 in blot-buffer. The blots were washed, incubated in PBS for 5 min and further processed with the ECL substrate system (ECL, Amersham Biosciences) according to the procedures described by the manufacturer. Films (KODAK X-omat AR) were exposed for five minutes before development.

DENV antigen detection EIA. The presence of DENV antigen in the serum/plasma samples was measured using a newly described, commercially available kit (Globio Co., MA, USA) based on an EIA method according to the procedures of the manufacturer. Briefly, diluted samples are incubated for two hours in micro-titre wells, followed by a one hour incubation step with a DENV specific antibody. When DENV antigen was present in the serum samples, it was bound by the detecting antibody and the antibody-antigen immune complexes were then detected after the addition of an enzyme-labeled conjugate and subsequent substrate reaction.

Detection of viral RNA. DENV RNA was isolated from 200 µl of patients' serum or plasma using the High Pure RNA Extraction Kit (Roche, Mannheim, Germany) according to the procedure described by the manufacturer. DENV serotype specific reverse transcriptase followed by PCR (RT-PCR) was performed as described previously [7,16]. Patients' samples were considered positive when a band of the correct size (DENV-1 482 bp, DENV-2 119 bp, DENV-3 290 bp, DENV-4 392 bp) was observed using an UV-illuminator after 2% agarose gel electrophoresis and subsequent staining with ethidium bromide.

Calculations. For the determination of DENV specific IgM and IgG antibody ratios, the cut-off values provided by the manufacturer were used. Samples were considered positive with the dot-blot assay when a clear spot could be visualized on films, in the absence of signal in the conjugate control. Readings of the dot-blot assays (DBI) were performed by two independent observers. Samples were considered positive using the denKEY kit when an O.D. value of greater than 0.150 was obtained as described by the manufacturer.

RESULTS

Detection of DENV NS-1 antigen in dissociated and non-dissociated samples. The DENV NS-1 detection sensitivity of the DBI was higher when the patients' samples were treated with acid to dissociate their Ab-Ag immune complexes as shown in Figure 1. Of the 181 samples tested using the DBI, DENV NS-1 antigen could be detected in only 32 of them without Ab-Ag dissociation (NDIS-DBI), whereas this antigen could be detected in 113 of these samples after acid treatment (DIS-DBI). Twenty-four of the 32 NDIS-DBI positive samples were also positive in the DIS-DBI.
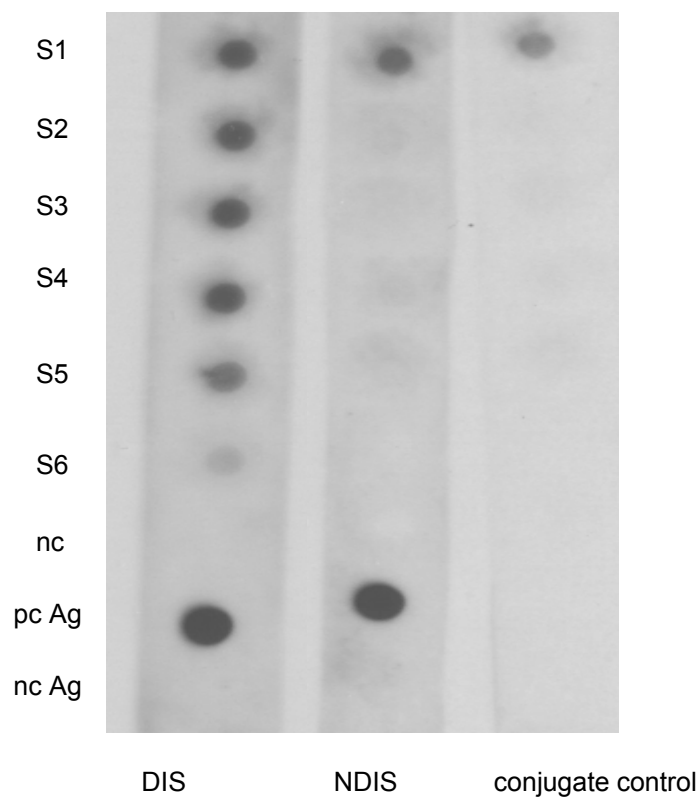

Figure 1: Detection of DENV NS-1 antigen using the DBI in serum/plasma samples after dissociation (DIS) of immune-complexes, or without dissociation (NDIS) of immune-complexes. pc Ag: positive control (DENV antigen); nc Ag: negative control antigen; nc: negative control human serum. Acetone extracted positive and negative antigen controls have been prepared as previously described [1].

Detection of DENV antigen in the presence of DENV specific IgM antibodies. The DENV antigen detection level of the denKEY kit was low since only 10 out of the 181 patients' samples obtained O.D. values higher than the cut-off value. Nine of the 10 denKEY antigen positive patients were positive on day 0 post admission to the hospital, whereas one patient was DENV antigen positive on day 7 post admission to the hospital. The denKEY kit identified DENV antigen in five patients who were suffering from primary DENV infections and five patients who were suffering from secondary DENV infections (Table 1). There was no correlation between the very low DENV antigen detection level in these patients' samples using this assay and the presence of DENV specific IgM antibodies in these samples (Figure 2), although most of these DENV antigen positive samples had relatively low or negative titers to IgM antibodies. The DENV antigen detection level of the DBI was significantly higher than that obtained using the denKEY kit, especially when samples were treated to dissociate immune-complexes (DIS-DBI). In 22 of the 55 DENV infected patients, DENV NS-1 antigen could be detected using the DBI without the dissociation step being necessary. Of these patients, 12 were experiencing primary DENV infections and 10 were experiencing secondary DENV infection (Table 1). When the patients' samples were dissociated, 43 of them were found to be positive for DENV NS-1 antigen, 14 of which were suffering from primary DENV infections and 29 from secondary DENV infections (Table 1). Detection of DENV NS-1 antigen was not influenced by the presence of DENV specific IgM antibodies when using NDIS-DBI or DIS-DBI (Figure 2).

Table 1: Detection of DENV (NS-1) antigen and viral RNA in patients with primary or secondary DENV infections. NDIS: samples not treated with acid for dissociation of Ab-Ag immune complexes. DIS: samples treated for dissociation of Ab-Ag immune complexes.

Assay	Primary infections (n=18)	Secondary infections (n=37)	Total (n=55)
denKEY kit	5 (28%)	5 (13%)	10 (18%)
RT-PCR	12 (67%)	20 (54%)	32 (58%)
NDIS-DBI	12 (61%)	10 (27%)	22 (40%)
DIS-DBI	14 (77%)	29 (78%)	43 (78%)

Detection of DENV NS-1 antigen in patients' samples from different DENV endemic areas. The DENV NS-1 antigen detection level, using the NDIS-DBI in samples from patients with primary DENV infection was similar when patients were from Indonesia (64%) or the Dutch Antilles (72%). The DENV NS-1 antigen detection level was increased in samples from primary Indonesian patients (91%) but slightly reduced in samples from primary Antillean patients (57%) when using the DIS-DBI (Table 2). In samples obtained from patients with secondary DENV infection, the DENV NS-1 antigen detection level was low among Antillean samples, 44% using NDIS-DBI and 33% using DIS-DBI. In contrast, the

detection level of DENV NS-1 antigen in samples from Indonesian patients with secondary DENV infections increased dramatically from 22% using the NDIS-DBI to 93% using the DIS-DBI (Table 2).

Table 2: Detection of DENV NS-1 antigen using the NDIS-DBI and DIS-DBI in samples obtained from Indonesian and Antillean patients with primary and secondary DENV infections.

	Indonesia n=39 (11 primary and 28 secondary)		Antilles n=16 (7 primary and 9 secondary)	
	NDIS	DIS	NDIS	DIS
Primary	7 (64%)	10 (91%)	5 (72%)	4 (57%)
Secondary	6 (22%)	26 (93%)	4 (44%)	3 (33%)

Comparison of DENV type specific RT-PCR and DENV antigen detection assays. A comparison of DENV antigen and viral RNA detection using the denKEY kit, the DBI and the RT-PCR in serially collected samples obtained from patients who lived in DENV endemic areas is shown in Figure 3. Using the RT-PCR, DENV RNA was detected in 32 of the 55 patients with acute DENV infections. Eight patients were infected with DENV 1 (7 Indonesian and 1 Antillean patient), four patients were infected with DENV 2 (all Indonesian patients), 19 patients were infected with DENV 3 (4 Indonesian and 15 Antillean patients) and one patient from Indonesia was infected with DENV 4 (Table 3). All but one of the 32 RT-PCR positive patients were positive on day 0 (the day of first presentation to the clinician) whereas five patients remained positive for viral RNA on day 1 and 2 post admission to the hospital. Only one patient was positive by RT-PCR on day 7 after admission to the hospital (Figure 3). Of the 16 RT-PCR positive Indonesian patients for whom clinical records were available, six patients were suffering from DSS, while 10 were suffering from DHF.

Table 3: Comparison of methods for detection of viral antigen (denKEY antigen kit and DBI) versus viral RNA (RT-PCR), in samples obtained from 55 DENV-infected patients. NDIS: non-dissociated samples, DIS: Ab-Ag immune complex dissociated samples.

Assay	PCR (n= positive patients)				
	DENV- 1 (n=8)	DENV- 2 (n=4)	DENV- 3 (n=19)	DENV- 4 (n=1)	Total (n=32)
denKEY kit	1	0	8	1	10
NDIS DBI	6	1	10	1	18
DIS DBI	6	4	11	1	22
Total NDIS and DIS DBI	8	4	14	1	27

Using the denKEY kit, DENV antigen was detected in 1 of the 8 DENV 1 infected patients, in none of the DENV 2 infected patients, in 8 of the 19 DENV 3 infected patients and in the single DENV 4 infected patient (serotypes as determined by RT-PCR). The one patient who was positive by the RT-PCR on day 7 after admission to the hospital was also found to be positive using the denKEY kit (Table 3). Using the NDIS-DBI, DENV NS-1 antigen was detected in 6 of the 8 DENV 1 infected patients, in 1 of the 4 DENV 2 infected patients, in 10 of the 19 DENV 3 infected patients and in the single DENV 4 infected patient. After dissociation of the patients' immune-complexes (DIS-DBI) DENV NS-1 antigen was detected in 6 of the 8 DENV 1 infected patients, in all four DENV 2 infected patients, in 11 of the 19 DENV 3 infected patients and in the DENV 4 infected patient (Table 3). In addition, DENV NS-1 antigen was detected by NDIS and DIS-DBI in 19 patients, who were negative for the presence of viral RNA using the RT-PCR (Figure 3).

Table 4: Overview of the results for the detection of DENV (NS-1) antigen and viral RNA in 181 samples obtained from DENV-infected patients using the denKEY kit, the DBI (NDIS: non-dissociated samples, DIS: dissociated samples) and the RT-PCR, respectively. +: sample scored positive in the respective test, - : sample scored negative in the respective test.

		RT-PCR	DENV NS-1 DBI	
			NDIS	DIS
denKEY kit	+ +	10	9	6
	+ -	0	1	4
	- +	32	24	107
	- -	139	147	64
NDIS DBI	+ +	23		25
	+ -	9		7
	- +	19		88
	- -	130		61
DIS DBI	+ +	28	25	
	+ -	85	88	
	- +	14	7	
	- -	54	61	

Overall comparison. Table 4 summarizes the overall comparison of the DENV (NS-1) antigen detection assays and the RT-PCR for the 181 serum/plasma samples tested. Forty-two serum samples tested positive for the presence of viral RNA using the RT-PCR, of which only 10 (23%) tested positive using the denKEY kit, 23 (55%) were positive using the NDIS-DBI and 28 (67%) tested

positive using the DIS-DBI. All of the 139 serum samples that tested negative using the RT-PCR, were also negative using the denKEY kit, 130 (93%) tested negative using the NDIS-DBI, whereas only 54 (39%) tested negative using the DIS-DBI (Table 4).

DISCUSSION

Routine diagnosis of DENV infections is mainly based on serological assays for the detection of DENV specific IgM and IgG antibodies. Detecting DENV antigen in serum/plasma samples with a simple, rapid and easy to perform assay such as an EIA or a dot-blot assay with a similar sensitivity and specificity of the RT-PCR, would be an excellent alternative for routine diagnosis of acute DENV infections.

In this study we describe a DBI for the detection of the DENV NS-1 antigen since high concentrations of this antigen were previously shown to be present in patients' samples obtained during the early acute phase of either primary or secondary DENV infections [9,17]. Since antibodies are generated to the DENV NS-1 protein, particularly during secondary DENV infections in patients from Indonesia (area where DHF/DSS is common), when compared to those from Puerto Rico (area where DHF/DSS is less common) [18] we also choose to compare the relative sensitivities of these assays in both primary and secondary DENV infections in patients from Indonesia and the Dutch Antilles before and after dissociation of the immune complexes. The predominant DENV serotype circulating during the outbreaks in Indonesia and the Dutch Antilles was DENV-3 [19]. All patients from Indonesia were children suffering from DHF and/or DSS, whereas the patients from the Antilles were all adults with symptoms of DF. For our assay, we used a monoclonal antibody (3D1.4), which detects the minimal peptide sequence, YSWKTWG, present in the NS-1 proteins of three DENV serotypes. The potentially higher specificity of this monoclonal antibody for the NS-1 protein of DENV-1 was not, however reflected in the relative NS-1-detection sensitivities for the other DENV serotypes [15]. This monoclonal antibody was more reactive with the corresponding sequence form the NS-1 protein of DENV-1, which contained a single amino-acid substitution (YSWKSWG) [15].

In the present study, we demonstrated that non-dissociated DENV NS-1 antigen was detected in 61% of DENV primary cases compared to 27% detection in secondary cases (Table 1). Further more we demonstrated that non-dissociated DENV NS-1 antigen was detected in the majority of the patients with primary DENV infection from Indonesia and the Dutch Antilles, but could be further increased in the Indonesian patients' samples using the DIS-DBI. Dissociation of DENV NS-1 Ab-Ag immune complexes proved to be essential for the detection of DENV NS-1 antigen in serum/plasma samples, particularly amongst samples obtained from Indonesian patients with secondary DENV infections since the sensitivity of the DBI increased dramatically (up to 93%) when these dissociated samples were tested. These results are in agreement with a previous study in which it was demonstrated that anti-NS-1 antibodies are mainly generated during secondary DENV infections in patients from Indonesia (where DHF/DSS is more common) than in patients from the Caribbean where DHF/DSS is not common (10). Those pre-existing anti-NS-1 antibodies possibly result to the formation
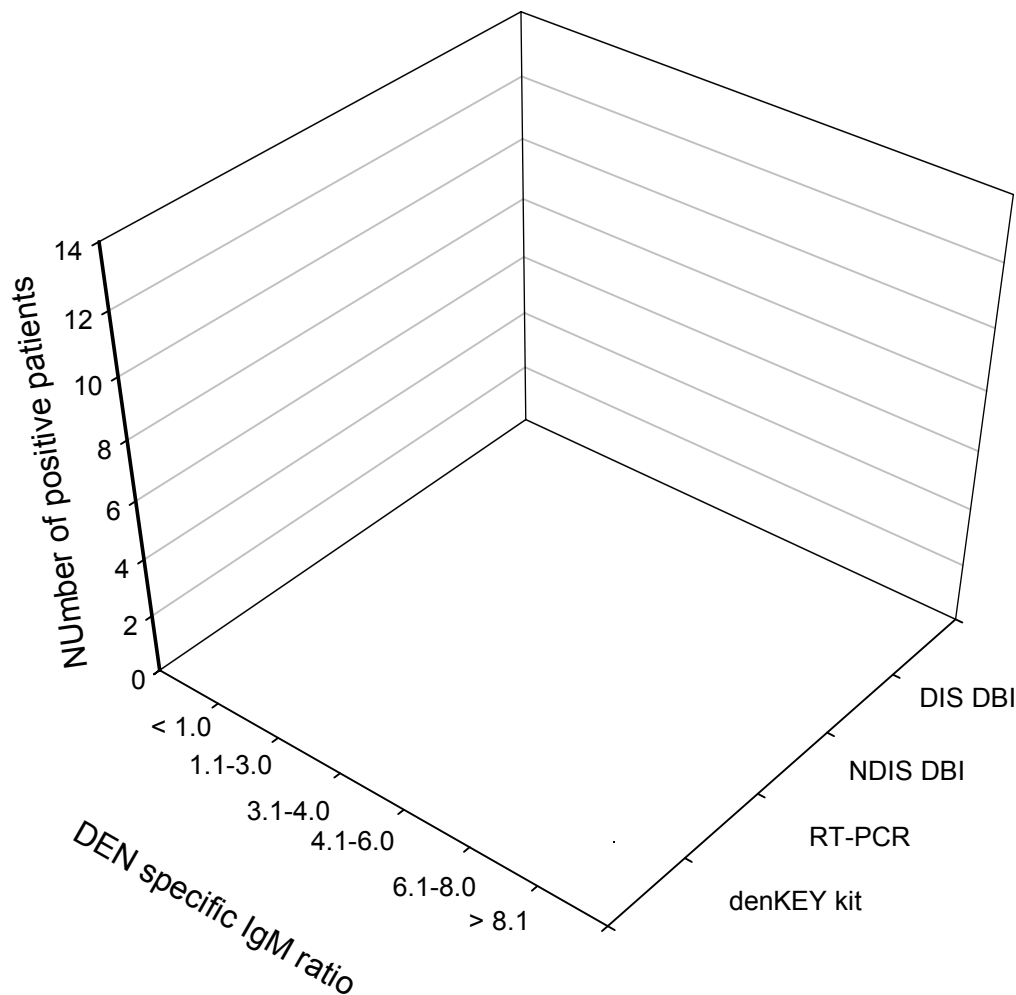
Figure 2: Detection of DENV (NS-1) antigen and viral RNA with different assays in comparison with the levels of DENV-specific IgM antibodies in samples obtained from patients with acute DENV infection. The Z-axis represents the number of positive patients using each assay from samples obtained on day 0.

of Ab-Ag immune complexes and therefore reduce the sensitivity of the DENV NS-1 antigen detection assay in particular when using the NDIS-DBI.

The sensitivity of the denKEY kit was very poor since only 10 samples tested positive for the presence of DENV antigen with this assay. However, both denKEY kit and DBI of non-dissociated samples detected with greater sensitivity DENV (NS-1) antigen in samples from primary infections, whereas the DBI of dissociated samples detected DENV NS-1 antigen with the same sensitivity in samples from primary or secondary infections. In a previous study it was demonstrated that DENV viral RNA could be detected in the presence of DENV specific IgM antibodies in serum/plasma samples of DENV infected patients [8]. In our study we confirmed these results and furthermore we demonstrated that also DENV NS-1 antigen could be detected in the presence or absence of DENV specific IgM antibodies. However the number of NS-1 antigen positive patients in the presence of IgM was significantly higher in dissociated samples at different levels of IgM antibodies (Figure 2)

We also studied the number of DENV antigen positive patients at several time-points after infection. Recently it was demonstrated that DENV NS-1 antigen could be detected between day 0 and 9 with a slight peak on day 4 post onset of illness [17]. Our findings are in agreement with this previous study since we demonstrated that the majority of positive samples for DENV NS-1 antigen were obtained in patients between days 0 and 2 post admission to the hospital (i.e. three to six days post

onset of symptoms). Further more we were able to detect the DENV NS-1 antigen using the DBI during the early convalescence phase of DENV illness in 15 of the 55 DENV patients (Figure 3). Viral antigens circulate in patients' blood for longer periods than viral RNA and therefore we were able to detect DENV NS-1 antigen in many patients' samples in which viral RNA was undetectable (i.e. RT-PCR negative). The results we obtained with the RT-PCR showed that the highest percentage of positive samples was detected on day 0 in both cohorts (97%). However, on days 1 to 7 RT-PRC positive results were also obtained.

The specificity of the DENV NS-1 antigen detection DBI did not seem to be influenced by the infecting DENV serotype. In contrast, the denKEY kit seems to detect DENV-3 with a higher sensitivity (43% of the DENV-3 infected patients were also positive with the denKEY kit) than the other three serotypes. However, it is difficult to draw any reliable conclusions about the serotype specificity of the assays. Our sample size of DENV positive patients using the RT-PCR was rather small and there was not a similar incidence of each of the four DENV serotypes among our patients (e.g. we were able to detect DENV-4 viral RNA in only one patient). We could not, therefore, perform a statistical analysis on the serotype-specificity of the DENV (NS-1) antigen detection assays in more detail. Furthermore, from the clinical data available we could not find any significant differences associated with disease severity and the presence of DENV NS-1 antigens in acute and/or convalescent samples.

In conclusion, in this study we demonstrated that the combination of NS-1 antigen detection by DBI in both non-dissociated and dissociated serum/plasma samples from primary and secondary cases of DENV infection results in the highest number of DENV antigen positive patients compared to the RT-PCR and the denKEY kit. We also showed that dissociation of immune-complexes is essential for the detection of DENV NS-1 protein in secondary cases of DENV infection. This newly developed method also offers the opportunity for rapid identification of dengue infections among many other flavivirus circulating in DENV endemic areas and causing similar clinical symptoms and additionally overcomes several problems of the RT-PCR. For example, patients' serum/plasma samples could be adequately stored at 4°C for subsequent testing using this assay since the DENV NS 1 antigen is more stable than viral RNA and the problems due to contamination of the RT-PCR products is not an issue in antigen detection assays. In addition, DBI is rapid, easy to perform and can be applied in less equipped laboratory settings. The high sensitivity of the DBI in detecting DENV NS-1 antigen in samples from acute primary or secondary DENV-infected patients makes it a valuable diagnostic tool and a good alternative of the RT-PCR. In contrast, we found the denKEY kit to be unsuitable for DENV diagnosis due to its low sensitivity.
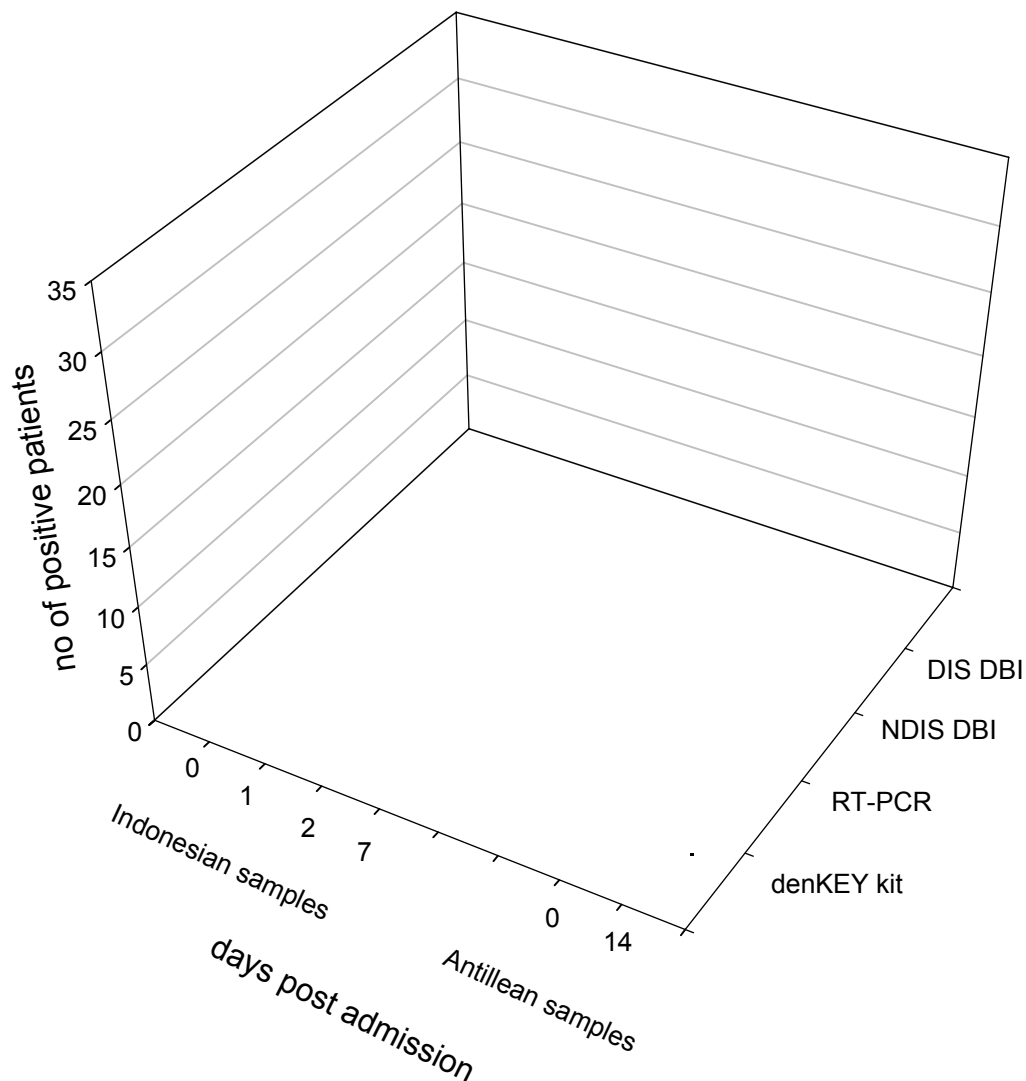
Figure 3: Kinetics of DENV (NS-1) antigen and viral RNA detected with the different assays on different time points post onset of illness. Day 0 is the day of presentation to the clinic and represents day 3-6 post onset of illness.

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Chapter 3

Humoral immune responses

of natural
dengue virus infections

Chapter 3.1

Kinetics of Dengue Virus Specific Serum Immunoglobulin Classes and Subclasses Correlate with Clinical Outcome of Infection

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ABSTRACT

The kinetics of dengue virus specific serum immunoglobulin classes (IgM and IgA) and subclasses (IgG 1-4) were studied in patients suffering from dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Serum samples from non-dengue febrile patients were included as controls. IgM, IgG 1 and IgG 3 serum antibodies were the predominant immunoglobulins throughout the course of illness in all three patient groups. In contrast, IgA antibodies were significantly higher in the acute phase in DSS patients compared to DF patients ($P<0.05$). The levels of IgG 1 differed significantly between patients with DF and those with DHF and DSS ($P<0.05$). A significant difference was also found in IgG 3 levels between DF patients and DHF patients ($P<0.05$) but not between DF patients and DSS patients. Finally, levels of IgG 4 antibodies differed significantly between DF patients and DSS patients ($P<0.05$). Collectively, these data show that increased levels of DENV specific IgA, IgG 1 and IgG 4 serum antibodies are risk markers for the development of DHF and DSS and that their measurement may provide valuable guidance for early therapeutic intervention.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne virus belonging to the family *Flaviviridae*. The four serotypes (DENV 1-4) are transmitted to humans through the bite of infected mosquitoes, which mainly belong to the *Aedes aegypti* and *Aedes albopictus* species. An estimated 50 million people are annually infected with DENV and over two billion people are at risk of acquiring DENV infection in tropical and sub-tropical regions [1]. Infection with DENV may either be asymptomatic, or be characterized by a variety of clinical manifestations. The majority of dengue patients develop an illness characterized by fever, chills, frontal headache, myalgia, arthralgia and a rash, which symptoms together form the clinical syndrome of dengue fever (DF). More severe manifestations of the disease are associated with the development of haemorrhagic phenomena with plasma leakage (dengue haemorrhagic fever [DHF]) and shock (dengue shock syndrome [DSS]) [2]. DHF and DSS are mainly affecting young children accounting for approximately 250,000 deaths annually [3,2]. The above mentioned features of DENV infection, as well as the fact that the mosquito vectors have a wide distribution in tropical and subtropical areas, have led to the emergence of DENV as one of the most important public health problems world-wide [4].

Despite decades of research, the pathogenesis of DENV infection remains poorly understood. Several hypotheses have been formulated to explain the development of DHF and DSS, with antibody-dependant enhancement (ADE) of infection [5,6] being the most widely accepted. It has also been speculated that viraemia plays an important role in the pathogenesis of severe DENV infections, however, it was recently demonstrated that the magnitude and duration of viraemia was not significantly different among patients with primary versus secondary DENV infections [7]. Other studies have demonstrated the indirect implication of circulating adhesion molecules in the pathogenesis of severe DENV infection [8,9].

Different IgG subclasses can fix and activate complement [10,11], and bind to Fcγ receptors [12,13,14,15,13,12]. These factors may also play an important role in the development of ADE and thus in the pathogenesis of DHF and DSS [16,17].

The laboratory diagnosis of DENV is based on virus isolation, detection of viral RNA, or on the detection of DENV specific IgM and IgG serum antibodies [18,2]. The ratio between acute phase IgM and IgG antibodies is indicative for primary or secondary infection [2]. Recent studies have indicated the diagnostic value of DENV specific IgA serum antibodies [19,20] and a relationship between levels of DENV specific IgG 1 serum antibodies and disease severity [21].

Here we have studied the possible correlation between the kinetics of DENV specific serum immunoglobulin classes and subclasses on the one hand and disease severity on the other. Besides having direct diagnostic and prognostic implications, the data contribute to our understanding of the pathogenesis of DENV infections of different severity.

MATERIALS AND METHODS

Serum samples. During the 1995-1996 DENV epidemic in Indonesia, serial serum samples were obtained from 171 patients with confirmed DENV infection and from 21 patients with non-dengue (ND) febrile illness to serve as controls. Table 1 summarizes the characteristics of the DENV infected patients and the controls. From the DENV

infected patients 72 had DF, 30 had DHF and 69 patients had DSS according to the criteria defined by the World Health Organization [2]. All patients had been admitted to the hospital on different days after onset of fever (range between: 0-20) and serial samples had been collected after admission. All patients were citizens of Yogyakarta and Semarang in Indonesia. The age varied between 7 months to 14 years (mean 7.6 years) and 53% of the patients were females. The mean duration of fever for DF, DHF I, DHF II, DHF III and DHF IV patients was 7.0, 8.6, 9.1, 9.6 and 11.8 days respectively. During this period all DENV serotypes were circulating of which DENV 3 was the most predominant serotype [22]. The non-dengue febrile patients were residents of the same areas of Indonesia and belonged to the same age group (mean 7.7 years, range 2-14 years). Of this group 43% were females and the mean duration of fever was 9.0 days. Non-dengue febrile patients were tested negative for malaria, epstein-barr virus, measles virus, rubella virus, influenza virus and rickettsia species, whereas only one of these patients tested IgM positive for chikungunya virus.

DENV antigens. DENV 1 (strain CDC), DENV 2 (strain N. Guinea C) DENV 3 (strain H 87) and DENV 4 (strain Hawaii 241) were used to infect C6/36 insect cells. The infected cells were cultured in Leibovitz medium supplemented with antibiotics and 1% fetal bovine serum (FBS), for 5 to 6 days. When more than 90% of the cells were infected by the virus (as determined by immunofluorescence assay), the culture supernatants were discarded. Subsequently the cells were harvested and acetone extracted as follows: one part of DENV infected cells suspension was mixed with 20 parts of ice-cold acetone (-20^oC). The mixture was centrifuged for 10 minutes at 1,000 rpm and the supernatant acetone was discarded. The procedure was repeated one more time and the pellet was left to dry in a 37^oC incubator for two hours. The pellet was resuspended in PBS and stored in aliquots in -70^oC until use in the DENV IgA and IgG subclasses capture enzyme immunoassay (c-EIA). The protein concentration of the DENV antigens was determined with the Bradford assay as 1 mg/ml for all DENV serotypes.

Serology. **(i) IgM EIA.** A commercial kit (Focus Technologies, CA, USA) was used for the measurement of DENV specific IgM antibodies in the serum samples of the patients. The test was performed according to the procedures described by the manufacturer [18]. **(ii) IgA EIA.** The assay used for the detection of DENV specific IgA antibodies was a modification of a method described earlier [19]. Briefly, commercially available plates coated with monoclonal anti-human IgA antibody (Meddens Diagnostics, Vorden, The Netherlands) were used for the assay. After blocking the plates with 10% (w/v) skim milk (ELK, Campina, Eindhoven, The Netherlands), in PBS for 1 hour at 37^oC, 50µl of serum samples were added on the plates. Serum samples were diluted 1:500 in PBS containing 2% (w/v) milk, 5% (v/v) normal goat serum (ICN Biochemicals Inc, Ohio, USA), and 5% (v/v) each normal rabbit and normal mouse serum (Dako, Glostrup, Denmark), (EIA-buffer). After one hour incubation at 37^oC, unbound antibodies were washed away and 50µl of DENV 1-4 antigens diluted 1:50 in EIA-buffer with 5% (v/v) normal human serum (NHS) were added to the plates. The plates were incubated for 2 hours at 37^oC, washed again to remove unbound antigen and an anti-flavivirus monoclonal antibody conjugated with horseradish peroxidase (Focus Technologies, CA, USA), diluted 1:2000 in EIA+NHS buffer was added to the wells for 1 hour. Plates were washed again and developed with 2,2,4,4 tetramethylbenzidine (TMB) as substrate. The reaction was stopped with the addition of 100 µl/well of 1N H_2SO_4 and the extinction was read at 450 nm (with 620 nm reference filter). **(iii) IgG subclass EIA.** An in-house c-EIA system was developed for the determination of DENV specific IgG subclasses in the DENV infected individuals and the control patients. Commercially available EIA microwells coated with monoclonal anti-human IgG 1, 2, 3 and 4 (C.L.B., Amsterdam, The Netherlands) were blocked with 10% (w/v) skim milk (ELK, Campina, The Netherlands), in PBS for 1 hour at 37^oC. The microwells were then washed three times with washing buffer (0.5% v/v Tween-20 in PBS). Serum samples were diluted 1:100 (or 1:1000 when tested for IgG 1) in PBS supplemented with 2% (w/v) skim milk (PBS-M). Fifty µl of diluted serum samples were then added into the microwells and incubated for 1 hour at 37^oC. The wells were washed again three times and a mixture of DENV 1-4 antigens, diluted 1:20 (50 ng/ml) in PBS-M supplemented with 5% (v/v) NHS was added into the wells (50 µl/well). The wells were incubated for 2 hours at 37° C, and washed again

as described above. An anti-flavivirus monoclonal antibody conjugated with horseradish peroxidase (Focus Technologies, CA, USA) diluted 1:2000 in PBS-M supplemented with 5% NHS was added to the wells for 1 hour. The wells were washed six times in the final step before they were developed with TMB as substrate. The reaction was stopped with the addition of 100 μl/well of 1N H_2SO_4 and the extinction was read at 450 nm (with 620 nm reference filter).

Table 1. Characteristics of DENV-infected and non-dengue (ND) febrile patients.

Disease severity	Grade	Sex n (%)		Mean age	Duration of fever
		Male	Female	Years (range)	Mean days
Febrile non-Dengue (ND)		12 (57)	9 (43)	7.7 (2-14)	9.0
Dengue Fever (DF)		36 (50)	36 (50)	7.4 (0.6-14)	7.0
Dengue Haemorrhagic Fever (DHF)	DHF I	8 (53)	7 (47)	8.7 (4-13)	8.6
	DHF II	7 (47)	8 (53)	8.1 (3-13)	9.1
Dengue Shock Syndrome (DSS)	DHF III	25 (44)	32 (56)	7.2 (3-14)	9.6
	DHF IV	4 (33)	8 (67)	5.4 (3-10)	11.8
Total		92 (48)	100 (52)	7.4 (0.6-14)	8.4

Calculations. The ratios of the DENV specific IgA and IgG subclass antibodies, were calculated with the following formula:

Ratio = (O.D. sample – O.D. blank)/3X(mean of the negative controls).

Where O.D.: optical density (extinction) of each sample.

For the calculation of IgM ratios the cut off serum of the manufacturer was used. For the calculation of statistical parameters a SPSS program was used. Since the distribution of the data were normal as determined by computer analysis, the significance of differences was calculated using the analysis of variance (one-way ANOVA) test. A two-sided P-value <0.05 was considered to represent a significant difference.

RESULTS

Kinetics of DENV specific Ig classes and subclasses in DF patients. The kinetics of DENV specific class and subclass serum antibodies in different patient groups are depicted in figure 1. In DF patients DENV specific IgM antibodies increased from mean ratio 2.2 ± 2.4 in the acute phase (days 2-4), to mean ratio 7.6 ± 5.0 in the early convalescent phase (days 8-11), reached the highest values on days 12-14 (mean ratio 8.9 ± 6.3), and decline (mean ratio 6.9 ± 6.0) in the convalescent phase (≥15 days). DENV specific IgA antibodies were only detectable in DF patients between days 8-11 after onset of fever (mean ratio 1.8 ± 1.7). The levels of DENV specific IgG 1 antibodies in DF patients were low in the acute phase (mean ratio 3.0 ± 4.3) to rise in the early convalescent (10.0 ± 6.7) and convalescent phases (9.4 ± 6.4). At days 2-4 after onset of fever, the levels of DENV specific IgG 2, IgG 3 and IgG 4 were low with mean ratios of 0.6 ± 1.2, 1.2 ± 2.0 and 0.6 ± 1.5 respectively, whereas these subclasses reached their highest levels on day 8-14 after onset of fever (mean ratios 3.2 ± 2.1, 4.4 ± 2.2 and 1.7 ± 2.5, respectively) to decline after 15 days (2.4± 2.0, 4.2 ± 2.6 and 1.3 ± 2.0 respectively).
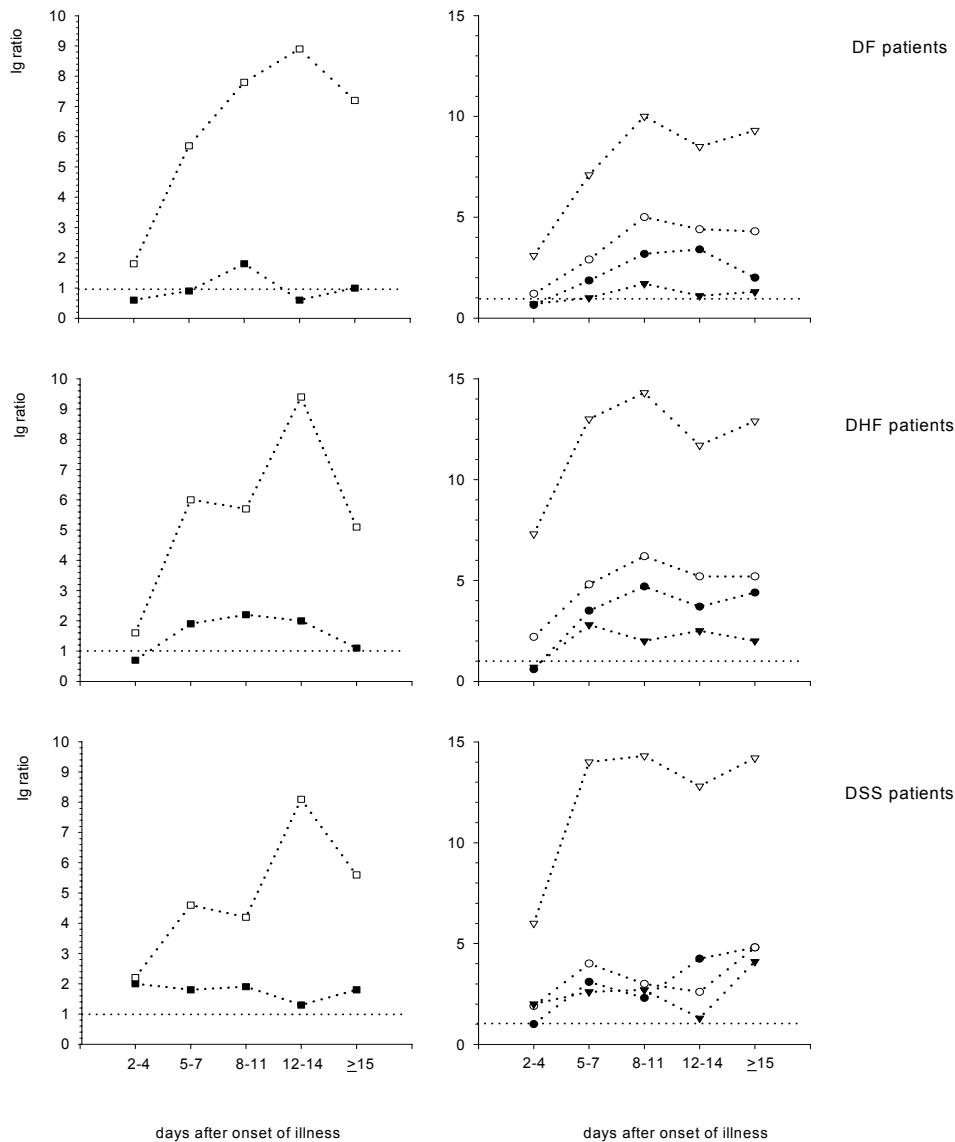
Figure 1: The kinetics of DENV specific IgA, IgM and IgG subclass responses in DENV infected patients with varying disease severity, over a period of two weeks after onset of fever expressed in mean ratios. Open squares: IgM responses, closed squares: IgA responses, open triangles: IgG 1 responses, closed circles: IgG 2 responses, open circles: IgG 3 responses and closed triangles: IgG 4 responses.

Kinetics of DENV specific Ig classes and subclasses in DHF patients. DENV specific IgM antibodies in the DHF patients had lower mean values (ratio 3.4 $\pm$ 4.0) on days 2-4 than on days 5-14 (mean ratio 9.4 $\pm$ 9.1) or from day 15 onwards (ratio 6.0 $\pm$ 5.5). The levels of DENV specific IgA antibodies were undetectable in the DHF patients in the acute phase of illness (days 2-4) (mean ratio 0.7 $\pm$ 0.7) but increased in the following early convalescent phase (days 5-14) to a mean ratio 2.2 $\pm$ 1.2, to decrease to a mean ratio 1.1 $\pm$ 0.8 from day 15 onwards. DENV specific IgG 1 was the predominant subclass in this patient group with ratios as high as 7.3 $\pm$ 5.2, on days 2-4, increasing to the highest levels (mean ratio 14.3) on days 8-11 and slightly declining from day 15 onwards (mean ratio 12.0 $\pm$ 5.2). DENV specific IgG 2 was undetectable in acute DHF patients (mean ratio 0.6 $\pm$ 0.6) but increased to highest levels on days 8-11 (mean ratio 4.7 $\pm$ 1.5) and slightly decreased 15 days after onset of fever (mean ratio 4.0 $\pm$ 2.1). In contrast, DENV specific IgG 3 antibody levels were

detectable in acute phase DHF patients (mean ratio 2.6 ± 2.3), increased to highest values in early convalescent phase to mean ratio 6.2 ± 0.6 and decreased in the convalescent phase to mean ratio 5.4 ± 2.0. DENV specific IgG 4 antibodies were not detectable in the acute DHF but increased rapidly to their highest values (mean ratio 2.6 ± 2.4) by days 5-7 after onset of fever and declined again to mean ratios 2.0 ± 2.2 in the convalescent phase (figure 1).

Kinetics of DENV specific Ig classes and subclasses in DSS patients. DSS patients showed low levels of DENV specific IgM antibodies on days 2-4 (mean ratio 2.2 ± 2.1), which had increased on days 12-14 to mean ratio 8.1 ± 5.0, and decreased to mean ratio 5.6 ± 4.0 on day 15. DENV specific IgA antibodies in DSS patients in the acute phase had mean ratio of 2.0 ± 2.5, increased to highest levels (mean ratio 1.9 ± 1.4) on day 8-11 and slightly decreased 15 days after onset of fever (mean ratio 1.8 ± 1.6). Low DENV specific IgG 1 antibody levels in the acute phase (mean ratios 6.0 ± 5.0), reached highest values in early convalescent phase (ratio 14.3) and remained at high levels in the convalescent phase (mean ratio 14.1 ± 0.5). DENV specific IgG 2 antibodies were hardly detectable on days 2-4 in DSS patients (mean ratios 1.0 ± 0.8) but the levels of DENV specific IgG 2 increased slowly to reach their highest levels on day 15 (mean ratio 4.8 ± 1.5). DENV specific IgG 3 antibodies were low in acute DSS patients (mean ratio 1.9 ± 1.6) and reached their highest levels ≥15 days after onset of fever (mean ratio 4.7 ± 1.7). The same pattern was seen in the levels of DENV specific IgG 4 antibodies (acute mean ratio 2.0 ± 2.1 and highest convalescent mean ratio 3.7 ± 2.4) (figure 1).

Statistical analysis of the kinetics of DENV specific Ig classes and subclasses. Statistical analysis revealed a significant increase between acute and convalescent phase of DENV infection in several Ig class ands subclass antibodies in the respective DENV patient groups (figure 2). In DF patients the levels of DENV specific IgM, IgA, IgG 1, IgG 2 and IgG 3 serum antibodies increased significantly in the convalescent phase (P=0.002, P=0.04, P=0.000, P=0.000 and P=0.000, respectively). In contrast, in DHF patients only DENV specific IgM, IgA, IgG 2 and IgG 3 increased significantly from the acute to the convalescent phase of illness (P=0.05, P=0.03, P=0.000 and P=0.002 respectively). Finally, in DSS patients DENV specific IgM, IgG 1 and IgG 2 revealed a significant increase in the convalescent phase of illness (P=0.006, P=0.000 and P=0.000, respectively).

Comparison of Ig class and subclass antibodies in patients with DENV infection and non-dengue febrile patients. Figure 3 depicts the comparison between patients with DENV infection and non-dengue febrile patients (controls) in the acute phase of infection. Non-dengue patients had significantly lower levels of DENV specific IgM antibodies compared with DF, DHF and DSS patients (P=0.006, P=0.004 and P=0.003 respectively). The levels of DENV specific IgA antibodies were significantly higher in DSS patients when compared with the non-dengue patients (P=0.007), but not between non-dengue patients and DF or DHF patients (P=0.814 and P=0.199 respectively). DENV specific IgG 1 and IgG 3 antibodies were significantly lower in controls than in all DENV patients (P=0.011 for DF, P=0.000 for DHF and P=0.000 for DSS, for both subclasses), whereas DENV specific IgG 2 antibodies were significantly lower in the controls when compared with DHF and DSS patients (P=0.017 and P=0.000 respectively) but not between non-dengue and DF patients (P=0.199). Finally, in the non-dengue febrile patients the levels of DENV specific IgG 4 antibodies were
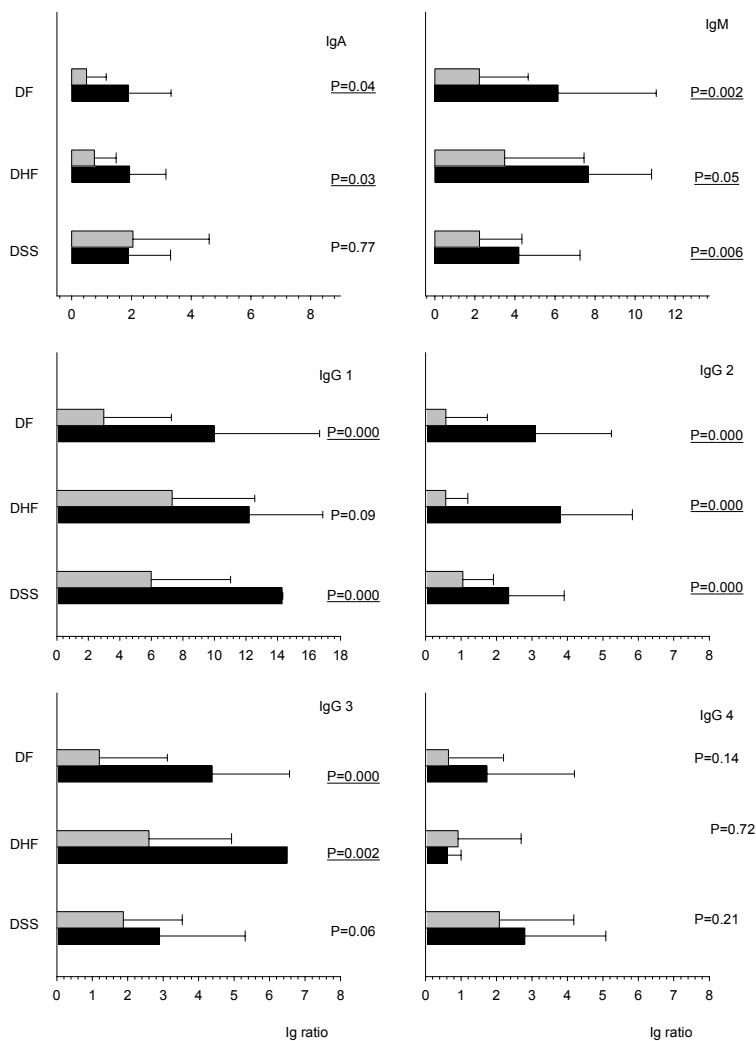
Figure 2: Comparison of DENV specific Ig class and subclass responses in DENV-infected patients with varying severity, in the acute phase of disease (light grey bars) and the convalescent phase (black bars) of dengue disease.

significantly lower than the levels of this subclass in the DSS patients (P=0.000) but were not significantly different between controls and DF or DHF patients (P=0.133 and P=0.057 respectively).
 Acute phase DENV specific IgM and IgG 2 antibodies were the only antibodies that did not differ significantly among the respective DENV patients (figure 3). DENV specific IgA and IgG 4 antibodies were significantly higher in the DSS group when compared with the DF group (figure 3). DENV specific IgG 1 antibodies differed significantly when DF patients were compared with DHF or DSS patients, whereas DENV specific IgG 3 antibodies differed only between DF and DHF patients (figure 3).

DISCUSSION

In the present study we measured the kinetics of specific IgM, IgA and IgG subclass antibodies against DENV in patients with varying disease severity, upon DENV infection. We demonstrated that DENV specific IgM, IgG 1 and IgG 3 antibodies were the predominant immunoglobulins throughout the course of illness in DF, DHF and DSS patients, whereas DENV

specific IgA, IgG 1 and IgG 4 serum antibodies were parameters associated with the development of DHF and DSS.

All DENV patients had significantly higher DENV specific IgM antibodies than non-dengue febrile patients in the acute phase of illness (days 2-4) and these levels increased significantly in the convalescent phase (days $\geq$15). These findings indicate that the presence of DENV specific IgM serum antibodies is rather associated with primary or secondary DENV infection than disease severity. Low levels of DENV specific IgM serum antibodies in some non-dengue patients may represent up to eight months past infection and may not be associated with present DENV infection [23].

Previous studies have underlined the importance of detection of IgA serum antibodies in patients with suspected DENV infections [20,19]. Acute DF patients display low or undetectable DENV specific IgA serum antibody levels. In the acute phase of the illness, patients who developed shock had significantly higher levels of DENV specific IgA when compared with DF or DHF patients.

It is known that IgG 1 and IgG 3 can fix complement most effectively, whereas IgG 2 can fix complement to a lower extent [10,11]. The complement system has been associated with shock syndromes [24]. In addition, activation of complement may induce clotting and hence may be implicated in intravascular coagulation, both complications seen in DHF and DSS. In our study we demonstrated that in the acute phase of DENV disease (days 2-4 after onset of fever), levels of DENV specific IgG 1 differ significantly between non-dengue patients and DF, DHF and DSS patients, between DF and DHF and between DF and DSS patients. These data suggest an important role for DENV specific IgG 1 in the development of severe form of DENV disease and as a prognostic marker for the development of severe disease. DENV specific IgG 1 was the predominant subclass throughout the course of DENV illness and remained at high levels during the convalescent phase of all DENV patient groups. DENV specific IgG 2 was present from day 5 after onset of fever onwards in all patient groups with no differences in the acute phase between any of the patients groups. However, the difference between controls and DHF/DSS patients was significant, differentiating febrile patients from those with haemmorhagic complications. DENV specific IgG 3, followed a similar pattern as IgG 1 in the acute phase as well as during the convalescent phase. Despite the lack of significant difference between acute DF and DSS and the rather low levels of DENV specific IgG 3 in DSS patients the results support the idea that high levels of DENV specific IgG 1 in combination with DENV specific IgG 3 levels may be used as a prognostic marker for severe DENV disease in the acute phase of illness. Production of IgG 4 serum antibodies is stimulated by production of IL-4 a Th2 cytokine [25]. Taken into consideration that Th2 responses are associated with severe pathology and with an exacerbation of many other viral infections such as HSV, influenza virus and HIV infection [26] and that a shift towards Th2 responses is seen in DSS [27], it is reasonable to speculate that elevated levels of DENV specific IgG 4 in acute DENV infection may be prognostic for development of DSS. We could confirm that DENV specific IgG 4 was present during acute and convalescent DENV infection, and more importantly that the levels of this subclass were significantly higher in DSS patients than in control or DF patients. Our findings are in agreement with findings of a previous study, where it was also demonstrated that IgG 1 and IgG 3 are the predominant subclasses in DENV infections [21]. Persistence of the complement fixing subclasses (IgG 1 and 3) and the cytokine induced IgG 4 in DHF
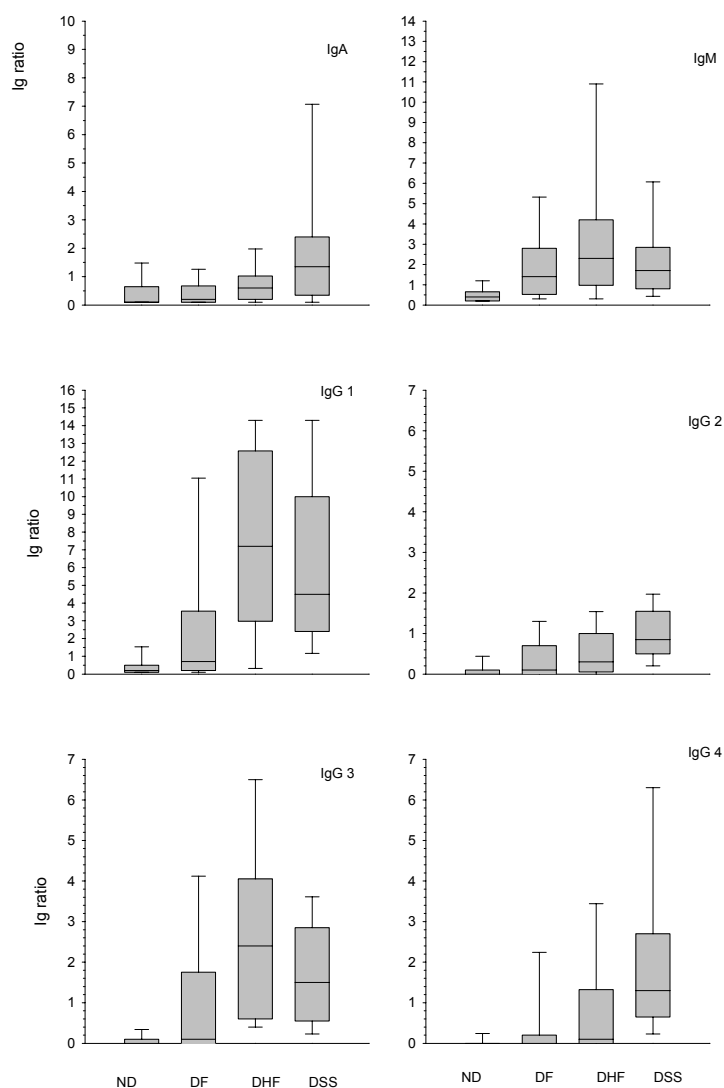
Figure 3: Comparison of DENV specific Ig class and subclass responses in the acute phase of illness in patients with varying disease and non-dengue (ND) febrile patients. Boxes indicate the interquartile range for each patient group. The median is indicated by the line within each box. The "whiskers" represent the extremes of Ig ratios.

and/or DSS patients supports the hypothesis that immunological factors such as ADE and complement activation products may contribute in dengue disease severity.

Taken together, our study showed that measurement of DENV specific IgA serum antibodies in the acute phase is a valuable tool in the diagnosis of DENV infection along with the traditional methods of measuring IgM or rise in IgG titers in patients suspected of DENV infection. Furthermore we showed that in addition to levels of DENV specific IgA, also those of DENV specific IgG 1, IgG 3 and IgG 4 correlate with severity of disease outcome. Therefore, measurement of the levels of these specific immunoglobulins may provide valuable guidance for decisions about intensity of treatment in the early stages of the infection, thus lowering disease fatality rates.

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Chapter 3.2

Elevated Levels of Total and Dengue Virus Specific Immunoglobulin E in Patients with Varying Disease Severity

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ABSTRACT

The kinetics of total and dengue virus-specific immunoglobulin E (IgE) were studied in serial serum samples obtained from 168 patients, 41 of which suffered from primary dengue virus infection and 127 suffered from secondary dengue virus infection. Seventy-one patients were classified as dengue fever, 30 as dengue hemorrhagic fever, and 67 as dengue shock syndrome. A control group included single serum samples from patients with a herpes virus infection (n=14), non-dengue febrile patients (n=10), and healthy blood donors (n=10). Patients with dengue virus infection had higher levels of total and dengue virus-specific IgE than non-dengue patients ($P < 0.05$). Patients with secondary dengue virus infections had not significantly increased levels of both total and dengue virus-specific IgE in the acute phase of disease compared to patients with primary dengue virus infections. Dengue virus-specific IgE was significantly higher in dengue hemorrhagic fever and/or dengue shock syndrome patients compared to dengue fever and non-dengue patients ($P < 0.05$). In conclusion, this study showed elevated total and dengue virus-specific IgE serum antibody levels in the acute stage of disease. Therefore, measurement of both total and dengue virus-specific IgE serum antibodies can be used as an additional prognostic marker in the development of severe complications in dengue virus infections. In addition, the presence and increase of dengue virus-specific IgE serum antibodies in patients with dengue virus infections, is suggestive of the pathogenetic role that IgE may play in the hemostatic disorders observed in dengue hemorrhagic fever and dengue shock syndrome.

INTRODUCTION

Dengue virus a member of the family *Flaviviridae* is a mosquito borne virus mainly distributed in the tropical and sub-tropical regions of the world [1]. So far, four serotypes (Dengue virus 1 to 4) have been identified, causing disease in man. The virus is transmitted to humans by the bite of infected mosquitoes mainly of the *Aedes aegypti* and *Aedes albopictus* species. The majority of dengue virus patients will develop dengue fever characterized by fever, chills, frontal headache, myalgia, arthralgia and a rash. In some cases the infection may lead to more severe dengue hemorrhagic fever with plasma leakage. Usually also conjunctival effusion, facial flushing and trucal erythema are present [2]. According to WHO criteria, dengue hemorrhagic fever is subdivided further into four grades of severity. Grades 1 and 2 correspond to dengue hemorrhagic fever, whereas grades 3 and 4 correspond to dengue shock syndrome characterized by circulatory failure and might become life threatening because of profound hypovolemic shock [2]. However, other severe forms of dengue virus have been reported that do not fit to the WHO classification [3,4]. The disease severity of dengue virus has so far mainly brought into relation with antibody-dependent enhancement mechanisms as a result of sequential infection with different dengue virus serotypes [5,6]. There is an increasing evidence that also other mechanisms are involved in the disease severity of dengue virus infections such as cytokines, viral load and virulence of certain viral strains [7,8,9,10].

Recently, it was demonstrated that IgM, IgG 1 and IgG 3 antibodies were the predominant immunoglobulins through the course of illness in dengue fever, dengue hemorrhagic fever and dengue shock syndrome patients. Increased levels of dengue specific IgA, IgG 1 and IgG 4 serum antibodies are risk markers for the development of dengue hemorrhagic fever/dengue shock syndrome [11]. High levels of total and virus-specific IgE are associated with an anaphylactic reaction [12]. In patients with hemorrhagic fever virus infections it was shown that both total and specific IgE levels are increased [13,14,15,16]. Recently, it was demonstrated that the total IgE levels in patients with a history of dengue virus infection, increased compared to individuals without a history of dengue virus infection [17]. This relationship between elevated total IgE and dengue virus infection may indicate the role of IgE in immunohomeostasis. Increased levels of total IgE in dengue virus infected patients may also indicate that a balanced immune response might lead to disease resolution, rather than the induction of a particular T-helper cell pathway. It is also known that infections with dengue virus induce interleukin 4 (IL-4) production [18] which is involved in IgE synthesis in humans [19].

The aim of the present study was to investigate whether there is a correlation between total and dengue virus -specific IgE response in relation to disease severity and if dengue virus -specific IgE antibodies could be used as a prognostic marker for disease severity.

MATERIALS AND METHODS

Serum samples. Serial serum samples were collected from 168 patients with clinically and serologically confirmed dengue virus infection. According to the criteria of the World Health Organization [2], from the 168 dengue virus infected patients, 71 patients were defined as dengue fever cases, 30 patients were defined as dengue hemorrhagic fever cases and the remaining 67 patients were defined as dengue shock syndrome cases. Samples had been collected during the 1995-1996 dengue virus epidemic in Indonesia, where all dengue virus

serotypes were circulating with Dengue virus 3 being the most predominant serotype [20]. The age of the patients varied between 7 months and 14 years and 52% of the patients were females. The characteristics of the dengue virus infected patients are summarized in Table 1. A control group included 14 samples obtained from patients with herpes virus infection (seven patients with an Epstein-Barr virus infection and seven patients with cytomegalovirus infection), ten samples obtained from patients with a febrile illness other than dengue virus, which were all residents of the same endemic areas in Indonesia as the dengue virus infected individuals and ten samples from Dutch healthy blood donors. In addition, eight samples were included from patients with elevated levels of IgE to serve as positive controls for the measurement of total IgE.

IgM/IgG enzyme immunoassay: Dengue virus-specific IgM and IgG antibodies were measured in the serum samples of the patients using a commercially available EIA kit (Focus Technologies, Cypress CA, USA), and performed according to the procedures described by the manufacturer. *Total IgE enzyme immunoassay:* Medium binding plates (Greiner, Fric Kenhausen, Germany) were coated with goat anti-human IgE antibodies (Cappel Cooper Biomedical, West-Chester, PA, USA) diluted 1:2,000 in carbonate buffer (pH 9.6) overnight at 4° C. After blocking the plates with 5% (w/v) skim milk (ELK Campina, Eindhoven, The Netherlands) in PBS for 30 min at 37° C, 100µl of diluted serum samples were added to the plates. Serum samples were diluted 1:100 in PBS containing 0.2% (w/v) milk, 0.1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo, USA), 0.1% (w/v) ovalbumin (Sigma Chemical Co., St. Louis, Mo, USA), 1% (v/v) fetal bovine serum (ICN Biochemicals Inc, Ohio, USA) and 5% (v/v) normal goat serum (ICN Biochemicals Inc, Ohio, USA) (EIA-buffer). After one hour incubation at 37° C, unbound antibodies were washed away and 100µl of rabbit anti-human IgE peroxidase labeled conjugate (DAKO, Glostrup, Denmark) diluted 1:500 in EIA-buffer were added to the plates. Plates were incubated for an additional hour at 37° C, and excess of conjugate was washed away. The plates were developed with 2,2,4,4 tetramethylbenzidine for 15 minutes at room temperature, the reaction was stopped with the addition of 1N H_2SO_4 and the extinction of the samples was read at 450 nm (with 620 nm reference filter). *Dengue virus-specific IgE enzyme immunoassay:* Plates were coated and blocked as described for the total IgE enzyme immunoassay. Serum samples were diluted 1:100 in EIA-buffer and added in 100µl volumes on the plates for one hour at 37° C. Unbound antibodies were washed away and a mixture of all four dengue virus serotypes prepared as previously described [11] was added to the plates diluted 1:20 in EIA-buffer supplemented with 5% (v/v) normal human serum. After overnight incubation at 4^oC plates were washed to remove excess of antigen and incubated for one hour at 37^oC with an anti-Flavivirus monoclonal antibody, peroxidase conjugated (Focus Technologies, Cypress CA, USA) diluted 1:2,000 in EIA-buffer with normal human serum. Plates were washed three times and developed as described in the procedure for total IgE.

Table1: Classification and characteristics of dengue virus infected patients included in the study. DF: dengue fever, DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome.

Classification		Patients	Sex		Mean age	Mean IgM/IgG
		n	Male	Female	years	ratio
Primary infections	DF	29	13	16	7.0	9.4
	DHF	5	4	1	6.8	9.7
	DSS	7	3	4	6.2	1.4
Subtotal		41	20	21	6.7	6.8
Secondary infections	DF	42	24	18	8.7	0.3
	DHF	25	11	14	8.7	0.4
	DSS	60	25	35	7.1	0.3
Subtotal		127	60	67	8.2	0.3

Calculations. For the calculation of IgM and IgG antibody ratios the cut-off serum of the manufacturer was used. Dengue virus infections were defined as primary when the ratio of dengue virus-specific IgM/IgG serum antibodies was higher than one, if the ratio of dengue virus-specific IgM/IgG serum antibodies was lower than one, the infections were defined as secondary dengue virus cases [2].

For the calculation of total IgE levels in the serum samples a standard curve was constructed using different dilutions of a positive reference serum sample. The concentration of the total IgE antibodies was calculated after adjustment of the dilution factor.

The ratios of the dengue virus-specific IgE antibodies were calculated according to the following formula:

Dengue virus-specific IgE ratio = (O.D. sample – O.D. blank) / (mean of negative controls + 3X S.D).

Where O.D.: Optical density (extinction) of each sample.

Univariate and multivariate statistical analysis was successively performed using BMDP software. The univariate statistical tests allow showing a relation between two variables but without adjusting for the influence of other variables. Making a multivariate statistical analysis and thus, after adjusting for the other variables and their interactions, it could be possible that a significant relation using univariate test disappear. When the variables are numerous, the results of the univariate analysis may be useful in order to select the variables to be included in the multivariate analysis. First, univariate analysis was performed. Using Spearman test, Student test and ANOVA, which allows to assess the significance of group differences of a grouping variable, the relation of the dependent variables, i.e. the level of dengue virus-specific IgE and the independent variables, i.e. age, sex and days post onset of illness of the patients, severity of infection, immunity to dengue virus (primary and multiple dengue virus infection) and the level of total IgE, IgM and IgG were assessed. Second a multivariate analysis was performed, using analysis of covariance (ANCOVA). Since the distribution of the data was normal as determined by computer analysis and in order to take into account the interactions of the independent variable in a global model, in a second time the independent variables were included in an ANCOVA descriptive model. An analysis of covariance can be viewed as a combination of analysis of variance (ANOVA) and regression. It was used to refine the univariate analysis by adjusting for covariates that may be related linearly to the outcome that is being investigated. Consequently, ANCOVA is conducted in order to show some variables, which could be significant using the univariate analysis and which have finally no significant role. A two-sided p-value <0.05 was considered to represent a significant difference. The results of the ANCOVA model allow assessing the real influence of the studied independent variable on the IgE specific level because this model takes into account the interactions of the different variables.

RESULTS

Classification of dengue virus infected patients. The clinical classification and the characteristics of the patients included in this study are summarized in Table 1. Based on the IgM/IgG ratio of the serum sample collected at the acute phase of illness, 41 out of the 168 dengue virus infected patients had a primary dengue virus infection (mean IgM/IgG ratio 6.8) and 127 patients had a secondary dengue virus infection (mean IgM/IgG ratio 0.3). Of the 41 primary infections, 29 patients had dengue fever (mean IgM/IgG ratio 9.4), five patients developed dengue hemorrhagic fever (mean IgM/IgG ratio 9.7) and seven patients developed dengue shock syndrome (mean IgM/IgG ratio 1.4). Of the 127 secondary infections, 42 patients had dengue fever (mean IgM/IgG ratio 0.3), 25 patients developed dengue hemorrhagic fever (mean IgM/IgG ratio 0.4) and 60 patients developed dengue shock syndrome (mean IgM/IgG ratio 0.3) (Table 1).

Table 2: Relation of the levels of dengue virus (DEN)-specific IgE antibodies and the day post onset of symptoms and the levels of total IgE and dengue virus-specific IgM and IgG.

	P value (Pearson test)
Day post onset of symptoms	0.0011
Level of total IgE	$<10^{-9}$
Level of DEN-specific IgM	0.53
Level of DEN-specific IgG	$<10^{-17}$

Kinetics of total and dengue virus-specific IgE serum antibodies in dengue virus infected patients. Levels of total IgE remained stable during acute dengue fever (days 1 to 7 post onset of symptoms), early convalescent dengue fever (days 8 to 13 post onset of symptoms) and convalescent phase of dengue fever (more than 14 days post onset of symptoms) with mean + standard deviation varying between 125+196, 108+162 and 124+203 U/L in the respective phases of illness (Fig. 1a). In the contrary, dengue virus-specific IgE serum antibodies increased from mean ratio 0.9+1.1 in acute dengue fever to 1.9+1.6 and 1.5+1.4 during early convalescent and convalescent dengue fever respectively (Fig. 1b).

Elevated levels of total IgE serum antibodies were also observed in the dengue hemorrhagic fever group of patients with mean U/L 154+206, 50+84 and 106+133 during acute, early convalescent and convalescent dengue hemorrhagic fever respectively (Fig. 1a). Dengue virus-specific IgE serum antibodies in dengue hemorrhagic fever patients were elevated at mean ratios 2.2+1.6, 2.0+1.3 and 1.8+1.1 during acute, early convalescent and convalescent phase of dengue hemorrhagic fever (Fig. 1b).

Patients that developed dengue shock syndrome had the highest mean values of total IgE serum antibodies in the acute phase of infection (331+285 U/L) and during early convalescent phase of infection (300+265 U/L), although the levels of total IgE decreased to 56+86 U/L in convalescence (Fig. 1a). Dengue virus-specific IgE serum antibodies in dengue shock syndrome patients was in rather stable levels during all stages of infection with mean ratios of 2.6+1.7, 3.3+2.1 and 2.7+2.3 during acute, early convalescent and convalescent phase of infection, respectively (Fig. 1b).

Levels of total and dengue virus-specific IgE serum antibodies in acute phase dengue virus infected patients. Results of the univariate analysis: dengue virus -specific IgE serum antibodies were significantly related to the day post onset of symptoms, severity of disease, immunity to dengue virus (primary vs. secondary infections) and the level of total IgE and IgG. Dengue virus-specific IgE serum antibodies were not significantly related to the age of the patients or to the levels of IgM antibodies (Tables 2, 3, 4). These results have to be confirmed by the results of the multivariate analysis.

Results of the ANCOVA after adjusting for covariates: Taken into account the influence of the other independent variables (Table 5), the ANCOVA showed that the level of dengue virus-specific IgE increased significantly over time (regression coefficient=0.04, P=0.027). The level of dengue virus-specific IgE increased significantly with the level of IgG (regression coefficient =0.16, P=0.0001) and

Table 3: Comparison of the level of dengue virus-specific IgE serum antibodies following the level of disease severity and the age category of the patients.

	mean	standard deviation	df[1]	F value	p value (ANOVA)
Dengue severity (WHO classification)			4	28.6	$<10^{-21}$
DF	1.06	1.27			
DHF 1	2.37	1.52			
DHF 2	1.89	1.47			
DHF 3	2.66	1.6			
DHF 4	3.78	2.89			
Age			2	1.13	0.32
0-5 years	2.27	2.17			
6-10 years	2.05	1.74			
11-15 years	1.89	1.58			

[1]: degrees of freedom

at less degree with the level of total IgE (regression coefficient =0.001, P=0.0006). Dengue virus-specific IgE decreased slowly but significantly with the age of the patient (regression coefficient =-0.06, P=0.016). The level of IgM had no influence on the level of dengue virus-specific IgE. The model also showed no influence of the immunological status of the dengue virus infections (P=0.19) and no significant interaction between the immunological status and the severity of dengue illness (P=0.38) (Table 5).

Table 4: Comparisons of the mean level of dengue virus (DEN)-specific IgE following the sex and the immunity status of the patient.

		mean	standard deviation	p value (Student test)
Sex	male	1.89	1.73	0.08
	female	2.19	1.82	
Immunity status	primary DEN infection	1.00	1.32	$<10^{-9}$
	secondary DEN infection	2.34	1.79	

Taken into consideration the other independent variables, the ANCOVA allowed us to calculate adjusted values of the dengue virus-specific IgE serum antibodies and it was demonstrated that the level of dengue virus-specific IgE was higher among dengue shock syndrome patients (Fig. 2). In detail, the level of dengue virus-specific IgE serum antibodies increased from mean ration 1.60 in primary dengue fever patients to mean ratio 2.03 and 1.24 in primary dengue hemorrhagic fever 1 and

2 patients respectively (dengue hemorrhagic fever patients) to reach highest levels in primary dengue shock syndrome patients (mean ratios 1.85 and 2.85 in dengue hemorrhagic fever grade 3 and 4 patients respectively). The trend of dengue virus-specific IgE serum antibodies during secondary infections was not significantly different with primary infections (Table 5), increasing from mean ratio 1.37 in dengue fever patients to mean ratios of 2.36 and 1.82 in dengue hemorrhagic fever patients (dengue hemorrhagic fever 1 and 2 respectively) and reached highest values in dengue shock syndrome patients with mean ratios of 2.35 and 3.72 among dengue hemorrhagic fever 3 and 4 patients, respectively.

Comparison of total and dengue virus-specific IgE serum antibodies among dengue and non-dengue febrile patients. Although the levels of total IgE serum antibodies between reference IgE serum (positive controls) and dengue fever, dengue hemorrhagic fever and dengue shock syndrome patients were not significantly different, the levels of dengue virus-specific IgE serum antibodies were significantly different between IgE reference serum and dengue hemorrhagic fever patients (P=0.03) or between IgE reference serum and dengue shock syndrome patients (P=0.002). Statistical analysis between control group (non-dengue febrile patients and blood donors) and acute phase dengue fever patients did not revealed a significant difference between these two groups (P=0.8 and P=0.4 for total and dengue virus-specific IgE respectively). The levels of total IgE antibodies did not differ significantly between non-dengue patients and acute phase dengue hemorrhagic fever patients (P=0.539), however, dengue virus-specific IgE antibodies was significantly different between acute phase dengue hemorrhagic fever patients and non-dengue patients (P=0.000). In acute phase dengue shock syndrome patients the levels of total and dengue virus-specific IgE serum antibodies were significantly higher than in non-dengue patients (P=0.000 for both total and dengue virus-specific IgE serum antibodies) (Fig. 3).
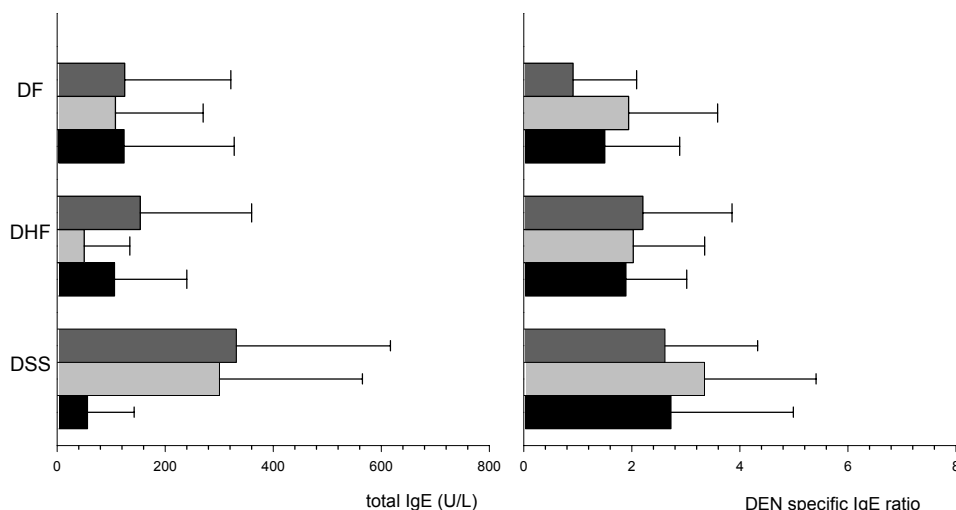

Figure 1: Kinetics of total and dengue virus (DEN)-specific IgE serum antibodies among the respective patient groups [dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)]. Dark gray bars represent the acute phase of disease, light gray bars represent early convalescent phase and black bars represent convalescent phase of the disease.

DISCUSSION

In the present study we measured the levels of total and dengue virus-specific IgE serum antibodies in patients with dengue virus infection of varying disease severity. We showed increased levels of total and dengue virus-specific IgE serum antibodies in the acute phase of illness in patients with primary and secondary dengue virus infections. The levels of total IgE serum antibodies in primary and secondary dengue virus infected patients measured in this study are in agreement with the results studied in similar patient groups, by Miguez-Burbano and colleagues [17]. The findings that total IgE serum antibodies are elevated in patients with primary and secondary dengue virus infections compared to non-dengue patients, indicates that dengue virus infections exert a strong stimulatory effect on IgE synthesis. The non-dengue group of patients included patients with acute Epstein Barr virus and cytomegalovirus infections, which also cause elevation of the levels of total IgE in the serum [21,22], resulting in a non-significant difference between the non-dengue patients and patients with a primary dengue virus infection. Increased levels of total IgE serum antibodies in the acute phase of disease of both primary and secondary infections may indicate that dengue virus infections also stimulate a non-antigen-specific activation pathway for IgE production.

It was also demonstrated that patients with dengue shock syndrome had significantly elevated levels of total IgE serum antibodies as compared to patients with dengue fever (P=0.000) or dengue hemorrhagic fever (P=0.002). In addition, all dengue virus infected patients had elevated levels of total IgE (when compared with total positive IgE reference samples), however patients with dengue virus infection had significantly higher levels of total IgE serum antibodies than patients with a non-dengue infection (P<0.05). Previous reports have suggested that elevated levels of total IgE serum antibodies in patients suffering from hemorrhagic fever virus infections, are associated with disease severity
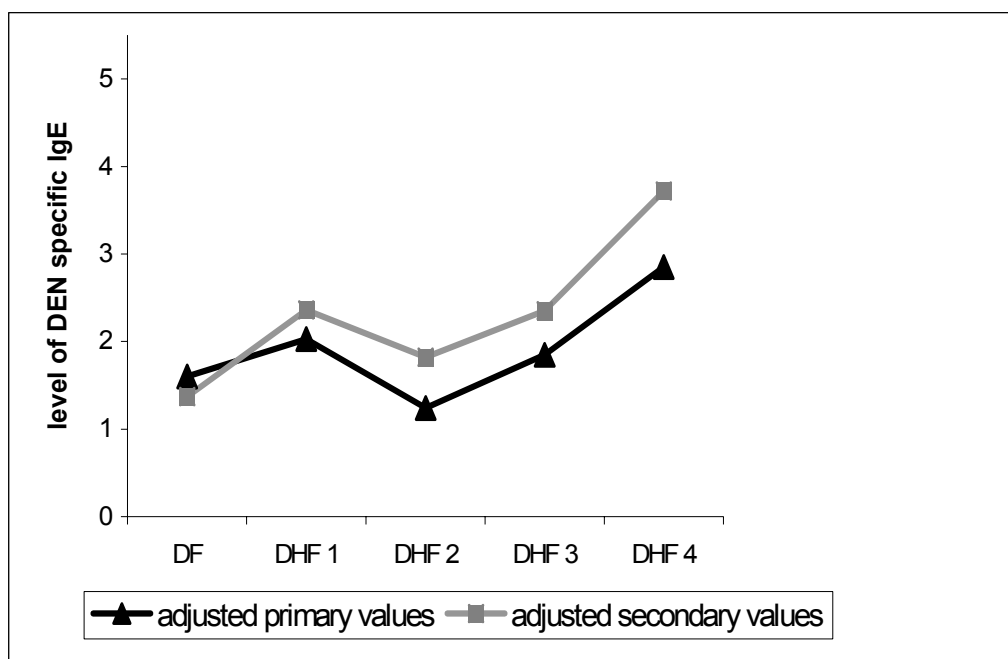

Figure 2: Levels of dengue virus (DEN)-specific IgE in correlation with dengue disease severity. The adjusted values resulted form the ANCOVA model took into account the influence of all variables in the model.

[17,15]. Pavri and colleagues have suggested that increased levels of total IgE serum antibodies are implicated in the pathogenesis of Kyasanur Forest disease, Japanese B encephalitis and dengue hemorrhagic fever [14,15,16]. Alexeyev and colleagues demonstrated elevated levels of total and Puumala virus specific IgE serum antibodies early in the course of hemorrhagic fever with renal syndrome suggesting that IgE may be involved in the pathogenesis of this disease [13].

Furthermore, the levels of dengue virus-specific IgE serum antibodies in patients with dengue virus infection were measured. The results showed that dengue virus-specific IgE was significantly elevated in dengue hemorrhagic fever and dengue shock syndrome patients than in non-dengue or dengue fever patients ($P<0.05$). In addition, all non-dengue patients and IgE positive reference serum samples were negative when tested for the presence of dengue virus-specific IgE serum antibodies, resulting in high specificity of the dengue virus-specific IgE serum antibody measurement assay. It was also demonstrated that dengue virus-specific IgE serum antibodies in the acute phase of dengue virus infection is significantly higher in patients that developed dengue hemorrhagic fever and/or dengue shock syndrome compared to dengue fever patients ($P<0.001$). In addition, the levels of dengue virus-specific IgE serum antibodies were significantly associated with the day post onset of symptoms of the patients as well as levels of total IgE and IgG. In contrast, the levels of dengue virus-specific IgE serum antibodies did not correlate with the age or the sex of the patients or the level of IgM serum antibodies or the immunological status. These findings suggest that these parameters could be used as prognostic markers for developing severe complications during infection with dengue virus.

Table 5: Level of significance of the independent variables included in the descriptive ANCOVA model.

Source of ANCOVA	sum of squares	Regression coefficient[1]	df[2]	F value	p value
Severity of DEN	53.7		4	6.36	0.0001
Immunity status	3.6		1	1.73	0.19
Interaction: severity and immunity status	8.9		4	2.22	0.38
sex	0.79		1	0.38	0.53
day post onset of symptoms	10.38	0.04	1	4.92	0.028
level of total IgE	25.6	0.001	1	12.14	0.0006
level of IgM	0.005	-0.0006	1	0	0.96
level of IgG	58.64	0.16	1	27.81	$<10^{-4}$
Age	12.41	-0.06	1	5.89	0.016

[1]: only available for the quantitative independent variables, i.e. the covariables

[2]: degrees of freedom

The implication of IgE in increased vascular permeability (a common clinical complication in severe dengue virus infection) has been reported in the past [23]. Proinflammatory cytokines such as IL-1β and tumor necrosis factor, triggered by IgE may contribute to this phenomenon [24]. Both these cytokines are produced during dengue virus infection [18]. Immunoglobulin switch to the IgE class is stimulated by Th2 cytokines namely IL-4 and IL-13 in a lower extent [25,26]. Considering that Th2 immune responses are correlated with severe pathology in several other viral infections [27] and that in dengue shock syndrome a shift from Th1 to Th2 responses is observed [18], we might speculate that elevated IgE serum antibodies may be associated with severity of dengue virus disease. Recently, we demonstrated that dengue virus infected patients that developed dengue shock syndrome had increased levels of dengue virus-specific IgG 4 serum antibodies compared to patients with dengue fever/dengue hemorrhagic fever [11]. Production of IgG 4 is also stimulated by IL-4 like the production of IgE serum antibodies. In addition, dengue virus-specific IgE serum antibodies may play a role in hemostatic disorders seen in dengue hemorrhagic fever, via induction and release of vasoactive factors such as histamine, which has been shown to be increased in dengue hemorrhagic fever patients [28].

In conclusion, the pre-existing levels of total and dengue virus-specific IgE serum antibodies in patients with dengue virus infections and in particular the significantly higher levels of these antibodies in the acute phase of dengue hemorrhagic fever /dengue shock syndrome, strongly suggest that IgE responses may be involved in the pathogenesis of disease or be a reflection of the underlying immunological pathogenetic mechanism. Moreover, the results of this study suggested that elevated levels of total and dengue virus-specific IgE serum antibodies in the acute phase of dengue virus infection may be prognostic for developing severe complication of disease.
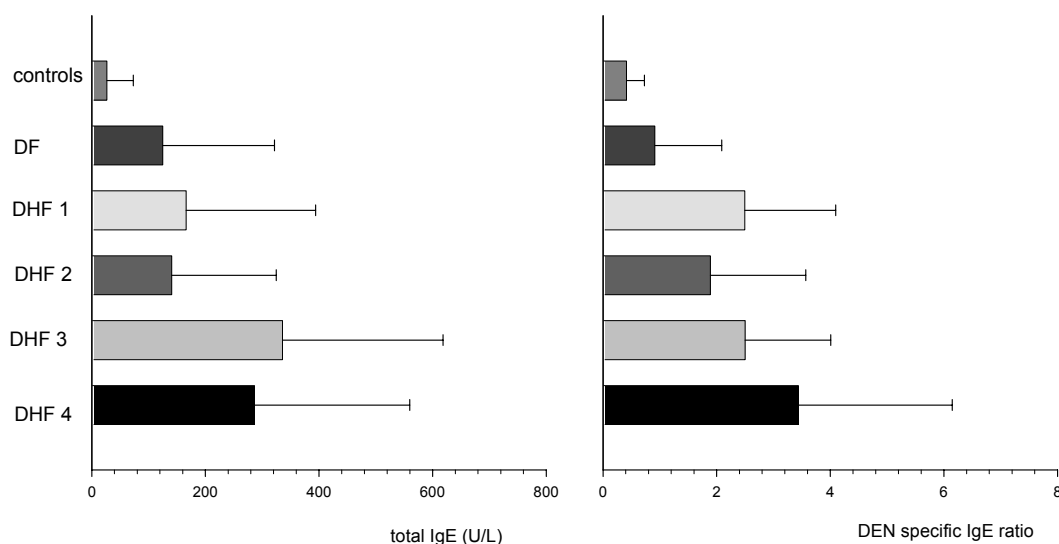
Figure 3: Levels of total and dengue virus (DEN)-specific IgE serum antibodies among the respective patient groups and controls in the acute phase of illness. DHF 1 and 2 patients compose the dengue hemorrhagic fever group of patients. DHF 3 and 4 patients compose the dengue shock syndrome group of patients. The control group includes patients with EBV and CMV infection, non-dengue febrile patients and patients with elevated levels of total IgE (positive controls).

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Chapter 3.3

Elevation of soluble VCAM-1 plasma levels in children with acute dengue virus infection of varying severity

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ABSTRACT

Approximately 1000 million infections with dengue viruses are estimated to occur annually. The majority of the cases develop mild disease, whereas only small proportion of the infected individuals develops severe hemorrhagic manifestations at the end of the acute phase of illness. In this study, the value of plasma levels of vascular cell adhesion molecule 1 (VCAM-1) in the pathogenesis and prognosis of dengue illness was investigated in children with dengue infections of varying severity. The plasma levels of soluble VCAM-1 (sVCAM-1) were measured in serial plasma samples obtained from 168 children aged between 7 months and 14 years with confirmed dengue infection. Of those children, 71 were suffering from dengue fever, 30 were suffering from dengue hemorrhagic fever and 67 were suffering from dengue shock syndrome. Plasma samples obtained from 21 patients with febrile illness other than dengue served as controls. A commercially available kit (R&D Systems, Oxon, UK) was used to measure the levels of sVCAM-1 in plasma samples. sVCAM-1 was elevated during acute dengue infection, and significantly elevated among dengue shock syndrome patients as compared to dengue fever or dengue hemorrhagic fever patients ($P<0.05$). Statistical analysis revealed that sVCAM-1 was associated with dengue disease severity and the time post infection (acute vs. convalescent phase) and not with age, sex or previous exposure of the patients to dengue infection. A significant difference was found in the plasma levels of sVCAM-1 between dengue shock syndrome and dengue fever patients, however the prognostic value of this marker in the acute stage of dengue illness proved to be limited. These data also favour to study the further elucidation of the role of sVCAM-1 in the pathogenesis of dengue infections.

INTRODUCTION

In the past decades dengue viruses have emerged as one of the most important vector-borne diseases worldwide. The transmitting mosquito is a member of the *Aedes* spp. and has a wide distribution in tropical and subtropical countries [1]. Infection with one of the four antigenically distinct dengue serotypes gives a wide spectrum of clinical symptoms varying from asymptomatic dengue infection, classic dengue fever a mild self-limited febrile illness, and dengue hemorrhagic fever with severe manifestations such as spontaneous haemorrhage and plasma leakage. When plasma leakage gets critical, patients with dengue hemorrhagic fever may develop shock, a condition known as dengue shock syndrome, which may be life threatening. Dengue viruses account for as many as 100 million infections annually with approximately 250,000 cases of dengue hemorrhagic fever with case fatality rates ranging from 0.1 to 15% [2].

Little is known about the pathogenesis of dengue virus infections. Interestingly, only a small proportion of patients experiencing secondary dengue virus infections will eventually develop dengue hemorrhagic fever/dengue shock syndrome, whereas only sporadically patients with primary dengue virus infection will develop dengue hemorrhagic fever/dengue shock syndrome [3],[4]. During acute phase of dengue virus infection, patients are febrile with flu-like symptoms (e.g. high fever, arthralgia, myalgia) and rash, whereas at the time of defervescence, some patients develop hemorrhagic manifestations and plasma leakage (dengue hemorrhagic fever/dengue shock syndrome). The plasma leakage is attributed to increased vascular permeability, however the mechanism for increased vascular permeability remains elusive. Previous *in vitro* studies have reported infection of endothelium with dengue viruses [5;6], although it has also been hypothesised that activation of the endothelium may be due to increased expression of adhesion molecules rather than due to infection of the cells *per se* [7].

Vascular cell adhesion molecule 1 (VCAM-1) is a member of the immunoglobulin super family and binds cells such as lymphocytes and monocytes. VCAM-1 is also found in cytokine-activated endothelial cells, macrophages and dendritic cells [8],[9]. Increased expression of sVCAM-1 as well as other adhesion molecules (such as intracellular CAM [sICAM]), results in enhanced binding of leukocytes and platelets and subsequent local inflammatory responses, endothelial cell damage and plasma leakage [10]. It is still unclear whether increased levels of soluble adhesion molecules are the cause or the result of activated endothelium. Elevation of sVCAM-1 has been observed in other infectious diseases such as cytomegalovirus infections, malaria or other acute bacterial or viral infections [11-13]. In a previous study, conducted in French Polynesia, it was reported that levels of sVCAM-1 were elevated in patients with dengue virus infections and among them sVCAM-1 levels were significantly higher in dengue hemorrhagic fever/dengue shock syndrome patients [14]. In this study we investigated further the prognostic role of sVCAM-1 as a diagnostic tool to predict severe outcome during the acute phase of dengue virus infection.

MATERIALS AND METHODS

Plasma samples. During the 1995-1996 dengue epidemic in Indonesia serial plasma samples from individuals with dengue virus infections (n=168) and from individuals with a febrile illness other than dengue (n=21) were collected. The characteristics of the dengue virus infected patients are described in Table 1. Classification of patients was based on clinical observations according to the WHO guidelines for grading dengue virus infections [15]. The plasma samples collected from individuals with infections other than dengue served as controls, and had been tested for diagnostic purposes and found negative for malaria, epstein-barr virus, measles virus, rubella virus, influenza virus and rickettsia species, whereas only one of these patients tested IgM positive for chikungunya virus. Controls matched dengue virus infected patients in age (mean 7.7 years), sex (53% male patients) and duration of fever (9.0 days) characteristics. All dengue and non-dengue patients were residents of the same endemic areas in Indonesia (Yogyakarta and Semarang), where DEN 3 was the predominant serotype circulating in the area during that epidemic [16]. Informed consent from the parents or legal gardians of all children included in the study was obtained.

Serological assays. Clinically suspected dengue virus infections were serologically confirmed with samples obtained from acute and convalescent phase of illness. Dengue virus-specific IgM and IgG antibodies were measured in the plasma samples of the patients using a commercially available EIA kit (Focus Technologies, Cypress CA, USA), and performed according to the procedures described by the manufacturer. Recently the performance, sensitivity and specificity of the dengue IgM and IgG EIA kit were described by Groen and colleagues [17]. Based on their serological profiles (IgM/IgG ratio) on day 7 post onset of illness, dengue patients were classified as having a primary (n=41) or secondary (n=127) dengue virus infection according to the criteria set by WHO [15]. A commercially available kit (R&D Systems, Oxon, United Kingdom) was used for the measurement of the sVCAM-1 in the plasma samples from the dengue and non-dengue patients, according to the procedures described by the manufacturer. Standard samples with known concentrations of sVCAM-1, provided by the manufacturer, were used for the creation of the standard curve and subsequently for the determination of the concentration of sVCAM-1 in each serum sample obtained from the dengue and non-dengue patients.

Table 1: Classification and characteristics of dengue virus infected patients. p.o.s: post onset of symptoms. DF: dengue fever, DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome.

		Classification		
		DF (n=71)	DHF (n=30)	DSS (n=67)
Patients (n=168)	Male	37	15	28
	Female	34	15	39
	Mean duration of fever in days (range)	7.0 (3-13)	8.8 (4-14)	10.7 (6-17)
	Mean age in years	7.8	7.7	6.6
Plasma samples (n=405)	Acute stage (days 1-7 p.o.s)	119	52	122
	Early convalescent stage (days 8-13 p.o.s)	12	7	50
	Convalescent stage ($\geq$14 days p.o.s)	18	14	11

Table 2: Comparison of the plasma level of sVCAM-1 (ng/ml) following sex and dengue virus specific immune status of the patients.

	mean	standard deviation	df[1]	F value	p value (ANOVA)
Sex			2	0.42	0.51
Male	860.3	650			
Female	813.4	785.1			
Immune status			2	3.04	0.08
Primary dengue infection	720	688			
Secondary dengue infection	869.7	729.7			

[1]: degrees of freedom.

Statistical analysis. Statistical analysis was performed using BMDP software. First we performed univariate analysis. Using correlation test (Pearson test) and ANOVA, we assessed the relation of the dependent variables, i.e. the level of sVCAM-1 and the independent variables, i.e. age, sex, day post onset of symptoms of the patients, severity of dengue disease (dengue fever, dengue hemorrhagic fever and dengue shock syndrome) and dengue immunity (primary or multiple dengue infections). Since the distribution of the data was not significant different from a normal distribution as determined by computer analysis (Koemogorov-Smirnof p= 0.06, chi^2 p=0.09), and in order to take into account the interactions of the independent variables, we included in a second time the independent variables in an ANCOVA descriptive model. A two-sided P-value <0.05 was considered to represent a significant difference. The results of the ANCOVA model allowed assessing the real influence of the studied on sVCAM-1 level because this model takes into account the interactions of the different variables. We used also a stepwise regression model in order to assess the exact influence of sVCAM-1 on the level of dengue severity, i.e. dengue fever, dengue hemorrhagic fever and dengue shock syndrome.

RESULTS

A kinetic analysis of sVCAM-1 plasma levels among patients with varying severity of dengue illness is depicted in Fig. 1. sVCAM-1 was elevated during acute dengue fever (days 1-7 post onset of symptoms) (mean $\pm$ S.D 808 $\pm$ 815 ng/ml) and declined thereafter to 487$\pm$617 ng/ml during early convalescent phase (days 8-13 post onset of symptoms) to reach the lowest levels (377$\pm$262 ng/ml) during convalescence (day 14 onwards post onset of symptoms). During acute dengue hemorrhagic fever sVCAM-1 was elevated to 774$\pm$773 ng/ml, declined during early convalescent phase (586$\pm$655 ng/ml) and reached lowest levels during convalescence (391$\pm$163 ng/ml). Acute phase patients with dengue shock syndrome had the highest sVCAM-1 levels (1083$\pm$686 ng/ml). Those levels declined during early convalescent phase (881$\pm$585 ng/ml) and reached lowest levels (345$\pm$262 ng/ml) during convalescent phase.

All dengue patients had statistically different elevated levels of sVCAM-1 during acute phase of dengue illness when compared to non-dengue febrile patients (P=0.000, P=0.001 and P=0.000 for

Table 3: Comparison of the plasma level of sVCAM-1 (ng/ml) following dengue disease severity and age of the patients. DF: dengue fever, DHF: dengue hemorrhagic fever.

	mean	standard deviation	df[1]	F value	p value (ANOVA)
Dengue severity			4	3.99	0.003
DF	729.9	769.3			
DHF I	612.3	507			
DHF II	744.3	832.2			
DHF III	964.6	573.4			
DHF IV	1099.1	1078.2			
Age			2	1.5	0.21
0-5 years	721.6	619.8			
6-10 years	889.6	750			
11-15 years	827.2	735.5			

[1]: degrees of freedom.

dengue fever, dengue hemorrhagic fever and dengue shock syndrome respectively using ANOVA). The differences of the means of the sVCAM-1 levels were significant among acute dengue fever and dengue shock syndrome patients (P=0.021) but not among dengue fever and dengue hemorrhagic fever or dengue hemorrhagic fever and dengue shock syndrome (P>0.05). The differences of the means of the sVCAM-1 levels were not significant among the respective groups of dengue patients during early convalescent or convalescent phase of dengue illness (P>0.05).

The univariate analysis showed that the level of sVCAM-1 was not influenced by the age, the sex and the immunity status of the patients while it was strongly related to the day post onset of symptoms of the patients and to dengue severity (Tables 2, 3 and 4). The ANCOVA model confirmed the results of the univariate analysis demonstrating that there was no influence of the age, sex and immunity status of the patients to the sVCAM-1 level (Table 5). The trend over time represented by the influence of the day post onset of symptoms of the patients was significant (Table 5) showing an increase of the sVCAM-1 level from day 1 to day 6 and 7 followed by a regular decrease. Most important was the influence of the dengue disease severity on the level of sVCAM-1 (Table 5). The stepwise regression model allowed us to demonstrate that it was the level of sVCAM-1 significantly higher among the dengue shock syndrome patients than among dengue hemorrhagic fever or dengue fever patients.

Table 4: Analysis of the evolution over time of the level of sVCAM-1. Results of the linear regression of the plasma level of sVCAM-1 (ng/ml) following days post onset of symptoms of the dengue virus infected patients.

	N	df[1]	Pearson r value	p value
Day post onset of symptoms	405	403	-0.2	$1.9*10^{-5}$

[1]: degrees of freedom.

DISCUSSION

Murgue and colleagues recently demonstrated that sVCAM-1 is associated with severity of dengue disease [14]. In this study 16 patients with dengue hemorrhagic fever were included of which five were grade III and IV (dengue shock syndrome). In our study we measured the levels of sVCAM-1 in serial plasma samples of 97 children with dengue hemorrhagic fever of which 67 children developed dengue shock syndrome. The additional information allowed us to confirm the results of the previous report and perform statistical analyses that revealed significant differences in the levels of sVCAM-1 among the respective groups of dengue patients.

We first demonstrated that all dengue patients had significantly elevated sVCAM-1 levels compared to patients with other febrile illness (non-dengue patients) during the acute phase of their illness. Secondly, we showed that all dengue patients in our study reached the highest levels of sVCAM-1 during the acute phase of dengue disease. However, only children who developed dengue shock syndrome had significantly elevated levels of this molecule during acute disease when compared with children with dengue fever or dengue hemorrhagic fever. This finding is in contrast with the findings of Murgue et al., demonstrating a significant elevation of sVCAM-1 levels among dengue hemorrhagic fever patients (including shock patients) compared with dengue fever patients. This discrepancy might be due to the sample size of the two different studies. Murgue and colleagues included 16 dengue hemorrhagic fever patients in contrast to the present study in which we included 97 dengue hemorrhagic fever patients. Further more, statistical analyses of these data (Tables 2-5) showed that the levels of sVCAM-1 were only associated with disease severity and day post onset of symptoms and not with immunity status, age or sex of the patients. These findings indicate that elevated levels of this molecule are associated with dengue infection and not with other parameters related to age, sex or previous exposure to dengue virus, of the patients. Although we were able to demonstrate the significance of elevated sVCAM-1 among different groups of dengue virus infected patients, we could not demonstrate a prognostic value of sVCAM-1 levels in individual patients (Fig. 1). The role of sVCAM-1 early in the pathogenesis of dengue virus infections was further investigated. This hypothesis is supported by the findings that sVCAM-1 levels decline soon after the patients pass the acute phase of dengue disease with no significant differences of the sVCAM-1 levels among the respective patient groups. The role of sVCAM-1 in the pathogenesis of dengue virus infections may be further speculated since the presence of sVCAM-1 in circulation is upregulated by cytokines such as TNF-α, IL-1β and IL-4 [11;18;19]. Those cytokines also play an important role in the pathogenesis of dengue virus infections [20],[21;22]. Previous studies have suggested that endothelial cells may be activated *in vitro* after infection with dengue viruses [7],[5;6]. Taken into consideration that elevated levels of VCAM-1 reflect activation of the endothelium [9] and that activated endothelial cells are involved in increased vascular permeability, a hallmark characteristic of dengue hemorrhagic fever and dengue shock syndrome, these findings further support the hypothesis of the implication of VCAM-1 in severe dengue virus infections. However it should be noted that activation of endothelial cells is an indirect effect of dengue virus infection, since dengue viruses primarily infect monocytes, which consequently have vasoactive effect on endothelial cells [7].

Table 5: Level of significance of the independent variables included in the descriptive ANCOVA model. p.o.s: post onset of symptoms

Source of ANCOVA	sum of squares	df[1]	F value	p value
Severity of dengue	5294596	4	2.74	0.028
Immune status	169256	1	0.35	0.55
Interaction : severity and immunity status	1846596	4	0.96	0.43
Sex	409939	1	0.85	0.36
Day p.o.s	10014048	1	20.72	$<10^{-4}$
Age	169054	1	0.35	0.55

[1]: degrees of freedom.

In conclusion, in the present study we confirmed that elevated levels of sVCAM-1 are observed in the acute phase of dengue illness, in particular among children who develop more severe manifestations of dengue. The elevation of this molecule in the plasma of patients with dengue is a result of the current infection with the virus independent of previous exposure to dengue viruses. Despite the significance of these results the use of sVCAM-1 as a prognostic marker for dengue shock syndrome proved to be of a limited value for individual patients. These data also support the hypothesis that sVCAM-1 plays a role in the pathogenesis of dengue virus infections.
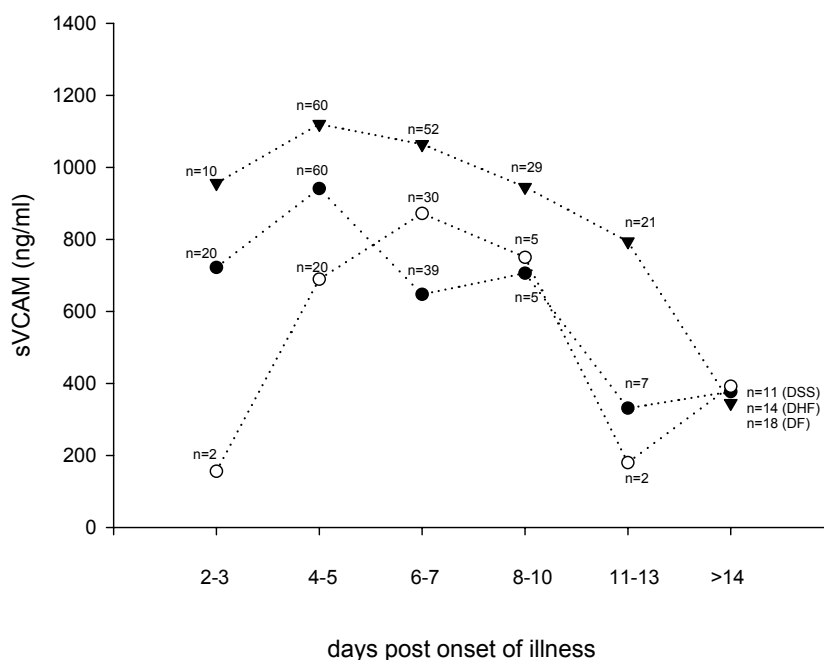

Fig. 1: Kinetics of sVCAM-1 levels over time of dengue illness. •: dengue fever (DF) patients, O: dengue hemorrhagic fever (DHF) patients, : dengue shock syndrome (DSS) patients. n: number of blood samples tested on the respective days post onset of illness.

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Chapter 4

Animal model to study dengue virus infections

Chapter 4.1

Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses

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ABSTRACT

Since studying the pathogenesis of dengue virus associated disease in humans has several limitations, an appropriate animal model is needed. Therefore, we investigated kinetics of viremia as well as humoral and cellular immune responses, after primary, secondary and tertiary heterologous dengue virus infections in cynomolgus macaques: these parameters were largely similar to those observed in natural human infection upon primary infection. Both antibody and T cell responses measured were largely cross-reactive. Upon secondary infection with a heterologous virus serotype, T cells responses specific for the primary infecting serotype were more pronounced especially when the immune system was primed with dengue 1 virus. Measurement of transcription levels of pro- and anti-inflammatory cytokines in white blood cells upon primary and secondary infection generally showed a balanced response. In addition, a region of the NS2A protein of dengue viruses was identified that induces T cell responses in macaques.

INTRODUCTION

The importance of specific B- and T-cell immune responses has been studied extensively in several acute and chronic viral infections. Activation of memory B and T cells that are able to cross-react with newly invading pathogens may either result in protective immunity or in predisposition for immune-mediated pathogenesis [1;2]. In dengue virus (DENV) infections such predisposition upon secondary infection with a heterologous serotype has been observed.

Infection with any one of the four antigenically distinct DENV serotypes usually results in mild flu-like illness (dengue fever [DF]). DENV infection induces protective immunity against re-infection with the same serotype [3]. However, subsequent infections with heterologous DENV serotypes have been postulated to leave the individual at risk of developing more severe clinical manifestations, including hemorrhages and severe plasma leakage, a life-threatening syndrome collectively known as dengue hemorrhagic fever (DHF) which may lead to dengue shock syndrome (DSS) [4;5]. Several studies have proposed a role for (sub-) neutralizing antibodies and T-cells in the development of DHF, implicating cross-reactive B and T-cells in the pathogenesis of enhanced disease seen in DHF and DSS [6;7]. Prospective studies in South East Asia offered the opportunity to analyze immune responses in small cohorts of children experiencing secondary DENV infections [8-10]. These studies, however, were limited in the number of subjects studied, the identification of the DENV involved in these infections and the measurement of the time span between sequential infections.

Several monkey species are known to be susceptible to DENV infection although they do not present clinical signs. In this study we investigated the potential of cynomolgus macaques (*Macaca fascicularis*) as an animal model to study DENV infections. To this end, we experimentally infected cynomolgus macaques with DENV-1 or DENV-4. The effect of pre-immunity to DENV on the immune responses triggered by subsequent heterologous infections, was studied upon secondary DENV-3 and tertiary DENV-4 infections in these animals. Serotype specific and cross-reactive humoral and cellular immune responses were studied.

MATERIALS AND METHODS

Animals and viruses: The following viruses were kindly provided from Dr. V. Deubel (Pasteur Institute, Lyon, France): DENV-1 strain 40514, DENV-2 strain Jamaica 1982, DENV-3 PaH881 and DENV-4 strain 28128. Virus titers expressed as $TCID_{50}$ /ml were determined by plaque assay on Vero E 6 cells as previously described [11]. Outbred population of *Macaca fascicularis* were infected subcutaneously with $10^{6.4}$ $TCID_{50}$ of DENV-1 (DENV-1 group, n=6) or $10^{6.2}$ $TCID_{50}$ of DENV-4 (DENV-4 group, n=6). Both groups were subsequently infected with $10^{4.8}$ $TCID_{50}$ of DENV-3 and a year later with $10^{5.8}$ $TCID_{50}$ of DENV-4. Peripheral blood samples were collected daily for 14 days after infection events and weekly or biweekly thereafter. Plasma was separated from cells by centrifugation and cells were collected either by red blood

cell lysis (white blood cells) or standard Ficoll extraction density centrifugation (PBMCs). All experiments done in primates were approved by the animal ethical committee of the Erasmus MC, Rotterdam, The Netherlands.

Virus detection: Presence of plasma viral RNA was measured by means of real time RT-PCR as previously described [12]. The threshold for detection of viral RNA was 10 $TCID_{50}$ equivalents/ml.

Antibody responses: DENV specific IgM and IgG antibodies were measured using commercially available kits (Focus Diagnostics, Cypress, CA, USA) according to the manufacturer's recommendations [13]. Samples with a ratio>1 were considered positive.

DENV neutralizing antibodies (VN) were measured as follows: plasma was 3-log titrated, allowed to neutralize 100 $TCID_{50}$ of each DENV serotype for 1h at $37^{o}C$ and transferred to a confluent monolayer of LLC-MK_2 cells. After incubation at $37^{o}C$ for 7 days, cells were fixed with absolute ethanol and developed with a fluorescent-based immunoassay using an in-house produced polyclonal DENV antibody and a rabbit anti-human IgG FITC-labelled conjugate (Dako, Glustorp Denmark). The cut-off titer for VN antibodies was set at 1:50. Statistical analysis was preformed using one-way ANOVA test of the GraphPad Prism version 4 software (San Diego, CA, USA) and P≤ 0.05 values were considered significant.

T cell responses against whole DENV: The frequency of DENV-specific IFN-γ-secreting cells was determined in PBMCs using an ELIspot kit (U-Cytech) according to the manufacturer's recommendations. Autologous B cells were infected with the respective DENV serotypes and used as antigen presenting cells for PBMCs at a ratio of 1:5 (per 200,000 PBMCs). Cell suspensions were transferred on ELIspot plates, and developed according to the manufacturer's recommendations. Spots were enumerated with an automatic spot reader (Bioreader 3000, Bio-Sys GmbH). For statistical analysis and graphical representation, spot numbers were considered after substraction of the background and the one-way ANOVA test was used (differences were considered significant when P ≤ 0.05).

T cell responses against DENV peptides: Short regions with high amino acid (aa) sequence diversity in the DENV polyprotein were selected for peptide design. A panel of 96 peptides were synthesized (PEPscreen© Sigma Genosys, The Woodlands Texas, USA), including some previously described human epitopes. The characteristics of the peptides are available from the authors upon request. PBMCs were enriched for DENV specific T cells and used in an IFN-γ ELIspot as described above with the following differences: Matrix peptide pools were prepared containing either 6 or 8 peptides per pool (each peptide at final concentration of 1μM, and each peptide was present in two different pools). Peptides that were positive in both pools in which they were present, were subsequently tested individually at a concentration of 5 μM, in triplicate using 100,000 PBMCs per well. Reactive peptides were identified as follows: mean of spot numbersof the negative controsl + 3x S.D (where negative controls were unstimulated PBMCs).

Measurement of expression of inflammation markers: Blood cells were separated from plasma by centrifugation and red blood cells were lysed (Roche Diagnostics, Almere, The Netherlands). Total RNA was isolated from white blood cells with phenol:chloroform (Fulka). An oligo dT primer (Invitrogen) was used for cDNA synthesis of mRNA using RT-superscript III (Invitrogen) according to the instructions of the manufacturer. Between 50-100 ng RNA was copied into cDNA. cDNA was used as a template for measuring expression of cytokines with real time PCR (primers available from the authors upon request) and a TaqMan Universal PCR kit (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) according to the instructions of the manufacturer. Transcript numbers were calculated relative to the internal

control (GAPDH) with the following formula: $2^{-\Delta ct} *10^5$, where Δct: $Ct_{cytokine}$- Ct_{GAPDH}. Data analysis was performed after log transformation of expression levels relative to GAPDH.

RESULTS

Outcome of primary DENV-1 and DENV-4 infections: Primary DENV-1 and DENV-4 infection resulted in variable levels of plasma viral RNA (between 18 and 850 $TCID_{50}$ equivalents/ml) in all 12 macaques on day 2 post infection, which had disappeared in all animals on day 8 and day 4, respectively (Table 1). All animals developed peak levels of DENV-specific IgM serum antibodies (up to 10 times the cut off value) within 2 weeks post infection, which declined to background levels within 15 weeks post infection. DENV-specific IgG serum antibodies reached plateau levels (2-3 times the cut off value) within 5 weeks post infection (Fig. 1). DENV neutralizing antibodies developed within 4 weeks post infection with homologous VN titers about 400 and 150, respectively. Heterologous VN titers upon primary DENV-1 infection were undetectable (against DENV-2), about 70 (against DENV-3) and about 200 (against DENV-4) (Fig. 2a) and upon primary DENV-4 infection about 200 (against DENV-1), undetectable (against DENV-2) and about 60 (against DENV-3) (Fig. 2b).

Table 1: Measurement of plasma viral RNA in animals infected with the different DENV serotypes after primary, secondary and tertiary infection. The mean $\pm$ standard deviation of plasma viral RNA levels expressed in $TCID_{50}$ equivalents/ml are given in brackets.

Primary infection							
Group	D0	D2	D3	D4	D5	D6	D8
DENV-1	0/6	6/6	6/6	4/6	4/6	1/6	0/6
		(400 ± 300)	(310 ± 230)	(130 ± 100)	(10 ± 0)	(10 ± 0)	
DENV-4	0/6	6/6	4/6	0/6	0/6	0/6	0/6
		(20 ± 2)	(10 ± 0)				
Secondary DENV-3 infection							
DENV-1	0/6	2/6	1/6	1/6	0/6	0/6	0/6
		(10 ± 0)	(10 ± 0)	(10 ± 0)			
DENV-4	0/6	2/6	1/6	0/6	0/6	2/6	0/6
		(10 ± 0)	(10 ± 0)			(10 ± 0)	
Tertiary DENV-4 infection							
DENV-1	0/6	0/6	0/6	2/6	2/6	1/6	1/6
				(10 ± 0)	(10 ± 0)	(10 ± 0)	(10 ± 0)
DENV-4	0/6	0/6	0/6	0/6	0/6	0/6	0/6

All macaques exhibited a DENV-specific T cell mediated IFN-γ response against the homologous DENV serotype after primary infection (Fig. 3a and 4a). Overall, the homologous T-cell responses measured in the DENV-1 group were higher than those in the DENV-4 group, with considerable variation between the individual animals. To study the breadth of these responses, PBMCs from each animal were also stimulated with the heterologous DENV antigens. After primary infection with DENV-1, macaques had the highest responses to DENV-1 antigens on week 7 post infection, which were significantly higher than the responses against DENV-2 ($P < 0.01$), DENV-3 ($P < 0.05$) and DENV-4 ($P < 0.01$) antigens. Macaques in the DENV-4 group reacted to the respective DENV serotype antigens at similar levels, with not more than 20 spots of IFN-γ producing cells at the majority of the time points tested. The highest responses after primary infection with DENV-4 were observed 11 weeks post infection and were higher (although not significantly higher in all cases) than those against DENV-1 ($P > 0.05$), DENV-2 ($P < 0.05$) and DENV-3 ($P > 0.05$) antigens.
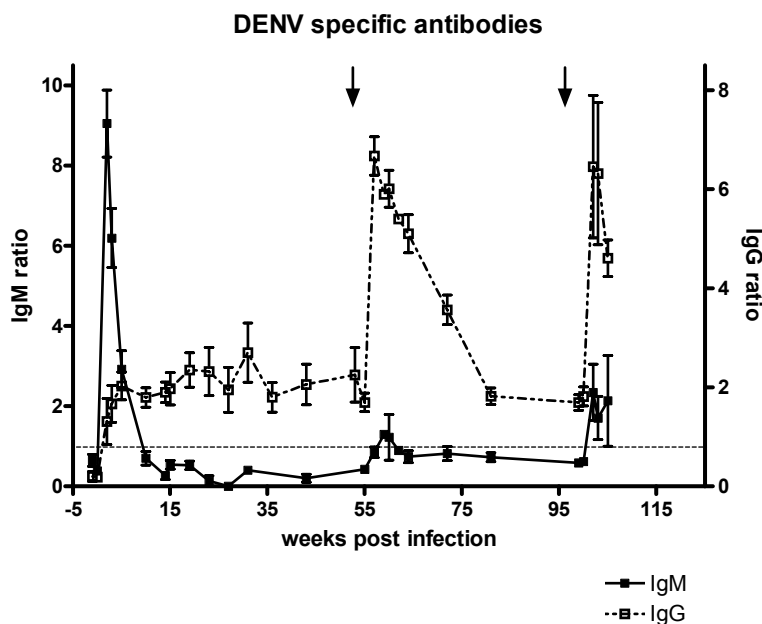

DENV specific antibodies

Figure 1: Antibody responses after sequential DENV infections in macaques. Development of DENV-specific IgM and IgG antibody responses following sequential DENV infections (indicated by arrows). Antibody ratios from both groups of macaques are depicted in one graph. Dashed line indicates the cut-off value of the assays.

Outcome of secondary DENV-3 infection: Next we tested the outcome of secondary DENV-3 infection in the DENV-1 and DENV-4 infected macaques. Secondary DENV-3 infection resulted in relatively low levels of plasma viral RNA (10 TCID$_{50}$ equivalents/ml) in three out of six macaques of the DENV-1 group from day 2 to day 4, and in three out of six macaques of the DENV-4 group from day 2 to day 3 (Table 1). A slight and short lived increase in DENV-specific IgM serum

antibodies was observed in one of the macaques of the DENV-1 group and four of the macaques of the DENV-4 group with levels never exceeding 1.5 times the cut off value (Fig 1). In contrast, DENV-specific IgG serum antibodies were boosted in all macaques to levels exceeding seven times the cut off value. Within 20 weeks the IgG levels returned to pre-boosting values (Fig. 1). Secondary DENV-3 infection of the DENV-1 group boosted DENV-1 VN serum antibodies to higher levels (titers between 1000 and 4000) than DENV-2, 3 and 4 VN serum antibodies ($P < 0.01$; $P < 0.01$ and $P < 0.001$, respectively) within 4 weeks post infection (Fig. 2a). Similarly, secondary DENV-3 infection of the DENV-4 group boosted DENV-4 VN serum antibodies to higher levels (titers 200-1000) than DENV-1, 2 and 3 VN serum antibodies (although the differences were not significant, $P > 0.05$) within 4 weeks post infection (Fig. 2b). Secondary DENV-specific T cell mediated IFN-γ responses in the DENV-1 group tended to be higher against DENV-1 than against DENV-2, 3 and 4 antigens ($P < 0.05$ on week 9 post infection) although considerable variation between individual animals was observed (Fig. 3b). Similarly these secondary cellular responses in the DENV-4 group tended to be higher against DENV-4 than against DENV-1, 2 and 3 antigens ($P > 0.05$), again with considerable variation between the individual animals (Fig. 4b).
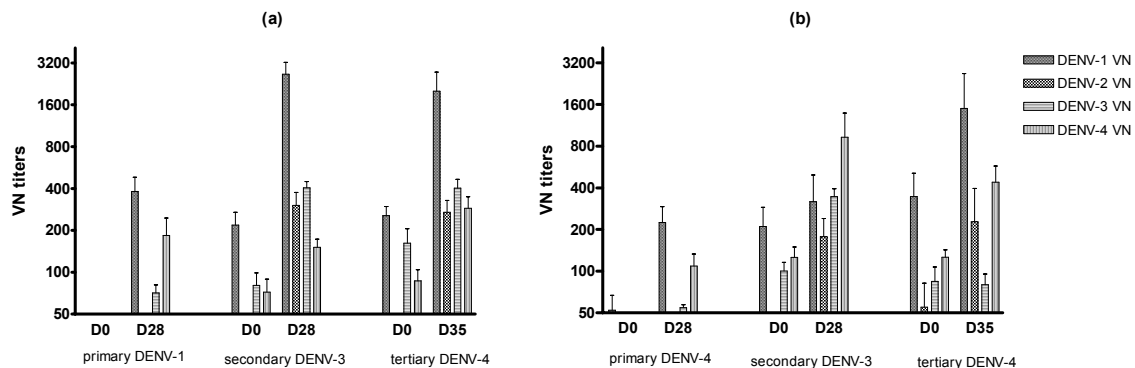

Figure 2: VN antibody responses after primary DENV, secondary DENV-3 and tertiary DENV-4 infection of macaques. (a): DENV-1 group, (b): DENV-4 group.

Outcome of tertiary DENV-4 infection: Tertiary DENV-4 infection resulted in relatively low levels of plasma viral RNA (10 $TCID_{50}$ equivalents/ml) in two of the macaques in the DENV-1 group from day 4 to day 8, whereas none of the macaques of the DENV-4 group showed detectable plasma viral RNA levels (Table 1). Again a slight and short lived increase in DENV-specific IgM serum antibodies was observed in one animal of the DENV-1 group and in two animals of the DENV-4 group, with levels never exceeding 3 times the cut off value (Fig. 1). DENV-specific IgG serum antibody levels were boosted again to levels comparable to those observed after secondary DENV-3 infection. Tertiary DENV-4 infection of the DENV-1 group, boosted DENV-1 VN serum antibodies to significantly higher levels (titers 1000-2000) than
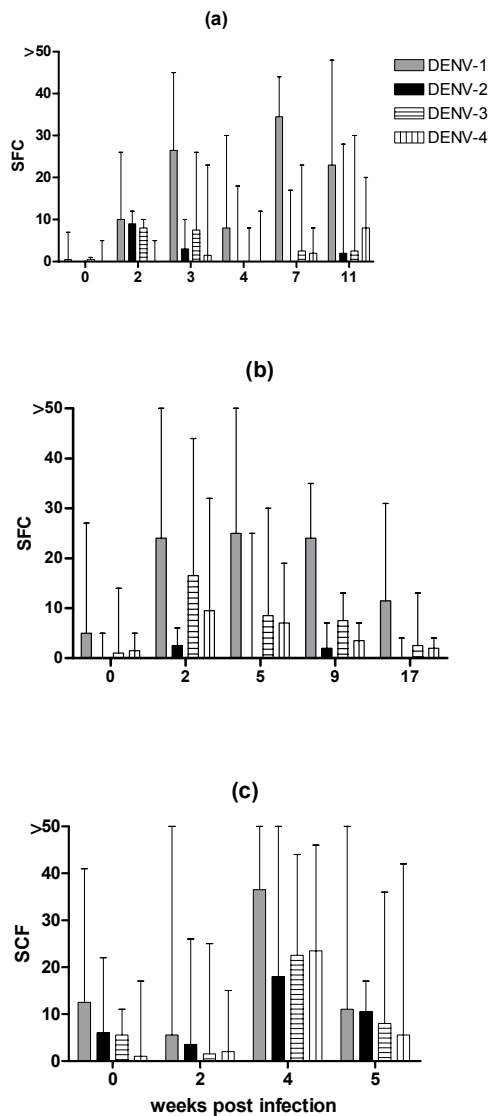
Figure 3: DENV-specific T cell responses among animals in the DENV-1 group. X-axes: weeks post infection after (a) primary DENV-1, (b) secondary DENV-3 and (c) tertiary DENV-4 infection. SFC: spot forming cells. Error bars represent the median and range of the group values.

DENV-2, 3 and 4 VN serum antibodies (P < 0.05) (Fig. 2a). This is in contrast to tertiary DENV-4 infection of the DENV-4 group, which did not result in a significant increase of DENV-1, 2 3 or 4 VN serum antibodies (Fig. 2b).

T-cell mediated IFN-γ responses in the DENV-1 group tended to be higher (although not significantly, P > 0.05) against DENV-1 than against DENV-2, 3 and 4 antigens at all time points tested (Fig. 3c). Cellular immune responses measured in the DENV-4 group upon tertiary DENV-4 infection were low, reminiscent of the low increase of VN serum antibody responses measured in this group upon tertiary DENV-4 infection (Fig. 4c).

Identification of a region in the NS2a protein eliciting DENV-specific T-cell responses: A secondary aim of this study was to identify T cell epitope regions in the NS2a protein of DENV. To this end we tested PBMC samples of macaques after primary infection with different DENV serotypes using the time points at which the highest bulk responses were initially measured. Previously identified epitopes described for humans were also found to be reactive for three of the macaques used in this study. One macaque of the DENV-4 group reacted (10 spots/100,000 PBMCs) to a CD4 epitope previously described (NS3 224-234 [14]). A second macaque of the DENV-4 group reacted to two different peptides in the capsid protein, presented in the context of HLA DPw4 and DR1 [15] (20 and 32 spots/100,000 PBMCs, respectively). In addition, we identified a region in the NS2A protein spanning aa positions 1250-1347, which apparently contains cross reactive T cell epitopes. Overlapping peptides designed in this region based on the aa sequences of the four different DENV serotypes were found reactive with PBMCs from macaques of the DENV-1 (two animals) and DENV-4 group (four animals).

Transcription levels of pro- and anti-inflammatory cytokines: Systemic cytokine responses elicited after primary (DENV-1 or DENV-4) and secondary (DENV-3) infection were analyzed. Therefore, expression levels of pro- (IFN-γ, TNF-α) and anti- (IL-10 and IL-4) inflammatory cytokines and the chemokine IL-8 were measured for 9 days after the respective infections. Neither primary infection with DENV-1 or DENV-4, nor secondary infection with DENV-3 led to clearly skewed cytokine expression levels. Both after primary and secondary infection a temporary decrease in IL-8 levels was observed whereas also IL-10 levels upon secondary DENV-3 infection of the DENV-4 group were slightly decreased (Fig. 5).

DISCUSSION

Since little is known about the pathogenesis of DENV infection in humans, an approach to address this issue would be to test existing hypotheses in a primate model. The primary aim of this study was to obtain information on the duration of viremia and the kinetics of humoral and cellular immune responses in primates sequentially infected with different DENV serotypes.

The most common clinical form of DENV infection in humans is DF. This illness is generally a self-limited disease, characterized by relatively low viremia that resolves soon after onset of symptoms [16]. We showed that the level and duration of viremia and the kinetics and magnitude of antibody responses observed in the experimentally infected macaques, were similar to those observed in most uncomplicated human DENV infections [17;18]. Previous studies have underlined the importance of VN antibodies and T cells in DENV infections and the role they may play in enhancement of infection and immunopathogenesis of DENV associated disease [19-23]. VN antibody and bulk T-cell mediated responses were found against homologous and heterologous viruses even after primary DENV infections, which was expected given that the
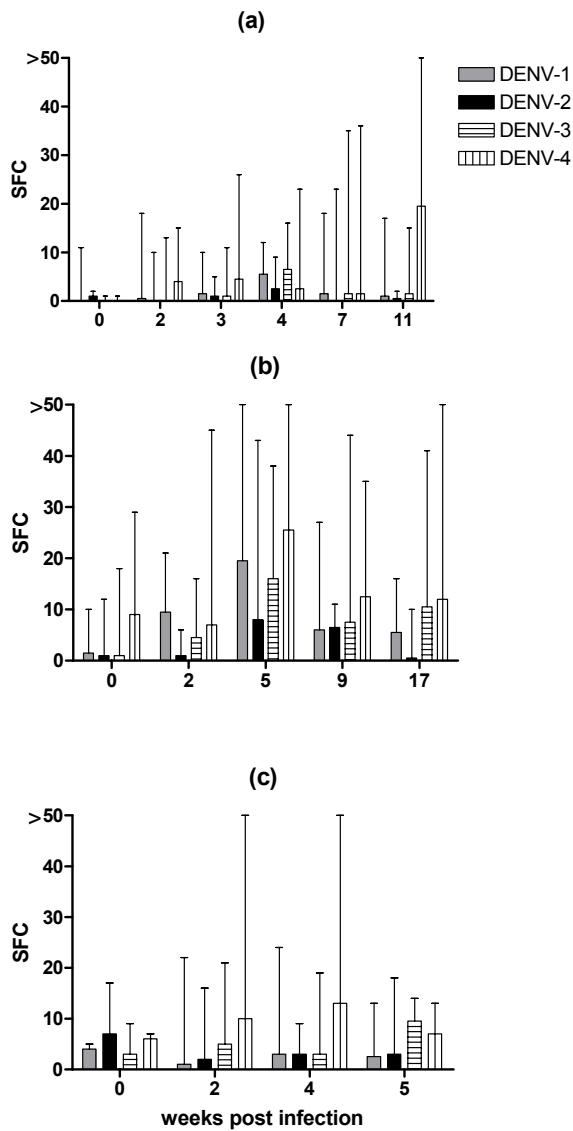
Figure 4: DENV-specific T cell responses among animals in the DENV-4 group. X-axes: weeks post infection after (a) primary DENV-4, (b) secondary DENV-3 and (c) tertiary DENV-4 infection. SFC: spot forming cells. Error bars represent the median and range of the group values.

DENV serotypes are closely related viruses that share B- and T-cell epitopes [24;25]. DENV serotypes may have different replication characteristics in the same animal species, whereas the same viruses or viral inoculums could have different replication characteristics in different animals of the same species. This may have resulted in the differences observed between and within the different groups of macaques studied.

The effect of priming the immune system with one serotype was studied upon heterologous sequential infections. Secondary heterologous DENV infections are a risk factor for development of DHF/DSS whereas tertiary or quaternary DENV infections are less likely to lead

to disease probably due to pre-existing immunity from the prior DENV infections [23]. We demonstrated that primary infection with DENV-1 in macaques had a significant effect on the immune response (VN antibodies and T-cell mediated IFN-γ production) measured after secondary DENV-3 infection. The phenomenon of original antigenic sin has been described for humoral [26] and more recently for cellular responses [27] in human subjects. Our data provide preliminary evidence that this phenomenon may be observed in macaques after primary DENV-1 followed by a secondary DENV-3 infection. Lack of significant anamnestic response triggered after tertiary DENV-4 infection may be explained either as a result of protective cross-immunity mounted during previous infections or as evidence that these immune responses have reached a plateau during tertiary infection so that significant difference could not be measured.

In contrast, priming the immune system with DENV-4 did not have the same significant effect in subsequent heterologous DENV-3 infection. Thus, this particular sequence of infection events does not provide evidence of original antigenic sin, possibly reflecting the differences by which two closely related, yet distinct viruses may influence responses during acute infections and possibly shape anamnestic responses to closely related viruses. However, lack of viremia and moderate increase of VN antibody titers and T-cell mediated IFN-γ production indicates protection of these animals upon reinfection with DENV-4 as has also been demonstrated to occur in natural human infections [28-30].

A secondary aim of this study was to identify regions in the DENV proteins with the potential to induce serotype specific T cell responses, or to identify DENV serotype specific T cell epitopes. The NS1.2A region of the DENV polyprotein has been shown to contain human T cell epitopes [31;32]. The NS2A protein of DENV has high variation at the aa level among the different serotypes therefore, we hypothesized that it is likely to include serotype specific T cell epitopes. However, the responses that we measured had a rather cross-reactive profile, suggesting that the aa's involved in epitope recognition may still be conserved and largely identical among the different serotypes. Nevertheless, to our knowledge, this is the first study to identify a 100 aa region in the NS2A DENV protein with high T cell immunogenic potential. Further investigations should provide more insight in the exact epitopes and their MHC restrictions.

The role of certain cytokines in the development of DHF/DSS has been proposed [33-35]. In general, the mRNA expression levels of the cytokines measured in this study did not tend to have a skewed profile and were relatively low, probably within the normal range. It is likely that within the time interval that we have studied cytokine responses, the immune response was largely compartmentalized within lymph nodes, with little spill over into the circulation. Therefore, in future studies local concentrations should be measured and later time points should be included.
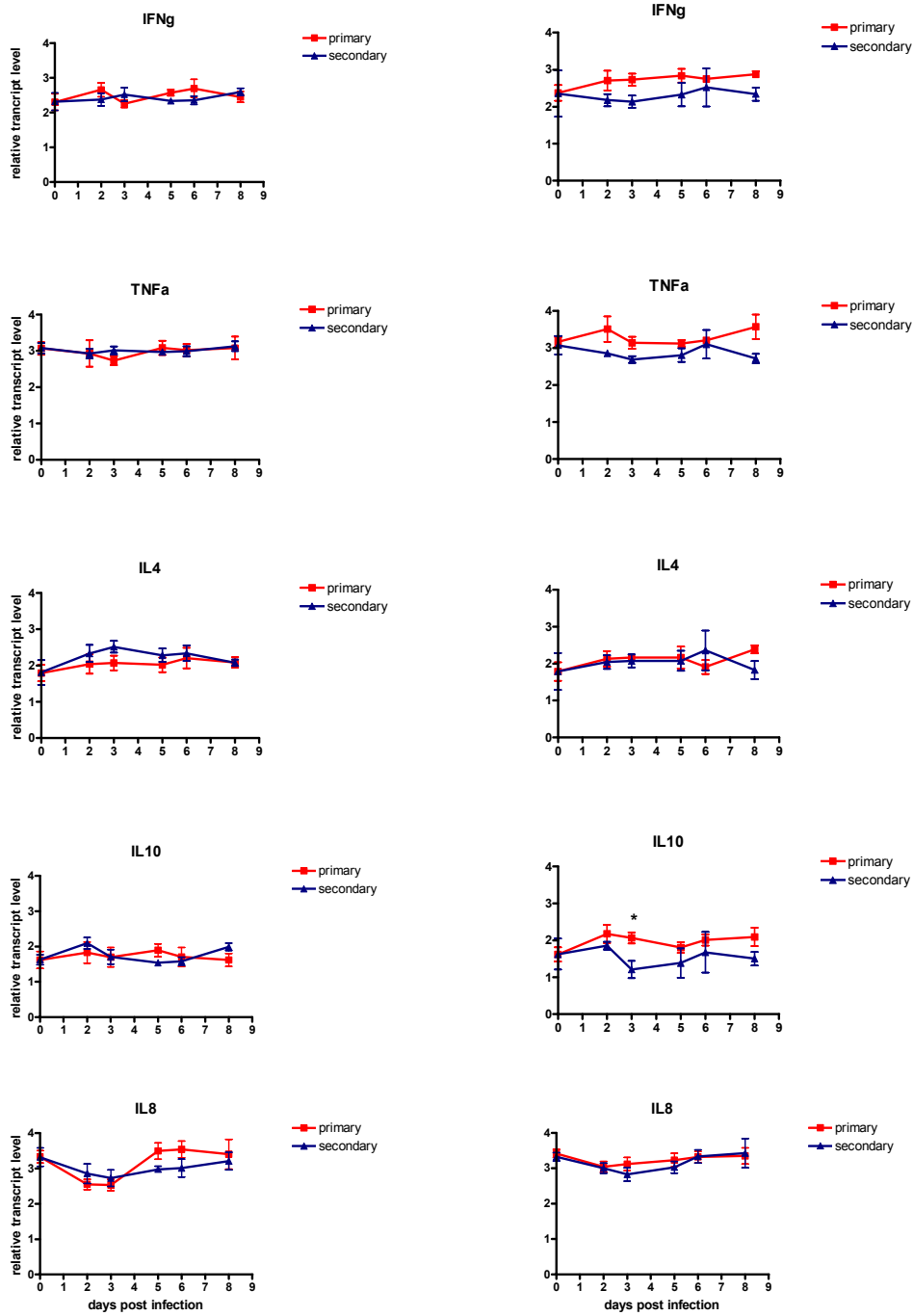
Figure 5: Expression of cytokines among macaques of DENV-1 group (left panel) and DENV-4 group (right panel). Expression levels are depicted relative to the GAPDH internal control. Asterisks indicate statistical differences (p<0.05) between primary and secondary infections on the same day post infection.

Taken together, the immune responses of macaques upon DENV infections proved to be similar to those observed in natural human infections. No indication of enhanced infection or pathology after sequential exposure of macaques to heterologous DENV serotypes was found.

ACKNOWLEDGMENTS

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Chapter 4.2

Efficacy of a live attenuated tetravalent candidate dengue vaccine in naïve and previously infected cynomolgus macaques

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ABSTRACT

The development of a safe and effective vaccine against dengue is a public health priority. Attempts to evaluate candidate vaccine formulations in human volunteers were largely unsuccessful, at least in part due to too high reactogenicity of some of the candidate vaccines tested. We evaluated a live attenuated tetravalent dengue vaccine candidate in flavivirus naïve and dengue virus type 3 immune non-human primates. Immune responses were measured both at the humoral and the cellular level and the efficacy of this vaccine candidate was evaluated by challenging the vaccinated animals with dengue virus type 4. Humoral and cellular immune responses upon vaccination were similar to those described after natural infection in humans. All animals were protected from developing viremia upon challenge infection. In addition, primary dengue virus type 3 infection of macaques neither influenced the immune response upon vaccination, nor interfered with vaccine induced protection from dengue virus type 4 challenge infection. The data suggest that the live attenuated tetravalent vaccine candidate used is promising and warrant further safety and efficacy testing in clinical trials.

INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* consists of arthropod-borne viruses many of which are important human pathogens such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV) and the four serotypes of dengue virus (DENV 1-4). DENV alone cause approximately 100 million infections per year, whereas two billion people are at risk acquiring DENV infections throughout the tropics and sub-tropics where DENV is endemic [1]. Since effective vector control programmes have been difficult to sustain and given the impact of flavivirus infections on human health, safe and effective vaccines against Flavivirus infections are needed. While effective human vaccines against YFV, JEV and TBEV have contributed significantly to the decrease of the associated human disease, effective human vaccines against WNV and DENV are still not available. The development of a safe and effective vaccine against dengue is one of the public health priorities defined by the World Health Organization (WHO).

Each of the four DENV serotypes may cause a variety of clinical manifestations, ranging from sub-clinical or mild flu-like infection, the commonly observed dengue fever (DF) characterized by fever and rash, to hemorrhagic manifestations. The latter are seen in about 2% of infections and are characterized by increased vascular permeability, internal haemorrhages and plasma leakage. This type of DENV associated disease is known as dengue haemorrhagic fever (DHF). In the most severe cases, when plasma leakage becomes critical, patients may develop dengue shock syndrome (DSS) associated with high mortality [2]. The pathogenesis of DENV infections is not fully elucidated. Epidemiological and *in vitro* studies have associated DHF and DSS with secondary DENV infection with a DENV serotype heterologous to that of the primary infection [3-6]. This led to the hypothesis that antibody dependent enhancement (ADE) of infection is involved in the pathogenesis of DHF and DSS [5]. Individuals with pre-existing sub-neutralizing antibodies are at greater risk in developing DHF and DSS during a secondary heterologous infection. It is now generally believed that the pathogenesis of DENV is multi-factorial and that besides viral factors also host factors, including both humoral and cellular immune responses, may play an important role [7-10]. The observation that almost 95% of DHF/DSS cases are associated with secondary DENV infection poses a special challenge to the development of a universal or multivalent DENV vaccine that should induce solid immunity against all or most of the four serotypes in both naïve and previously infected individuals.

Several vaccine candidates have been developed including live attenuated mono- and tetra-valent formulations, inactivated whole virus vaccines and recombinant subunit vaccines (for review [11;12]). Some of these vaccine candidates have reached phase 1 or 2 clinical trials, but so far none of them has been licensed for human use at least in part due to high reactogenicity [13;14].

In the present study, we evaluated the efficacy of a live attenuated tetravalent (LAT) DENV vaccine candidate in a vaccination-challenge cynomologous macaque (*Macaca fascicularis*) model. The influence of pre-immunity to DENV was studied upon vaccination with LAT DENV vaccine. Both naïve and pre-infected macaques were vaccinated with the LAT DENV vaccine candidate twice and subsequently challenged. For practical and ethical reasons we studied the influence of pre-immunity and challenge with only one DENV serotype using wild-type virus strains that were unrelated to the vaccine strains (DENV-3 for preinfection and DENV-4 for challenge). Kinetics of antibody and T cell responses as well as viremia were measured and clinical signs were monitored after primary infection, vaccination and challenge.

MATERIALS AND METHODS

Cells, viruses and vaccines: Monkey LLC-MK$_2$ cells and Vero E6 cells were grown at 37°C in a humidified atmosphere with 5%CO$_2$ in Eagle's minimal essential medium (EMEM) or Dulbecos MEM (DMEM) respectively, containing 100 U penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10% heat inactivated fetal bovine serum (FBS) (all reagents from BioWhittaker). Vero E6 cells were used from passage number 146 to 198 for plaque assays and LLC-MK$_2$ cells were used from passage number 348 to 351 in virus neutralization assays. Aedes pseudoscutelaris insect cells (AP-61) were grown at 27°C in a humidified atmosphere with 5%CO$_2$ in Leibovitz-15 medium (BioWhittaker) containing 100 U penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% heat inactivated FBS.

DENV-3 strain PaH881 (Thailand 1988) and DENV-4 strain 28128 (Tahiti 1987) were kindly provided from Dr. V. Deubel from Pasteur Institute, Lyon, France. Viruses were grown in AP-61 cells and passed three times before preparation of virus stocks that were used throughout the study for infection and challenge of animals and T cell studies. Virus titres expressed in TCID$_{50}$ were determined by plaque assay on Vero E6 cells for each serotype: DENV –3 10$^{6.63}$ and DENV –4 10$^{6.2}$

Monovalent live attenuated DENV vaccines were prepared at WRAIR (Silver Spring, MD). The candidate DENV vaccines were attenuated by serial passage of viruses on primary dog kidney cells and included the following viruses: DENV-1 (45AZ5) PDK-27 (Lot Number 1-1-90), DENV-2 (S16803) PDK-50 (Lot Number 1-1-90), DENV-3 (CH53A89) PDK-20 (Lot Number 2-1-91) and DENV-4 (341750) PDK-6 (Lot Number 1-1-90). The use of these cell-lines for attenuation of DENV has been previously established [15]. The vaccine lots were produced on FRhL2 cells. Vaccines were provided by GlaxoSmithKline Biologicals (Rixensart, Belgium) and shipped lyophilized to Erasmus MC on dry ice and immediately stored at –20°C until use. Prior to vaccination the appropriate amount of ampoules per serotype were reconstituted in water for injections according to the manufacturer's recommendations. Equal volumes of each monovalent vaccine were pooled together and mixed well to form the LAT vaccine. Each animal received 1 ml of LAT vaccine subcutaneously. Using a real time RT-PCR system [16], viral RNA titers were calculated: DENV-1: 10^5, DENV-2: 10^4, DENV-3: 10^4 and DENV-4 10^6 TCID$_{50}$ equivalents/ml. Virus titers were confirmed by plaque assay on Vero E6 cells as previously described [17].

Infection, Vaccination and Challenge: Two groups of six, three to five years old cynomolgus macaques (*Macaca fascicularis)*, were used in this study. Animals were kept in the animal facility of the Erasmus MC, under conditions specified by the faculty i.e. housed in group cages in groups of 6-8 animals per cage. During infection/vaccination/challenge animals were housed in pairs in BSL-3 isolator cages. The first group (DENV-3 group) was infected with 10^6 TCID$_{50}$ DENV-3 virus and six months later vaccinated with LAT vaccine. At this time the second group of animals (DENV-naïve group) received the first vaccination dose. Six months later both groups were boosted with one dose of LAT vaccine and six months later challenged with 10^6 TCID$_{50}$ of DENV-4. Two animals were included at this time point as challenge controls. For statistical comparisons a historical control group (n=6) was included. Animals of the historical control group matched in age and sex the animals of the present study and were infected, under the same experimental conditions as described in the present study, with the same DENV-4 strain, from the same virus stock that was used for challenge. The infection and sampling protocols were identical for DENV-3, DENV-naive and control group animals. Animals were anesthetised before each treatment with 15 mg/kg of ketamine and observed until recovery from anaesthesia for side effects. Viruses and vaccines were injected subcutaneously in the upper right back of each animal. Peripheral blood samples from infected animals were drawn on daily basis for the first 8 days of infection/vaccination/ challenge events and weekly or biweekly thereafter. Seven weeks post challenge animals were euthanized according to recommendations and protocols of the animal ethical committee. One of the animals in the DENV-3 group died on the day of the booster vaccination and one animal from the DENV-naive group died on day 2 post challenge. The deaths of both animals were due to fighting with their cage-mates and were unrelated to vaccination or challenge with DENV.

Telemetry: Temperature transponders were inserted in the peritoneal cavity of ten animals prior to challenge experiments. Four animals belonged to DENV-3 group, four animals belonged to DENV-naive and the remaining two were the challenge control animals. Temperature was recorded every ten minutes from the time of insertion of the transponder (one week prior to challenge), until the animals were euthanized.

Detection of dengue viral RNA: Two ml of citrate blood obtained on days 0, 2, 3, 4, 5, 6, 8, 10 and 14 post infection/vaccination/challenge, centrifuged at 1600x *g* for 20 min. Plasma was separated from total blood cells and directly stored in lysis buffer for subsequent viral RNA detection. Viral RNA was isolated with an automated method using MagnaPure© and commercially available reagent kit (Total nucleic acid isolation kit, Roche Diagnostics, Almere, The Netherlands), according to the manufacturer's instructions. Presence of virus RNA after each infection/vaccination/challenge was measured by means of real time RT-PCR using published primers [18].

DENV-specific antibody responses: DENV-specific IgM and IgG antibodies were measured using commercially available kits (Focus Diagnostics, Cypress CA, USA) according to the instructions of the manufacturer. The specificity of those kits for macaque samples has been established previously [19]. The ratio of each sample (Fig. 1) was calculated as follows: O.D.$_{sample}$/O.D.$_{cut\ off}$ (where cut off was provided by the manufacturer). Samples with a ratio>1 were considered positive.

DENV-specific neutralizing antibody titers (VN) were measured on monolayers of LLC-MK$_2$ cells. Serial 3-fold serum titration (starting from 1:30) was mixed with 100 TCID$_{50}$ of each of the DENV serotypes. Neutralization of virus was allowed to occur for 1 hour at 37°C and the virus-serum mixtures were transferred in triplicate wells of confluent LLC-MK$_2$ cells grown in 96-well plates, in 100µl volumes. On day 7 cells were fixed with absolute ethanol, washed with PBS and incubated with polyclonal monkey anti-DENV

antibodies (produced in-house) for 1 hour at 37°C. Plates were washed and incubated with rabbit anti-human IgG FITC conjugate (Dako, Glostrup, Denmark) for 1 hour at 37°C. Positive wells were scored under a fluorescent microscope by two independent investigators.

DENV-specific T cell responses: The frequency of DENV-specific IFN-γ-producing PBMCs was determined using an ELIspot kit (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's recommendations. Briefly, autologous herpes papio virus transformed B cells (B-LCLs) were infected with each of the DENV serotypes at an m.o.i. of 1 for DENV 1, 3 and 4 or 0.5 for DENV 2. Infection of B-LCLs was demonstrated by FACS analysis 48 hours later (data not shown). At this time point 30-50% of B-cells were infected with DENV and co-cultivated for one hour with viable PBMCs at a ratio of 1:5. Cell suspensions ($2x10^5$ PBMCs/well) were transferred to an ELIspot plate, pre-coated with anti-monkey IFN-γ specific monoclonal antibody. After overnight incubation at 37°C ELIspot plates were developed and spots were enumerated with an automatic spot reader (Bioreader 3000, Bio-Sys GmbH).
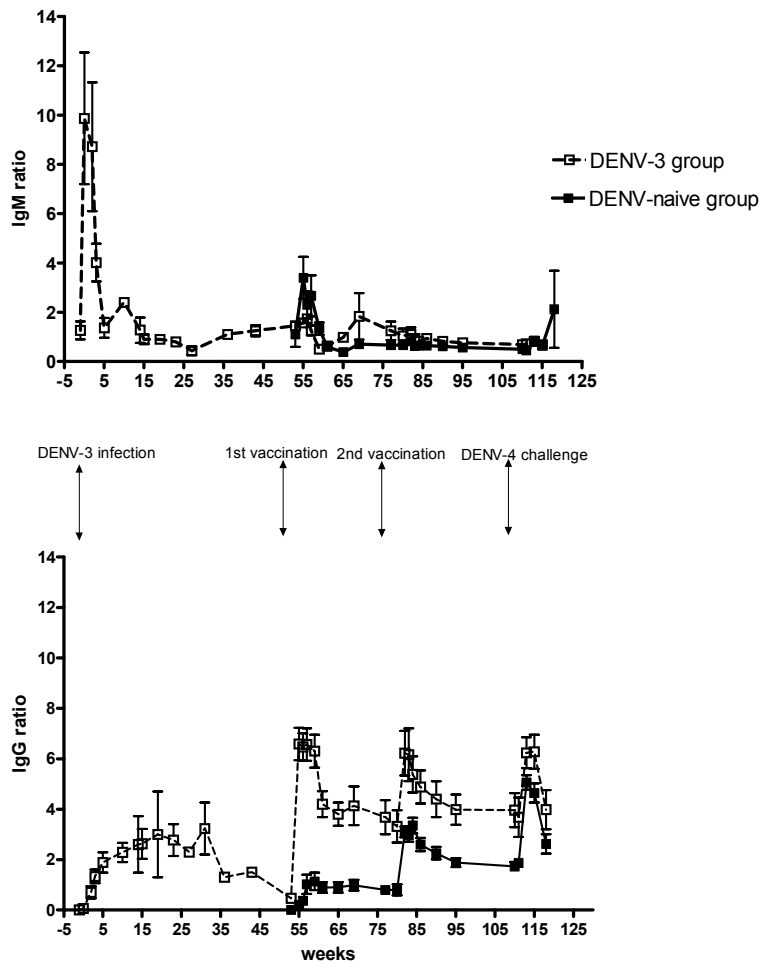

Figure 1: DENV-specific IgM and IgG antibody responses in DENV-3 (open squares) and DENV-naïve (closed squares) macaques. The time points of infection with DENV-3, vaccination and challenge with DENV-4 are indicated with arrows.

The phenotype of DENV-specific IFN-γ producing cells (CD8+ or CD3+/CD8- cells) was determined as follows: PBMCs were stained with anti-CD8 beta-PE antibody (Immunotech, Marseille, France) and magnetically separated from the CD8- cells using anti-PE magnetic beads (Miltenyi Biotec, Utrecht, The Netherlands). The CD8- cell population was stained with anti-CD3-APC antibody (BD Pharmingen, San Diego, CA, USA) and magnetically separated from the CD8-/CD3- cells using anti-APC magnetic beads (Miltenyi Biotec). All cell populations (CD8+, CD8-/CD3+ and CD8-/CD3-) were used in an IFN-γ ELIspot assay as described above.

Statistical analysis: VN titres and T cell responses to whole virus were compared between groups of animals upon challenge with one-way ANOVA using the statistical package of the Graph Pad Prism software (version 4). P values <0.05 were considered significant.
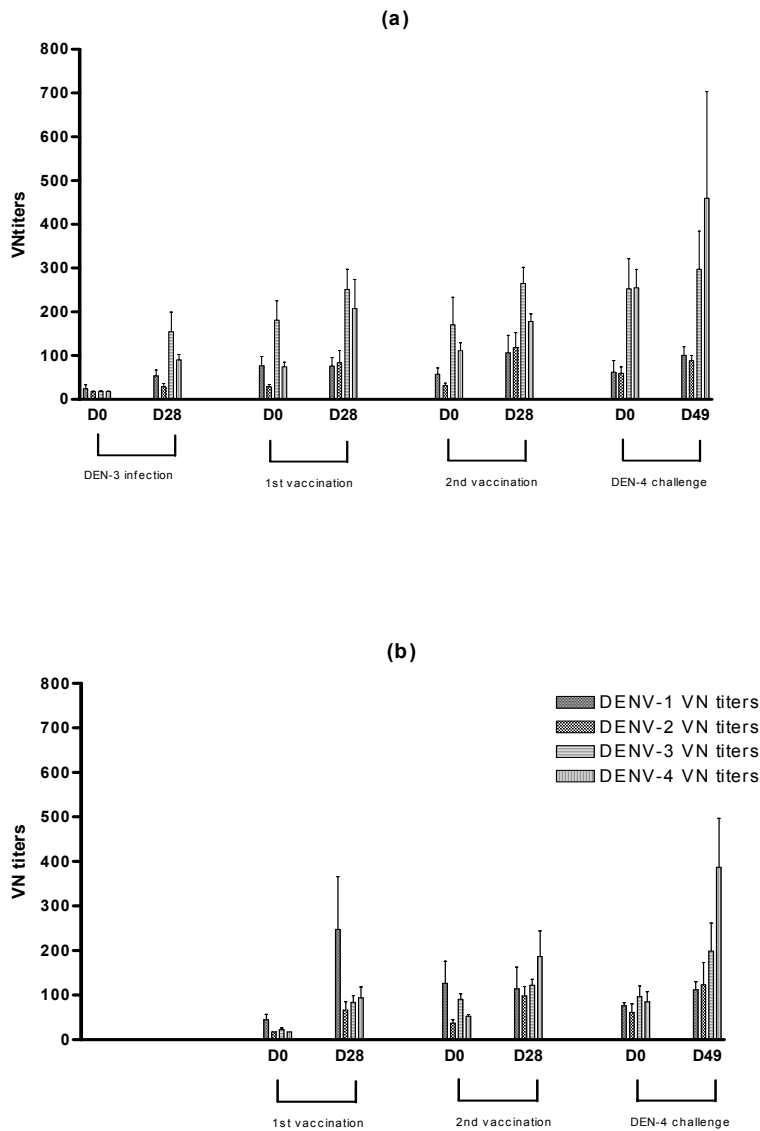

Figure 2: VN titres against the four DENV serotypes in (a) DENV-3 group and (b) DENV-naïve group, after DENV-3 infection (only DENV-3 group), first and second vaccination and challenge with DENV-4.

RESULTS

Clinical signs: No clinical signs indicating dengue virus associated disease (such as fever or rash) were observed in any of the macaques upon DENV-3 infection, LAT DENV vaccination or DENV-4 challenge.

Viremia: Primary DENV-3 infection resulted in moderate to high levels of plasma viral RNA (between 20 and 33,000 $TCID_{50}$ equivalents/ml) in all six macaques of the DENV-3 group on day 2 post infection, which had disappeared in all animals by day 5. No viral RNA was detected after LAT DENV vaccination, booster vaccination or challenge with DENV-4 in the DENV-3 or DENV-naïve group of macaques. Challenge with DENV-4 resulted in moderate levels of plasma viral RNA (up to 100 $TCID_{50}$ equivalents/ml) in the control macaques on day 2, which had disappeared in all macaques on day 4.

Antibody responses: Sera from all animals were tested for DENV-specific IgM and IgG antibodies in ELISA's and VN antibodies were measured with the respective DENV (types 1-4) neutralization assays.

All animals of the DENV-3 group developed peak levels of DENV-specific IgM antibodies four weeks post DENV-3 infection, which declined to background levels 15 weeks post infection. DENV-specific IgG antibodies reached plateau levels within five weeks post infection (Figure 1). All DENV-3 group animals showed a boost in DENV-specific IgG antibodies after first and second vaccination and upon challenge with DENV-4, whereas DENV-specific IgM antibodies were absent in all but one macaque after this challenge (Figure 1).

Similarly, all animals in the DENV-naïve group developed peak levels of DENV-specific IgM antibodies four weeks after first vaccination, which declined to background levels within 10 weeks after vaccination. DENV-specific IgG antibodies reached plateau levels within five weeks after vaccination. Second vaccination of the DENV-naïve animals had no effect on the level of DENV-specific IgM antibodies whereas DENV-specific IgG antibodies were boosted to levels two to three times higher than after the first vaccination (Figure 1). One macaque of DENV-naïve group developed detectable DENV-specific IgM antibodies seven weeks after challenge with DENV-4, whereas DENV-specific IgG antibodies were boosted in all animals after challenge with DENV-4 (Figure 1).

All animals in the DENV-3 group developed VN antibody titers within four weeks after primary infection, which were higher against the homologous DENV-3 (200 ± 100) than against the heterologous DENV-1 (50 ± 30), DENV-2 (<30) and DENV-4 (90 ± 30) serotypes. LAT DENV vaccination resulted in 2 to 3 fold increase of the VN titres against DENV-1 (100 ± 40), DENV-2 (100 ± 60), DENV-3 (300 ± 100) and DENV-4 (200 ± 100) compared to pre-vaccination titres (Figure 2a). Second vaccination resulted in further increase of VN titers against DENV-1 (150 ± 80), DENV-2 (150 ± 70), DENV-3 (300 ± 70) and DENV-4 (250 ± 40). Upon challenge with DENV-4 VN

antibody titers against DENV-4 reached the highest levels (500+200) compared to VN antibodies against DENV-1 (100+40), DENV-2 (100+30) and DENV-3 (400+150).

First vaccination of DENV-naïve animals induced moderate levels of VN antibodies against DENV-1 (about 300+150), DENV-2 (100+40), DENV-3 (100+40) and DENV-4, (100+60). Second vaccination resulted in a more equal response against the four DENV serotypes (150+60 for each of the DENV serotypes). On the day of challenge with DENV-4, all animals of the DENV-naïve group had moderate titres of VN antibodies against all four serotypes (approx. 120+40), which increased in the weeks after challenge for all four serotypes (150+40, 200+70 and 300+100 against DENV-1, 2 and 3, respectively) but in particular against DENV-4 (500+200, P < 0.001) as expected (Figure 2b).
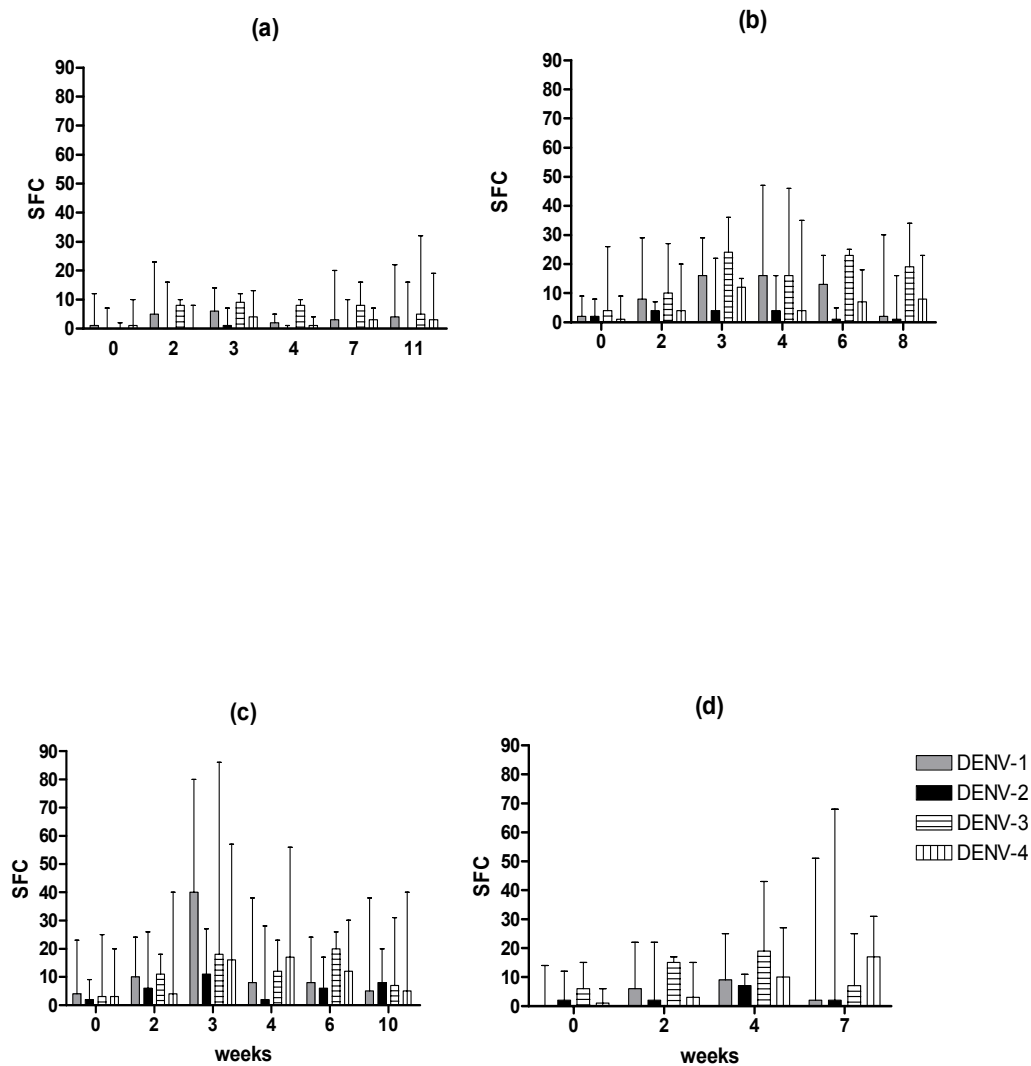

Figure 3: T cell mediated IFN-γ responses measured in the DENV-3 group of macaques after (a) infection with DENV-3 (b) first vaccination, (c) second vaccination and (d) challenge with DENV-4. Spot forming cells per 200,000 PBMCs tested against whole DENV. Error bars represent the median and range of the group values.

Seven weeks after challenge control animals developed homologous DENV-4 VN antibodies (150+40) and heterologous DENV-1, 2 and 3 VN antibodies (80+30, <30 and 60+20, respectively) (data not shown).

Virus specific T cell responses: Upon infection all macaques of the DENV-3 group exhibited a DENV-specific T cell mediated IFN-γ response against homologous DENV-3 and heterologous DENV-1, 2 and 4 antigens (Figure 3a). Upon vaccination of those animals with LAT DENV vaccine, the responses to DENV 1, 3 and 4 were relatively high, whereas the responses to DENV-2 were relatively low when detectable (Figure 3b). Second vaccination of the DENV-3 group resulted in boosting of T cell mediated IFN-γ responses against all four DENV serotypes (Figure 3c), whereas challenge with DENV-4 did not result in a significant increase in the responses to any of the DENV antigens (Figure 3d).

In contrast, DENV-naïve animals that received the same vaccine formulation, responded against all four DENV serotypes upon first and second vaccination with considerable variation between the individual animals (Figure 4a and 4b). Challenge of these animals with DENV-4 induced moderate T cell mediated IFN-γ responses against all four DENV serotypes (Figure 4c).

DENV-specific IFN-γ producing cells were further characterized for CD8/CD3 markers using PBMCs from primary DENV infection (DENV-3 group) or first vaccination (DENV-naïve group). IFN-γ producing cells were mainly of the CD8-/CD3+ phenotype, whereas a low number of CD8+ cells produced IFN-γ (data not shown).

Effect of pre-immunity to DENV-3 on immunogenicity of LAT DENV candidate vaccine: Using data of humoral and cellular immune responses, the influence of pre-immunity to DENV-3 on the outcome of vaccination and subsequently on protection upon challenge was studied. By comparing post vaccination and post challenge VN titres against all DENV serotypes, no significant differences were found between the DENV-3 group and the DENV-naïve group (P > 0.05). No significant differences in IFN-γ responses were observed between DENV-3 and DENV-naïve groups after LAT DENV vaccination (P > 0.05), indicating that immunity to DENV-3 did not influence the immunogenicity of the candidate LAT DENV vaccine.

DISCUSSION

The aim of this study was to evaluate a LAT DENV vaccine candidate in an animal model, both in DENV-3 immune and DENV-naïve animals. Non-human primates are probably the most relevant animals to study human infections. Although they do not develop overt clinical signs after DENV infection, they remain the most closely related species to be naturally infected with DENV and display markers of infection similar to humans (i.e. viremia, specific antibody and T cell responses). We have previously demonstrated the potential of cynomolgus macaques as an animal model to study DENV candidate vaccines [20].
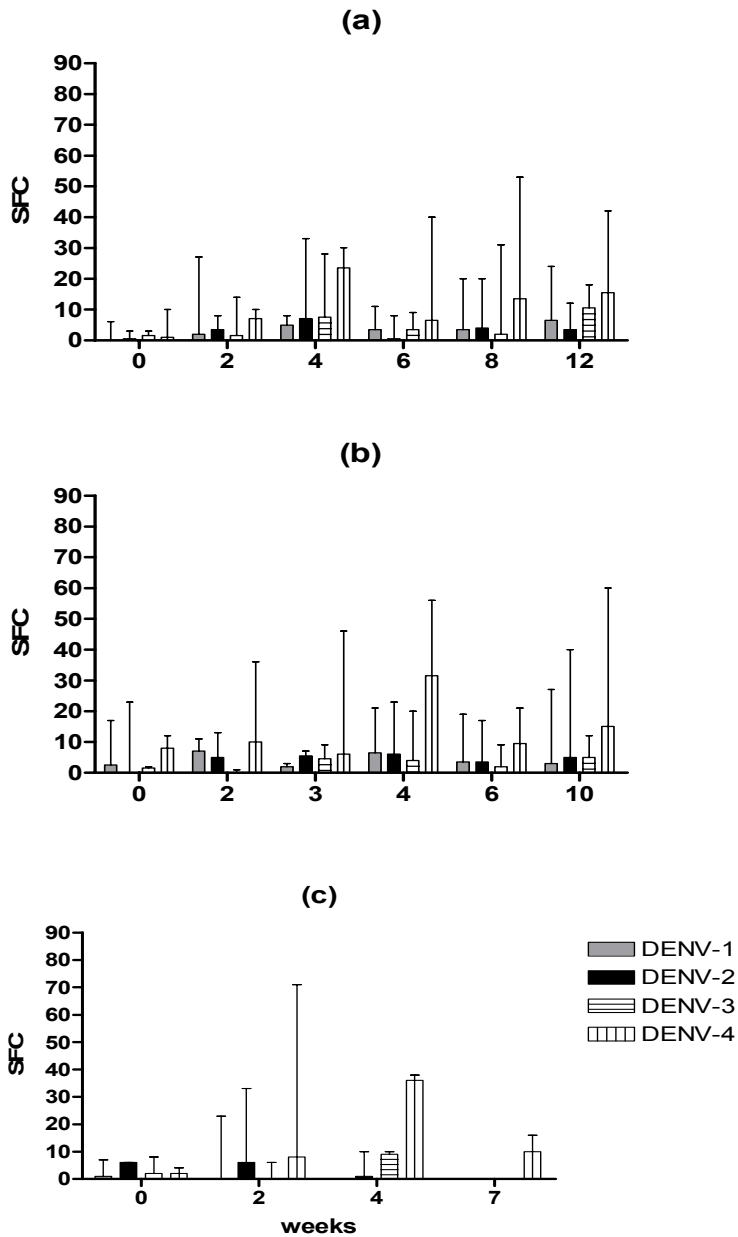
Figure 4: T cell mediated IFN-γ responses measured in the DENV-naïve group of macaques after (a) first vaccination, (b) second vaccination and (c) challenge with DENV-4. Spot forming cells per 200,000 PBMCs tested against whole DENV. Error bars represent the median and range of the group values.

Evaluating DENV vaccines has proven to be difficult. The correlates of protection are not well defined [12]. Neutralizing antibodies are believed to play an important role for protection against DENV infection, however the endpoint titres that confer protection have not been determined. In addition, neutralizing antibodies may (in sub-optimal titres) be involved in enhancement of infection [21;22].

Lack of viremia after vaccination in both DENV-3 and DENV-naïve groups suggests that attenuation of the vaccine strains was successful, whereas lack of viremia of vaccinated animals after challenge with DENV-4 suggests the protective potential of the LAT DENV vaccine. These data are supported by the finding that the same DENV-4 virus stock, administered to the naïve animals of the same species (control group) under the same experimental conditions replicated in macaques and induced plasma viral RNA and DENV-specific antibody and T cell responses (Koraka et al., Microbes and Infection *in press*). Previously, rhesus macaques have been used in studies receiving the same vaccine preparations in the respective monovalent formulations. In this study only two out of four rhesus macaques had a one day viremia upon vaccination with DENV-2 vaccine and one rhesus macaque had a 0.5 day viremia upon vaccination with DENV-4 vaccine [23]. Similarly the DENV-1, 2 and 3 components of the vaccine used in our study were also used as monovalent vaccines in human volunteers [24]. Only two of the six volunteers receiving the DENV-3 vaccine candidate had short lived, low titers viremia. The differences observed between our study and the previous ones might be due to differences in assays used to detect viremia, differences in primate species or differences in the vaccine formulations (monovalent vs. tetravalent vaccine candidates). To our knowledge the same tetravalent vaccine formulation has not been used in other studies with human volunteers or non-human primates.

Induction of humoral responses upon vaccination differed between immune and naïve animals. Macaques in the DENV-3 group showed an antibody response typical to secondary DENV infection upon first vaccination with LAT DENV vaccine whereas DENV-naive animals followed a pattern of antibody responses typical to primary DENV infection upon first vaccination with LAT DENV vaccine. These findings, as well as the titres of VN antibodies, are consistent with previous studies with candidate DENV vaccines in humans or non-human primates [25-27]. Uniform VN responses against all four DENV serotypes were induced only after second vaccination, which suggests the necessity of a booster dose for equal immunogenicity of this candidate vaccine against all four serotypes in the cynomolgus macaque model. In a recent study Sun et al., showed that using a WRAIR-derivative of a LAT DENV vaccine, a booster vaccination was necessary in rhesus macaques to induce seropositivity rates of 100%, 100%, 90% and 70% against DENV 1, 2, 3, and 4, respectively [28]. Immunity to DENV-3 did not significantly influence the VN antibody titer to any DENV serotype upon vaccination. On the day of challenge with DENV-4 all animals had VN antibody titres against all serotypes greater than 100. Since there was no significant increase in DENV-4 specific VN antibody titres after challenge in the DENV-3 group, it is likely that the virus did not replicate efficiently upon challenge.

Cellular immune responses have been less well characterised upon vaccination of non-human primates with candidate DENV vaccines [29]. Vaccination of macaques in the DENV-3 group resulted in a high IFN-γ response against DENV-1 and DENV-3 whereas the responses against DENV-2 and DENV-4 (especially after first vaccination) were considerably lower. These

findings indicate that DENV-3 specific (presumably memory) T cells were readily available upon vaccination and therefore those animals were able to mount a more efficient DENV-3 immune response compared to the other serotypes. The observation that the DENV-1 responses were similar to DENV-3 may reflect the similarity of these two serotypes at the amino acid level [30] and therefore it is more likely that DENV-3 elicited T cells will cross-react with DENV-1 antigens more efficiently than with DENV-2 or DENV-4 antigens. In contrast, T cell responses observed in the DENV-naïve group upon vaccination and challenge were more uniform. T cell responses of these animals were higher against DENV-4 antigens than against DENV 1-3 antigens after first and second vaccination and challenge, possibly reflecting the fact that DENV-4 had the highest titre in the vaccine formulations.

Collectively the data show that the LAT DENV vaccine induced protection in both DENV-immune and naïve non-human primates. Lack of viremia in vaccinated animals and induction of humoral and cellular immune responses after challenge with DENV-4, support the conclusion that vaccinated animals were protected from challenge with wild-type DENV. In addition, immunity to DENV-3 did not significantly influence the immune response induced by the candidate LAT vaccine. The observed humoral and cellular immune responses as well as the induced protection from challenge warrant further investigation of safety and efficacy of this candidate vaccine in human trials, including challenge with more DENV serotypes.

ACKNOWLEDGEMENTS

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Chapter 5

Summarizing discussion

Dengue viruses (DENV) are the most abundant mosquito-borne viruses worldwide. Epidemics of dengue fever occurred throughout the 19th and early 20th century with the emergence of dengue hemorrhagic fever in the mid 1950's. Since then, DHF has become the leading cause of hospitalization and death among young children in South east Asia. DHF re-emerged in Latin America in the late 1970's where it is predominantly a disease of adults. In the beginning of the 21st century dengue is an international public health problem affecting not only residents of endemic areas but also travellers to the tropics, putting more than 2 billion people at risk of acquiring DENV infection.

This thesis summarizes the commercially available serological methods for diagnosis of DENV infections and highlights the potential and limitations of these assays for accurate diagnosis of dengue infections. In addition, a newly described diagnostic method is proposed as an alternative to conventional assays. Furthermore, this thesis describes the humoral immune responses during natural dengue infections in patients with varying disease severity.

The pathogenesis of dengue infections largely remains to be elucidated. Several factors have been postulated to be associated with disease severity including sequential infections with heterologous dengue serotypes, viral virulence and host genetic factors. In the present thesis, also the evaluation of the potential of a primate model for DENV infections is described. The humoral and cellular immune responses to DENV in cynomolgus macaques were studied after sequential infections with heterologous dengue serotypes. Subsequently, this macaque model was used to evaluate a DENV vaccine candidate.

LABORATORY DIAGNOSIS OF DENV INFECTIONS

DENV infections are often presented as an un-differentiated syndrome, similar to diseases caused by viruses or other vector-transmitted agents. Epidemiological studies have shown that DENV are endemic in most of the tropics and sub-tropics, often co-circulating with closely related viruses of the genus *Flavivirus* such as JEV, WNV and YFV (Figure 1). Typically, a bite of an infected mosquito will result in the infection of peripheral blood mononuclear cells and development of viremia within days post infection (primary viremia). Wide spread infection coincides with the appearance of fever lasting two to seven days. During fever the individual experiences a secondary viremia during which DENV can be isolated from the blood [3]. Individuals naïve to flaviviruses will mount a strong IgM antibody response with neutralizing activity against the infecting DENV serotype. IgM antibodies will appear soon after onset of fever and remain at high levels for a relatively short time (2 to 3 months and in some rare instances up to 8 months) [4,3]. IgG antibodies will rise after the peak of IgM antibodies and can be detected in the blood for a prolonged period of time compared to IgM antibodies [3]. Since the respective immune responses are largely serotype specific early after infection, the primary infecting serotype can be identified with serological tests. In the late convalescent phase however, cross-reactive antibodies are produced and the antibody response in this phase is to a much larger extend a mixture of type specific and cross reactive antibodies [5]. Thus interpretation of serological tests in the convalescent phase should be considered with caution.
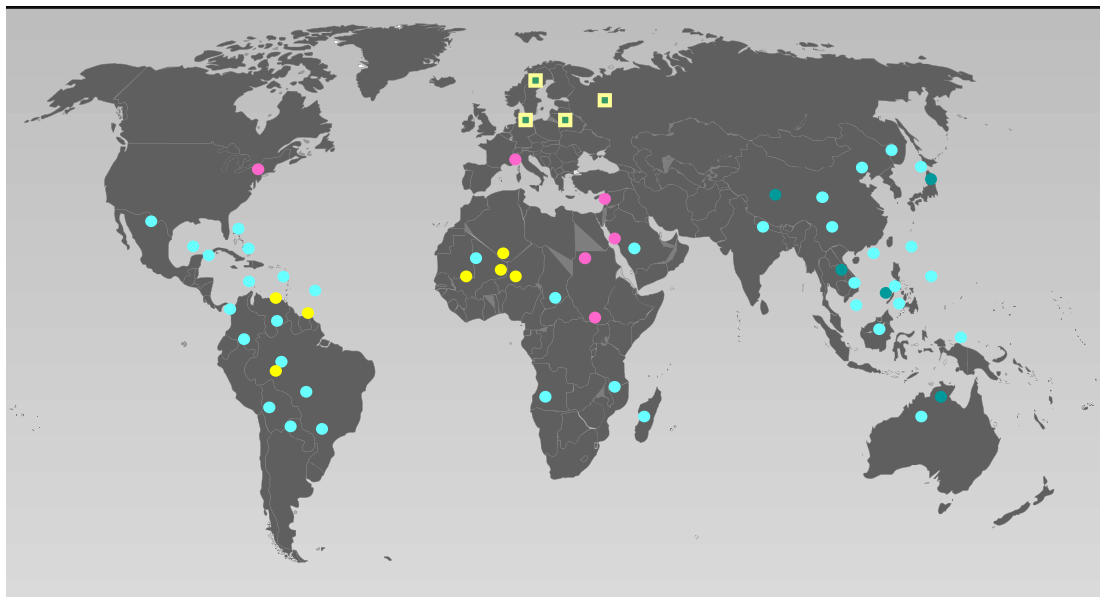
Figure 1: World-wide distribution of flaviviruses. Light-blue dots: dengue, dark blue dots: Japanese encephalitis virus, pink dots: West Nile virus, yellow dots: yellow fever virus, green squares: tick-borne encephalitis virus.

In contrast to what is observed during primary infection, a secondary DENV infection has a different serological profile. Although IgM antibodies are produced with similar kinetics of appearance as in primary responses, they are usually of much lower level, or even undetectable, as compared to the levels of primary IgM antibody response. The predominant antibodies during secondary DENV infection are of the IgG isotype, which increase soon after re-infection at levels usually higher than those measured after primary infection [6,7,3]. Antibodies with neutralizing activity to DENV contribute to virus clearance. Measurement of virus neutralizing (VN) antibodies has been the only serological method to determine the infecting DENV serotype. To date, a variety of diagnostic tests have been developed for the detection of DENV infections. Depending on the laboratory, the availability of equipment or personnel and the needs of each laboratory for accurate and timely diagnosis of DENV infections, a wide variety of assays has been developed for this purpose (Table 1).

Table 1: overview of diagnostic assays that have been developed and/or have been in use for routine diagnosis of dengue virus infections.

Test	Target	Specificity	Time required	Simplicity/requirements
HI	antibodies	low	hours	simple
PRNA	antibodies	high	days	BSL3, laborious
EIAs	antibodies	moderate	hours	simple
EIA	antigen	high	hours	simple
(RT)-PCR	viral RNA	high	hours	Special equipment, laborious
Virus isolation	infectious virus	high	days	BSL-3, laborious

Serological assays detecting DENV-specific antibodies: Historically, the haemagglutination inhibition (HI) test and the plaque reduction neutralization assay (PRNA) have been used for serological diagnosis of DENV infections [8,9]. HI was widely used for routine diagnosis because of its simplicity, sensitivity and reproducibility. Reagents can be prepared locally and the test can differentiate between primary and secondary infections. However, cross-reactions with other members of the genus *Flavivirus*, lack of specificity, need of paired serum sample for accurate diagnosis, and requirement for chemical pre-treatment of the sample before use, led to the overall abandonment of this tests for routine diagnosis of DENV infections [10]. Different cell lines and read-out methods have been used for the measurement of VN antibodies with the PRNA [11,9]. The sensitivity of PRNA is based on the induction of VN antibodies in the early convalescent phase of primary infection, specific for the infecting serotype. As we demonstrated in chapter 4.1, during sequential heterologous DENV infections, VN antibodies will be specific to at least two but usually all four DENV serotypes or even to other flaviviruses [3]. Therefore, the specificity of this test is questionable and interpretation of PRNA results after secondary infection should be done with caution. In addition, PRNA is laborious, time consuming and requires handling of live viruses in BSL3 facilities. In the era of serological diagnosis based on enzyme immunoassays, numerous diagnostic tests were developed and became commercially available for detection of DENV-specific IgM and IgG antibodies (reviewed in [12]). The different formats that were developed include classical indirect and capture ELISA tests, rapid immunochromatographic tests, dipsticks and blots, all based on the same principal of interaction between DENV antigens and DENV-specific antibodies present in the sera of infected patients (Table 2). The majority of the tests available for detection of DENV-specific IgM antibodies are based on the capture principle, whereas IgG antibodies are usually detected either with capture or indirect ELISA systems. To date, none of these tests can discriminate between the four DENV serotypes and cross-reactions with other flaviviruses are observed. According to the WHO, presence of DENV-specific IgM antibodies is indicative of a recent DENV infection whereas a four-fold rise in IgG titres between acute and convalescent serum samples is diagnostic for DENV infection [3]. Also the ratio between IgM and IgG titres is used to distinguish between primary and secondary DENV infections. Routine laboratory diagnosis of DENV is mainly based on the presence of IgM antibodies in acute phase samples in combination with increase in IgG titres between acute and convalescent samples. In chapter 2, evaluation of several serological assays for DENV diagnosis is described (chapter 2.1). In contrast to previous evaluation studies in which individual tests were evaluated [13,14,15], usually by the manufacturer, without a widely accepted gold-standard method for comparison, we evaluated the sensitivity and specificity of six different assays using one, carefully chosen, standard serum panel of DENV infected and non-infected individuals. The overall performance of each test was subsequently calculated.

Among a plethora of commercially available diagnostic tests, each laboratory has to choose the best one that fits its needs, facilities and available personnel, therefore studies

such the one described in chapter 2.1 are needed for accurate evaluation of the available assays. The four DENV serotypes share many cross-reactive antigenic determinants. Cross-reactions are particularly observed during secondary infections also with other members of the genus *Flavivirus*. Co-circulation of two or more DENV serotypes as well as other flaviviruses in a single area (Figure 1) makes serological diagnosis complicated. An example of mis-diagnosing closely related flaviviruses was evident during the introduction of WNV in the USA in 1999 when encephalitis cases were initially thought to be due to the closely related SLE virus [16]. The envelope protein of flaviviruses consists of many antigenic determinants, e.g. the flavivirus group reactive epitopes (chapter 1). Upon infection with any of the four DENV serotypes, an individual will mount antibody responses to flavivirus specific, DENV group specific and serotype specific epitopes. Therefore serological assays that use

Table 2: Commercially available diagnostic tests for detection of DENV antibodies. Sensitivity and specificity of each assay is given in % as described in the respective references.

Company	Type of Assay	Antigen	Principle	Sensitivity/ Specificity	Ref.
Focus Diagnostics Cypress, CA, US	IgM EIA	DENV 1-4	Capture	96/91	Chapter 2
	IgG EIA	DENV 1-4	Indirect	100/88	
PanBio Brisbane, Australia	IgM EIA	DENV 1-4	Capture	87/96	Chapter 2 and [13]
	IgG EIA	DENV 1-4	Indirect	100/98	
	IgM/IgG RIT	DENV 1-4	Capture	100/92 (IgM) 52/100 (IgG)	
	IgM/IgG Duo kit	DENV 1-4	Capture	95/100 (IgM) 50/93 (IgG)	
Progen Biotechnik, Heidelberg, Germany	IgM IFA	DENV-2	Indirect	71/89	Chapter 2
	IgG IFA	DENV-2	Indirect	77/86	
Venture Technologies	IgM Blot		Capture	100/78	[13]
	IgG Blot		Indirect	75/55	
Integrated Diagnostics (INDX), Baltimore, MD, USA	IgM Dipstick EIA	DENV-2	Indirect	96/86	Chapter 2
	IgG Dipstick EIA	DENV-2	Indirect	97/98	
Genelabs Diagnostics, Singapore	IgM Blot	DENV 1-4	Capture	100/92	Chapter 2
	IgG Blot	DENV 1-4	Indirect	85/95	

whole virus as antigen for detection of DENV specific antibodies will give cross-reactions with other members of the genus *Flavivirus*. The limitations of those assays are described in chapter 2.2 where assays against different flaviviruses are evaluated using serum samples from individuals with different flavivirus infections. The cross-reactions observed in this study underline the need for a better system to detect DENV infections in areas where more flaviviruses co-circulate. The use of different methods and serum samples of various origins other studies [17] has demonstrated the limitations of serological diagnosis of DENV infections, underlining the need for improved methods to overcome cross-reactions (see also below).

Detection of virus and/or viral RNA and viral antigens: Virus isolation from clinical samples by cell culture or direct inoculation into mosquitoes is the most accurate and specific method for laboratory confirmation of DENV infection [9,18]. Originally, intracerebral inoculation of newborn mice was used for isolation of DENV, however, the majority of DENV strains are not neurotropic, and therefore this approach lacks sensitivity [19]. Direct inoculation of mosquitoes with clinical specimens is the most sensitive method for isolation of DENV. Given the limitations of using live mosquitoes for diagnostic purposes, the difficulty to maintain mosquito colonies and the need for specialized facilities for such an approach, this method was soon replaced by inoculation of samples on mosquito cell lines. The most widely used cell lines are the ones derived from *Aedes albopictus* (C6/36) and *Aedes psedeuscutelaris* 61 (AP-61), which have high sensitivity in recovering DENV, especially when compared with mammalian cells (e.g. VERO). Infected cells are usually visualized with the aid of a monoclonal or polyclonal DENV antibody and staining with a fluorescence conjugate [20,21]. With the use of appropriate serotype specific monoclonal antibodies, virus isolation can even provide information on the infecting serotype. Some studies have indicated the need for careful selection of the antibodies to be used in the IFA staining, whereas others have developed flow-cytometric assays for the detection of DENV antigens in infected cell cultures [22,23]. This approach is faster in obtaining results than the IFA. Although sensitivity of virus isolation on mosquito cell cultures during primary infections varies, during secondary infections DENV circulating in the blood may be associated with antibodies, forming immune complexes, making virus isolation an insensitive method. Another disadvantage of this method is the time interval needed for obtaining diagnosis. Isolation of DENV from cell culture might take from several days to weeks before definite diagnosis can be achieved, which undermines the usefulness of this method as a routine diagnostic tool. Alternatively, detection of viral RNA has become a powerful tool in DENV diagnosis since it has the advantages of virus isolation and in addition, is faster and is independent of the presence of antibodies in the circulation and the formation of immune complexes. Only limited numbers of copies of viral RNA are needed for amplification and detection of DENV in serum or plasma. Thus detection of viral RNA has higher sensitivity than virus isolation techniques. Several molecular amplification assays have been developed for the detection of viral RNA including traditional reverse transcriptase PCR, and more recently real-time RT-PCR as well as nucleic acid

sequence based amplification (NASBA) techniques (reviewed in [24]). These methods are very sensitive and highly specific, allowing the identification of the infecting serotype in just a few hours compared to virus isolation. Different groups have developed different primer sets, using conserved regions of the DENV genome and employed them as diagnostic tool for DENV infections. However, molecular amplification and detection of viral RNA requires well equiped laboratories and well trained personnel to perform the assays. In addition, such techniques are more sensitive to contamination, leading to false positive results and are rather expensive to be used as routine diagnostic tools in endemic areas. Crucial for isolation of virus/viral RNA is the timing of sampling and the handling of the specimen. In chapter 4 we demonstrated that macaques experimentally infected with DENV have low levels of plasma viral RNA, and this for only a few days. Short, low titer viremia has also been shown during natural human infections [25,3,26]. In other words a patient will seek medical advice, usually after the viremic phase. In addition, DENV are temperature labile therefore transportation and storage of samples require low temperatures for preservation of the virus or the viral RNA.

Alternatively, detection of DENV antigens in the serum of infected patients is gaining a growing interest in the field of DENV diagnosis. In chapter 2.3 we propose an alternative method for detection of DENV NS1 antigen in sera from patients with primary and secondary infections. This dot-blot based assay proved to be more sensitive than RT-PCR. The presence of antibodies in flavivirus immune individuals might pose a problem for detection of DENV antigens during secondary infections since pre-existing antibodies will form immune-complexes with circulating antigens. We have shown that dissociation of such immune-complexes increased the sensitivity of the assay to 95% compared to RT-PCR, providing a valuable alternative tool for routine DENV diagnosis (chapter 2.3). Attempts to detect DENV antigens with IFA or radio-immunoassays in the 80's were abandoned due to the low sensitivity [27,28]. More recently attention was drawn to the detection of DENV NS1 antigen and some assays have already been commercialized [29,30,31]. Detection of DENV antigens has several advantages compared to other diagnostic techniques: is highly sensitive and specific, rapid and simple to perform, does not require specialized equipment or well trained and experienced personnel. Technically detection of DENV antigen has all the advantages of ELISA antibody assays, overcoming the problems of specificity and cross-reactivity observed with serological assays detecting DENV specific antibodies. Furthermore, timing of collection and handling of samples is not as crucial as for virus isolation or detection of RNA since DENV antigens are more stable than virus/viral RNA and circulate in the blood for longer time after onset of symptoms.

HUMORAL IMMUNE RESPONSES DURING NATURAL HUMAN DENV INFECTIONS

Humoral immune responses are presumed to be the most important during acute infections, leading to clearance of the invading virus. However in the case of DENV infections, many antibody-mediated mechanisms have been proposed to explain pathogenesis of severe dengue disease (Table 3). The predominant theory to explain pathogenesis of severe DENV

infections has been the antibody dependent enhancement of infections. Retrospective studies have demonstrated the presence of antibodies in the serum of patients that experienced infection with only one DENV serotype up to 40 years post infection [32]. These findings support the hypothesis that infection with one DENV serotype provides life-long immunity to that particular serotype. Despite the high antigenic similarities of the four DENV serotypes, antibodies produced during primary infections do not adequately protect upon heterologous secondary infection. In contrast, it is believed that one of the risk factors to develop severe disease is due to ADE upon secondary infection with a heterologous DENV serotype [33,34,35].

In chapter 3, we measured the magnitude and kinetics of different types of immunoglobulin class and subclasses during primary and secondary DENV infections. Primary infection with any of the four DENV serotypes leads to production of high IgM antibodies [4,3], whereas upon secondary DENV infection, little or no production of IgM antibodies is observed [7,3]. Upon sequential heterologous DENV infections, fewer new epitopes are presented by the newly infecting serotype which may explain the limited or absent levels of IgM. In chapter 3.1 we showed that the kinetics of IgM responses during secondary infection are different than during primary infections. They appear late during secondary infection (are usually measured simultaneously with IgG) and decline much faster than after primary infection, usually within one month post symptoms.

IgG antibodies are measured at low to moderate levels during early convalescence after primary DENV infections. Upon secondary infection, IgG rise rapidly during the acute phase, to high levels sustained for several months [3]. The subclass distribution of IgG has not been well characterized during DENV infections [36]. In chapter 3.1 we describe the distribution of IgG subclass antibodies in relation to disease severity. We showed that IgG 1 and 3 are the predominant subclasses during acute and early convalescent disease, whereas IgG 4 is significantly elevated in DSS patients. Other studies have demonstrated that IgG 1 and 3 are the predominant antibodies during acute infections with measles [37], RSV [38] and

Table 3: Antibody mediated mechanisms hypothesised to be related to dengue virus pathogenesis and development of DHF/DSS (for references see text).

Mechanism	Antibodies implicated	Effect
Enhancement of infection	Neutralizing and non-neutralizing	Increased viral load via increased cell infection
Formation of immune-complexes	Mainly non-neutralizing	Increased vascular permeability via compliment activation
Cross reactivity	Cross reactive with endothelial cells, NS1, coagulation proteins	Increased cell apoptosis, inflammatory activation and haemorrhage

hantavirus [39]. Activation of complement is hypothesized to be crucial in DENV pathogenesis (see Introduction, Figure 7) via production of the C5-C9 complex, either by triggering production of inflammatory cytokines or production of clotting factors, they contribute to increased vascular permeability and plasma leakage, a hallmark of DHF/DSS. IgG 1 and 3 are able to activate the complement system whereas IgG2 and 4 are poor activators [40,41,42]. Consistently, IgG 1 and 3 are elevated in patients with DHF/DSS, whereas IgG 2 levels are not (chapter 3.1). IgG 4 may play an inhibitory role in complement activation [43], therefore the significant increase in DSS patients described in chapter 3.1 might seem contradictory. However, production of IgG 4 is stimulated by IL-4, a typical Th2 cytokine [44]. A shift of the T-helper responses from Th1 to Th2 during defervescence has been described to occur during DENV infections and is associated with severe disease [45]. In addition, Th2 responses are commonly associated with increased severity of disease for many other acute viral infections including influenza and HIV infections [46].

In chapter 3.1 we also studied the kinetics of DENV-specific IgA antibodies. Low levels of IgA antibodies have been described during primary and secondary DENV infections ([47,48] and chapter 3.1). However, the magnitude of IgA responses is lower than that of the IgM responses measured in DENV patients during acute infection and the kinetics are much shorter, since IgA is only detected during acute and early convalescent phase and usually declines soon afterwards. This immunoglobulin exhibits high cross-reactivity within the DENV serocomplex, similar to the cross-reactivity observed for IgG [49]. Although the biological significance of elevated IgA responses is not well understood, this immunoglobulin may probably serve as an accurate diagnostic marker for acute DENV infections.

Finally in chapter 3.2 we describe the levels and kinetics of total and DENV-specific IgE antibodies during primary and secondary DENV infections. We showed a significant increase of IgE during secondary infections confirming previous studies [50,45]. High levels of total IgE antibodies have been associated with increased pathogenicity in flaviviral infections including DENV and JEV and other viral infections such as hantavirus infection [51,52,53]. Production of IgE is triggered by IL-4 and IL-13. Supported by the notion that shift to Th2 responses is associated with increased severity of DENV disease, elevation of IgE antibodies is expected in DHF/DSS patients. Moreover, IgE is associated with induction of IL-1β and TNF-α, both cytokines implicated in severe DENV disease.

Even though current techniques cannot discriminate between type specific Ig antibodies, humoral Ig responses appear to be crucial and directly or indirectly involved in the development of DHF/DSS. Many questions need to be answered to elucidate DENV pathogenesis but the studies described in chapters 3.1 and 3.2 have increased our understanding of the humoral arm of the immune system during DENV infections and of the implications that these responses have in the development of severe DENV disease.

The role of antibodies in enhancement of DENV infections has been considered central for decades. Enhancement of infection of susceptible cells will result in increased virus replication triggering a burst in cytokine production and subsequently damage of the vascular

endothelium and plasma leakage. Although ADE may explain in part the immune-mediated pathogenetic phenomena seen during DHF/DSS, it is not likely to be the only factor associated with immune-mediated pathogenesis. It is likely that the quantity as well as the quality of the antibody response are both important when studying DENV pathogenesis. The levels of "optimal" or "sub-optimal" VN antibodies are not well defined and it is likely that even in the presence of high levels of VN antibodies ADE may still occur. On the other hand, non-VN antibodies will not necessarily be detrimental for the host but may have a protective effect. In a recent study it was shown that severity of DENV-3 secondary infection is associated with pre-existing VN antibodies to DENV-3 virus [54]. The authors of this study suggested that pre-existing DENV-3 antibodies will have a protective effect when at high levels upon secondary infection with DENV-3. This study supports the ADE hypothesis when assuming that the low levels of DENV-3 VN antibodies are sub-optimal. However, this effect was not observed during secondary DENV-2 or DENV-1 infections. These findings suggest that the sequence of infection events is crucial for development of DHF/DSS due to shaping of the immune response by a particular serotype. In chapter 4.1 we showed that priming the immune system with DENV-1 had a significant effect on the immune responses measured after secondary DENV-3 infection, whereas such an effect was not measured with priming the immune system with DENV-4. OAS originally described for influenza virus [55] has now been observed in association with the humoral and cellular immune responses upon secondary DENV infections ([56,57] and chapter 4.1). Whether OAS is involved in DENV pathogenesis remains unclear however, recent evidence supports the notion that the quality of the immune response triggered after primary DENV infections is crucial for subsequent heterologous infections.

In addition to antibodies produced during DENV infections, plasma concentrations of other mediators involved in hemostasis are changed during infection and have been shown to be associated with disease severity. In chapter 3.3 we showed a significant association of plasma levels of sVCAM-1 and disease severity. Activation of the coagulation and fibrinolytic pathway during (secondary) DENV infections as evident by the presence of soluble VCAM, ICAM, thrombomodulin, F1+2, D-dimer ([58,59] and chapter 3.3) have been implicated in the development of DHF and DSS. Endothelial cell activation, reflected by the presence of VCAM, ICAM and thrombomodulin, is hypothesized to be involved in the onset and regulation of coagulation in patients with DHF/DSS ([60] and chapter 3.3). Significant coagulation abnormalities have been demonstrated to occur during DENV infections and are associated with either a loss of coagulation factors due to increased vascular permeability or disseminated intravascular coagulation (DIC)([59] and A.T.A. Mairuhu et al., submitted for publication). One of the main roles of PAI-1 in vivo is to inhibit fibrinolytic activity, therefore increased concentrations of PAI-1 may contribute to a procoagulant state leading to increased deposition of fibrin and subsequent multiorgan failure and death. Inhibition of the fibrinolytic cascade has been demonstrated with increased levels of PAI-1 in patients with poor clinical outcome of DENV infection [1,59]. PAI-1 is secreted upon stimulation of a variety of cells (i.e. endothelial, hepatocytes, platelets) by IL-1 or TNF-α. Infection with DENV leads to activation

of T cells [61] and induction of such proinflammatory cytokines [2] that may contribute to subsequent production of PAI- 1 and inhibition of fibrinolysis is believed to have detrimental effects on the host.

MACAQUE MODEL TO STUDY DENV INFECTION AND THE POTENTIAL OF VACCINE CANDIDATES

Although the majority of DENV infections will be sub-clinical or mild febrile illness (DF), a substantial proportion of infected individuals will develop DHF characterized by increased vascular permeability, a condition that might be life-threatening especially when progressing to DSS. Despite the importance and impact of DENV infections on public health and the years of studying the mechanisms for developing DHF/DSS, the pathogenesis of DENV infections remains poorly understood. As stated above, current hypotheses to explain the pathogenesis of DENV infections include ADE and the immune-mediated pathogenesis due to abnormal T cell activation [62,35,63]. Other studies have identified the importance of OAS and viral virulence as well as host factors in the development of severe dengue disease [56,64,57,65,66]. The lack of an appropriate animal model in which disease signs are displayed upon infection with DENV, has hampered research on DENV pathogenesis and therefore fundamental questions on the role of humoral and cellular immune responses, host factors and viral virulence in the pathogenesis of DENV infections remain largely unanswered. Animal models have been used extensively to study infectious diseases and have been established for many viral infections [67,68,69,70]. Small animals such as rodents that are widely used in research laboratories to study other infectious diseases have not proved useful for studies on DENV infections: several murine models of dengue disease have been reported. Mice infected with cell-cultured or mouse-brain adapted strains of DENV displayed signs of paralysis which is not a typical clinical sign of DENV infections [71]. More recently NOD/SCID mice displayed more relevant signs of dengue disease such as fever, rash, thrombocytopenia and erythema [72]. On the other hand several non-human primate species have been used with varying degrees of success in experimental DENV infections and the characteristics of infections largely proved to resemble the characteristics of natural human DENV infections [73,74,75]. During the early 1970's Halstead and colleagues infected a number of different monkey species with DENV and concluded that rhesus and cynomolgus macaques had the better potential to study DENV infections. Both these species were subsequently used in other studies mainly to evaluate DENV vaccine candidates. In chapter 4 we describe the evaluation of the cynomolgus macaque model to study DENV infections. The experimental set up described in chapter 4.1 was designed to address the existing hypotheses for DENV pathogenesis, namely the effect of primary DENV infections on immune responses upon subsequent heterologous infections. Both humoral and cellular immune responses were studied. Sequential infections with heterologous DENV serotypes have been associated with increased risk of developing severe disease both due to (adaptive) humoral responses as well as T cell responses. Primary infection with DENV would lead to induction of

DENV-specific T cells with specific and cross-reactive properties. When acute infection is resolved, the majority of the DENV specific T cells will undergo apoptosis whereas a small proportion of these cells will contribute to the memory pool of T cells of the individual. Upon secondary infection with a heterologous DENV serotype (or even a different strain of the same serotype), the DENV specific T cells of the memory pool will be more readily available and become rapidly activated to combat the invading serotype. A proportion of these cells will be specific to shared epitopes among the different serotype (thus being DENV "specific" and bind to their antigens with high avidity) but other memory T cells will have cross-reactive properties (thus being DENV cross-reactive and bind to their antigens with lower avidity). In contrast, naïve cells will be activated at a later time if at all to become specific for the new serotype. The overall effect that is measured is a boost in T cell responses against antigens of the first serotype encountered and not the most currently infecting one. This phenomenon of OAS has been described during natural human infections[57]. In chapter 4.1 we provide preliminary indications that this phenomenon may occur in experimentally infected macaques after a particular sequence of infections (DENV-1 followed by DENV-3 infections, and apparently not after DENV-4 followed by DENV-3 infection).

In chapter 4.1 we observed that the immune responses induced upon tertiary DENV infection are more likely to be protective rather than pathogenic. DENV-specific antibodies and memory T cells induced during sequential infections against shared and cross-reactive epitopes are likely to be adequate for combating the virus before the induction of serotype specific T cells.

Theoretically the development of a protective vaccine against all DENV infections should be considered feasible. Infection with DENV is usually mild, with short viremia and life long immunity to the infecting serotype. However, an effective vaccine against all four DENV serotypes is still not licensed for human use, despite the many efforts of several groups to develop a safe and protective DENV vaccine (see Table 3 of Introduction). In chapter 4.2, we describe findings of an evaluation of a live attenuated tetravalent DENV vaccine candidate in the cynomolgus macaque model. In addition, the effect of specific pre-existing immunity of macaques upon vaccination with the candidate DENV vaccine is described. Vaccination against DENV should confer protection against dengue disease and not predispose for severe infection upon subsequent challenge. If pre-exposure to DENV may predispose the individual to higher risk in developing DHF/DSS then an effective vaccine should be protective for both DENV naïve and DENV immune individuals. Taking into consideration that DENV is endemic in most of the tropics where DHF/DSS is more often seen, such an evaluation is necessary before a vaccine reaches the stage of clinical trials. The candidate vaccine that we tested in chapter 4.2, proved to be immunogenic, eliciting both humoral and cellular immune responses in cynomolgus macaques after a single dose. However, the immunogenecity of the vaccine was not within the same range for all four DENV serotypes after one dose of vaccine. Therefore a second vaccination was essential to elicit more uniform immune responses against all four serotypes. Similar results were previously obtained after live attenuated

tetravalent DENV vaccination of rhesus macaques, in which equal immunogenicity was obtained after careful evaluation of the dose of each monovalent vaccine component [76]. Undetectable viremia in all macaques after challenge with wild type DENV-4 highlights the protective potential of the candidate vaccine described in chapter 4.2. Macaques also did not show a significant increase in DENV-4 VN antibodies after challenge with DENV-4 suggesting that the challenge virus did not replicate to such an extent that a significant boost of DENV-4 specific VN antibodies could be obsereved. In other words, VN antibodies present at the moment of challenge were probably sufficient to control infection with wild-type DENV. These experiments should be followed by further challenge experiments of vaccinated macaques to include all four DENV serotypes. In addition, pre immunity to DENV (DENV-3 in chapter 4.2) did not influence the immunogenecity or the efficacy of the candidate vaccine. Although differences in humoral and cellular immune responses were observed among DENV-naïve and DENV-immune macaques receiving the same formulations of DENV vaccine, these differences did not show a significant effect of pre-existing immunity to DENV after vaccination and challenge, making the particular vaccine formulation a good candidate for further evaluation.

FUTURE STUDIES

Sixty years after the discovery of DENV and 30 years of continuous research to elucidate the different manifestations of DENV pathogenesis, progress in this field has remained limited. At the beginning of the 21st century there seems to be general agreement that progression to DHF/DSS is a multifactorial event.

We hypothesize that primary DENV infection will induce a wide range of immune responses: serotype-specific (unique epitopes found only on one specific serotype), serotype-shared (epitopes that are found on all four serotypes, are likely to be 100% identical on their primary amino acid sequence and are recognized by all four serotypes with the same avidity) and serotype cross-reactive (epitopes with amino acid sequences with more than 30% homology between the different serotypes that are recognized by heterologous DENV with lower affinity). Primary immune responses are likely to alter immune responses to a secondary infection, with considerable consequences for the outcome of subsequent infection with a heterologous serotype. Serotype specific immunity induced upon primary infection, will play a limited role upon secondary infection with a heterologous virus. On the contrary, serotype-shared and serotype cross-reactive responses may lead to increased pathogenesis. Thus identification of mediators of such responses would be crucial in the elucidation of DENV pathogenesis. In addition, studies aiming to identify cross-reactive, possibly pathogenic, epitopes encoded by the DENV genome will provide powerful tools in the rational design of diagnostic tools and protective vaccines against DENV. Well designed prospective studies in areas where different DENV serotypes circulate will probably be required to address questions about DENV pathogenesis. Given the limitations of field studies the alternative to study DENV pathogenesis would be the use of animal models. In the absence

of animals displaying disease upon DENV infection, characterization of their surrogate markers as parameters (including molecules of the fibrinolytic and coagulation pathways) leading to human disease seems to be necessary.

Despite the indications that OAS might contribute to DENV pathogenesis both at the B and T cell level, little research has been done to understand the role of OAS in DENV pathogenesis. Although the immunological mechanisms to explain OAS are not completely elucidated, OAS will occur if cross-reactive memory B and T cells that were induced during a primary infection are dominant over naïve responses to a heterologous serotype upon secondary infection. As a consequence of OAS-mediated B cell response during a secondary or tertiary infection with a heterologous DENV, high levels of low avidity cross-reacting antibodies will be produced. Under certain conditions, these antibodies could increase infection of target cells via ADE of virus infection. Further studies on the role of OAS in DENV infections and it's implication in pathogenesis may lead to better understanding of DENV infection and associated disease.

Considering the studies described in the present thesis some important areas of future research could be identified:

- Diagnostic procedures for DENV infections can be improved in research as well as in routine diagnostic settings. Accurate and timely diagnosis is crucial for the management of patients with DENV-associated disease. Alternatively to the antigen detection assay proposed in chapter 2.3, further development of specific assays employing serotype-specific peptides should be considered. Identification of serotype-specific epitopes eliciting antibody responses and their use in development of diagnostic assays would greatly increase the specificity of DENV diagnosis, overcoming the challenges of cross-reactions observed among DENV and related flaviviruses. A simple, inexpensive, rapid and accurate test (such as a peptide-based EIA) would be ideal for tropical developing countries where DENV is endemic. In addition, elucidating the antibody response reactivities during sequential DENV infections and identifying serotype-specific linear and conformational B cell epitopes would be crucial for our understanding of their role in primary and anamnestic B cell responses.

- Elucidation of the role of DENV proteins, especially NS1 as a biomarker for DHF/DSS and it's involvement in autoimmune mediated pathogenesis, could seriously be considered.

- Indication exist that OAS occurs during heterologous secondary DENV infection ([56,57] and chapter 4.1). Understanding the role of B and T cell mediated-OAS on sequential DENV infections, elucidating the mechanisms, factors and epitopes underlining B and T cell mediated OAS, and it's role in promoting ADE seems to be necessary.

- Prognosis of DHF/DSS could be improved when patients are managed in a timely way. In chapter 3 of this thesis and in reference [2], we identified factors with

predictive potential for development of DHF/DSS. Further studies to identify factors associated with development of severe dengue disease are necessary and may include molecules involved in the fibrinolysis and coagulation pathways. In addition, DENV-associated abnormalities in coagulation and endothelial cell activation (A.T.A. Mairuhu et al., submitted for publication) provide a good model to study the effect of viral infections in the coagulation cascade. Prospective studies would increase our understanding of the humoral responses during DENV infections and possibly identify factors with predictive value. Such achievements would assist in the decrease of DENV-associated mortality.

- Prevention of DENV infections through vaccination would be the most effective way to reduce morbidity and mortality. A safe and effective vaccine against infections with all four DENV serotypes should confer protection without predisposing the individual to development of severe disease. In order to develop such a vaccine a better understanding of the immune responses elicited during DENV infection and their role in immunopathogenesis is necessary.

- Virus virulence has also been proposed as a factor contributing to disease severity (see Introduction). Structural differences between strains of the same serotype have been associated with virulence. For example, the secondary structure of the UTR's of the DENV genome have been hypothesized to play an important role in virulence. Such structures are located at the distal part of the 3'UTR, are conserved among all flaviviruses and independent from the proximal part of the UTR. This model is now challenged with the finding that the proximal part of the UTR may influence the formation of secondary or tertiary structures in the distal part and thus influence replication (Koraka et al., in preparation). Such findings warrant further investigations to define the role and the implication of such structures in virus replication and virulence.

Taken together, our increasing knowledge in immunology and the application of new tools and advanced laboratory techniques will significantly contribute to the design of more effective approaches aiming to further elucidate DENV pathogenesis, development of improved diagnostics and safe and effective immunization strategies.

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Nederlandse samenvatting

Infecties met dengue virussen (DENV) zijn wereldwijd de meest voorkomende door geleedpotigen overgedragen infecties en veroorzaken jaarlijks ongeveer 250.000 dodelijke slachtoffers. Elk van de vier nauw verwante, maar in antigenetisch opzicht verschillende serotypes (DENV 1-4) veroorzaken een breed spectrum aan ziektebeelden. Van milde ongedifferentieerde ziekte met koorts (dengue fever: DF) tot dengue (een influenzachtig ziektebeeld gekarakteriseerd door een maculopapulaire uitslag) of tot de meer ernstige dengue hemorragische koorts (DHF), die gekarakteriseerd wordt door verhoogde vasculaire permeabiliteit en interne bloedingen. In de gevallen dat de bloedingen kritisch worden, kunnen de patiënten dengue schok syndroom (DSS) ontwikkelen, dat vaak een dodelijke afloop heeft wanneer niet op tijd behandeld wordt.

Dit proefschrift is het resultaat van een onderzoeksprogramma uitgevoerd binnen de afdeling virologie van het Erasmus MC te Rotterdam, voor een deel in samenwerking met het Dr. Kariadi Ziekenhuis in Semarang, Indonesië (hoofdstuk 3) en in overleg met de Wereldgezondheidsorganisatie (hoofdstuk 4).

Het doel van het onderzoek was om virologische en immunologische aspecten te bestuderen van natuurlijke DENV infecties bij mensen met verschillende ernst van de ziekte en bij experimenteel geinfecteerde makaken.

DENV zijn endemisch in het grootste gedeelte van de tropen en zijn verantwoordelijk voor tenminste 50 miljoen menselijke infecties per jaar. In vele delen van de wereld zijn DENV co-endemisch met andere nauw verwante flavivirussen hetgeen differentieel diagnostiek moeilijk maakt. Hoewel de meerderheid van DENV geinfecteerde individuen nauwelijks of slechts milde ziekteverschijnselen vertoont, ontwikkelt ongeveer 5% de meest ernstige vorm van de ziekte. Daarom is een snelle en juiste diagnose van het grootste belang voor de juiste behandeling van geinfecteerde patiënten. In hoofdstuk 2 wordt een overzicht gegeven van commerciëel verkrijgbare testen voor de laboratorium diagnose van DENV infecties. De beperkingen van deze veelal gebruikte testen worden beschreven en alternatieve diagnostische methoden worden geëvalueerd. In hoofdstuk 2.1 worden zes verschillende commercieel verkrijgbare testen voor het aantonen van DENV infecties geëvalueerd, gebruikmakend van een zorgvuldig gekozen panel van serummonsters afkomstig van individuen met en zonder DENV infecties. De kwaliteit van elke test m.b.t. sensitiviteit en specificiteit werd bepaald. In hoofdstuk 2.2. worden de beperkingen van serologische methoden om infecties met DENV of andere flavivirussen aan te tonen, aangegeven. Serummonsters afkomstig van patiënten met bevestigde flavivirus-infecties (inclusief DENV, West-Nile virus, Japanese encephalitis virus en tick-borne encephalitis virus) werden getest op de aanwezigheid van specifieke IgM en IgG antistoffen, gebruikmakend van twee veel gebruikte serologische tetsen (immunofluoresentie en ELISA). In alle serummonsters

werden kruisreactieve antilichamen aangetoond, hetgeen de beperkte specificiteit van deze testen benadrukt. In hoofdstuk 2.3. wordt een alternatieve methode voor de laboratoriumdiagnostik van DENV infecties voorgesteld. Het aantonen van DENV antigenen in serum van patiënten die een acute infectie doormaken bleek gevoeliger en specifieker dan het aantonen van IgM antistoffen in dezelfde serummonsters. Deze nieuw ontwikkelde antigeen-detectie test bleek ook betere resultaten op te leveren dan een commerciëel test.

De pathogenese van DENV infecties is slechts ten dele opgehelderd ondanks drie decennia van onderzoek. Het is algemeen geaccepteerd dat bij de pathogenese van DENV infecties meerdere factoren betrokken zijn, zoals cytokine-, antilichaam- en T cel responsen alsmede andere gastheer factoren en virologische parameters. Er wordt verondersteld dat humorale immuunresponsen een belangrijke rol spelen by DENV infecties en dat deze direct of indirect betrokken zijn bij de pathogenese van ernstige ziekte. In hoofdstuk 3 worden humorale immuunresponsen beschreven die werden gemeten in DENV geinfecteerde patiënten met verschillende ernst van ziekte. In hoofdstuk 3.1 worden de IgM, IgA en IgG subklassen van DENV specifiek serumantilichamen vergeleken: (i) in het serum van patiënten met verschillen in de ernst van ziekte en (ii) in gepaarde controles van individuen zonder DENV infecties. De meest voorkomende specifieke immuunglobulines gedurende het beloop van de DENV infectie waren IgM, IgG1 en IgG 3. De DENV-specifieke IgA, IgG 4 en IgG 1 antilichaamtiters waren significant hoger in patiënten met DSS in vergelijking met patiënten met DF. Dit suggereert dat deze antilichamen een risicofactor zijn voor de ontwikkeling van DSS. In hoofdstuk 3.2 wordt de kinetiek van de hele en de DENV specifieke IgE antilichaamresponse beschreven in patiënten met primaire en secundaire DENV infecties. De DENV specifieke IgE antilichaamtiters waren significant verhoogd in patiënten met DHF en DSS ten opzichte van patiënten met DF die primaire of secundaire infecties doormaakten. In hoofdstuk 3.3 werd van dezelfde groep patiënten de hoeveelheid oplosbaar VCAM-1 eiwit (sVCAM-1) in het plasma bepaald. Tijdens de acute fase van DSS was de sVCAM-1 concentratie in het plasma significant verhoogd, maar niet gedurende de convalescente fase of in patiënten met DF. Dus in hoofdstuk 3, werden markers voor een verhoogd risico voor het ontwikkelen van ernstige dengue geassocieerde ziekte geïdentificeerd en hun voorspellende waarde geëvalueerd. Deze risicomarkers zijn niet alleen belangrijk voor de prognose van dengue geassocieerde ziekte, maar ze spelen mogelijk ook een rol bij de pathogenese van DENV infecties.

Er zijn geen diermodellen beschikbaar waarmee de ontwikkeling van ziekteverschijnselen na DENV infectie effectief bestudeerd kunnen worden. De beschikbaarheid van een niet-humaan primaat model zou zeer wenselijk zijn om DENV infecties te bestuderen en om vragen t.a.v. de pathogenese te beantwoorden. In hoofdstuk 4 wordt de ontwikkeling beschreven van een dier-model om DENV infecties te bestuderen en het gebruik om DENV vaccin-kandidaten te evalueren. In hoofdstuk 4.1 werden cynomolgus makaken experimenteel geinfecteerd met

DENV-1 of DENV-4 gevolgd door een secundaire infectie met DENV-3 en een tertiaire infectie met DENV-4. De kinetiek van de viremie, van de humorale en van de cellulaire immuunresponsen werden bepaald en deze vertoonden grote overeenkomsten met wat beschreven is voor natuurlijke infecties bij mensen. Zowel de specifieke antilichaam- als de T celrespons vertoonden kruis-reactiviteit na opeenvolgende infecties met heterologe DENV. De immuunrespons na de secundaire en tertiaire infecties waren sterker tegen het DENV serotype dat voor primaire infectie werd gebruikt. In hoofdstuk 4.2 wordt beschreven hoe dit diermodel werd gebruikt voor de evaluatie van veiligheid en werkzaamheid van een levend verzwakt tetravalent DENV kandidaat-vaccin in naïve dieren en dieren die eerder een infectie met een dengue virus hadden doorgemaakt. Alle makaken ontwikkelden vergelijkbare antilichaam en T celresponsen tegen alle vier DENV serotypes na een of twee doses met het kandidaat-vaccin. Het was duidelijk dat beschermende immuniteit was geïnduceerd omdat in geen van de gevaccineerde dieren viremie kon worden aangetoond na challenge-infectie met het wild type DENV. Bovendien werd aangetoond dat de aanwezigheid van pre-existerende immuniteit tegen DENV-3 geen invloed had op de immuunrespons die door het kandidaat-vaccin was opgewekt of op de beschermende werking van het vaccin tegen de challenge-infectie. Tot slot wordt in hoofdstuk 4 een regio van 100 aminozuren in het NS2A eiwit van DENV beschreven die kruis-reactieve DENV-specifieke T cel-epitopen lijkt te bevatten.

ΠΕΡΙΛΗΨΗ

Ο δάγκειος πυρετός είναι ένα λοιμώδες νόσημα, το οποίο μεταδίδεται με το τσίμπημα κουνουπιού του γένους Aedes και συναντάται σε αστικά και περιαστικά κέντρα των τροπικών και υποτροπικών περιοχών (κυρίως στην Ασία και Λατινική Αμερική) . Τις τελευταίες δεκαετίες τα κρούσματα του δάγκειου πυρετού έχουν αυξηθεί ραγδαία. Η Παγκόσμια Οργάνωση Υγείας (Π.Ο.Υ.) εκτιμά ότι πάνω από τα 2/5 του παγκόσμιου πληθυσμού κινδυνεύουν σήμερα από τον δάγκειο, ο οποίος είναι ενδημικός σε περίπου 100 χώρες . Σύμφωνα με την Π.Ο.Υ. οι λοιμώξεις με δάγκειο ανέρχονται στις 50.000.000 ετησίως με 250.000 θανάτους.

Κάθε ένας από τους 4 διαφορετικούς ορότυπους του δάγκειου ιού (DENV 1-4) μπορεί να προκαλέσει ένα ευρύ φάσμα ασθενειών : (i) ήπιο αδιαφοροποίητο πυρετό, (ii) δάγκειο πυρετό (dengue fever [DF]), (iii) δάγκειο αιμοραγικό πυρετό (dengue hemorrhagic fever [DHF]), χαρακτηριζόμενο από αυξημένη αγγειακή διαβατότητα και εσωτερική αιμοραγία. Ο δάγκειος αιμοραγικός πυρετός μπορεί να προκαλέσει καταπληξία (dengue shock syndrome [DSS]), ένα σύνδρομο με αυξημένη θνησιμότητα εάν δεν αντιμετωπιστεί έγκαιρα.

Η παρούσα διατριβή είναι το αποτέλεσμα ερευνών που τελέστηκαν στο τμήμα Ιολογίας του Πανεπιστημίου Εράσμους του Ρόττερνταμ Ολλανδίας. Η έρευνα που περιγράφεται στο κεφάλαιο 3 τελέστηκε σε συνεργασία με το νοσοκομείο Δρ. Καριάντι της Σεμάρανγκ, Ινδονησία, ενώ η έρευνα που περιγράφεται στο κεφάλαιο 4 τελέστηκε σε συνεργασία με την Π.Ο.Υ. Ο στόχος αυτής της διατριβής ήταν να μελετηθούν οι ιολογικές και ανοσολογικές πτυχές των λοιμώξεων του δάγκειου.

Έγκαιρη διάγνωση του δάγκειου είναι απαραίτητη για την κατάλληλη θεραπευτική αγωγή των ασθενών (ειδικά αυτών που αναπτύσσουν DHF/DSS). Παράλληλα, η ακριβής διάγνωση του δάγκειου παραμένει προβληματική επειδή σε πολλές περιοχές ο δάγκειος είναι συν-ενδημικός με άλλους Φλαβιιούς, γεγονός που δυσκολεύει τη διαφορική διάγνωση.

Στο κεφάλαιο 2 παρουσιάζεται μια επισκόπηση των εμπορικά διαθέσιμων μεθόδων για την εργαστηριακή διάγνωση των λοιμώξεων του δάγκειου, τα μειονεκτήματα που μπορεί να έχουν αυτές οι μέθοδοι, καθώς επίσης και εναλλακτικές διαγνωστικές μέθοδοι. Στο κεφάλαιο 2.1 αξιολογήθηκαν έξι διαφορετικές εμπορικές μέθοδοι για την (ορολογική) εργαστηριακή διάγνωση λοιμώξεων του δάγκειου. Οι μέθοδοι αυτοί περιλαμβάνουν ενζυμικούς ανοσο-προσδιορισμούς, μεθόδους έμμεσου ανοσοφθορισμού, μεθόδους ανοσοαποτυπώματος western, κ.α. για τον προσδιορισμό αντισωμάτων της τάξης IgM και IgG. Για την αξιολόγηση αυτή χρησιμοποιήθηκε ένα προσεκτικά επιλεγμένο σετ οροδειγμάτων από ασθενείς με ή χωρίς λοιμώξεις δάγκειου. Κάθε μέθοδος αξιολογήθηκε ξεχωριστά για την ευαισθησία και ειδικότητά της, συγκρινόμενη με τις υπόλοιπες μεθόδους.

Στο κεφάλαιο 2.2 παρουσιάζονται τα μειονεκτήματα δύο ευρέως διαδεδομένων ορολογικών μεθόδων (ενζυμικός ανοσοπροσδιορισμός & έμμεσος ανοσοφθορισμός) για την ακριβή διάγνωση

των λοιμώξεων του δάγκειου και άλλων Φλαβιΐων. Δείγματα ορών από ασθενείς με λοιμώξεις Φλαβιΐων (συμπεριλαμβανομένων δάγκειου, ιού του δυτικού Νείλου, ιού της ιαπωνικής εγκεφαλίτιδας, κ.α.) εξετάστηκαν για την παρουσία IgM & IgG αντισωμάτων. Αντισώματα διασταυρούμενων αντιδράσεων (ειδικά της τάξης IgG) ανιχνεύθηκαν σε όλα τα δείγματα ορών, υπογραμμίζοντας τη χαμηλή ειδικότητα αυτών των μεθόδων.

Στο κεφάλαιο 2.3 προτείνεται μια εναλλακτική διαγνωστική μέθοδος για τις λοιμώξεις του δάγκειου. Η ανίχνευση αντιγόνων του δάγκειου χρησιμοποιώντας τη μέθοδο αποτύπωσης κηλίδας σε δείγματα ορών ασθενών με οξείες λοιμώξεις, αποδείχθηκε πιο ευαίσθητη και ειδική από την ανίχνευση IgM αντισωμάτων. Η μέθοδος αυτή που αναπτύχθηκε στο εργαστήριό μας, αποδείχθηκε καλύτερη από μια ανάλογη, εμπορικά διαθέσιμη, μέθοδο.

Οι γνώσεις μας για την παθογένεια των λοιμώξεων του δάγκειου παραμένουν ελλιπείς, παρά τις εκτεταμένες έρευνες που έχουν τελεστεί τις τελευταίες τρεις δεκαετίες. Σήμερα είναι ευρέως αποδεκτό ότι πολλαπλοί παράγοντες συντελούν στην παθογένεια του δάγκειου, όπως για παράδειγμα αντισώματα, Τ-λεμφοκύτταρα, κυτταροκίνες, παράγοντες που προέρχονται από τον ιό ή ακόμα και από τον ξενιστή. Επιδημιολογικές ενδείξεις έχουν οδηγήσει στην πιο διαδεδομένη θεωρία για την εξήγηση της παθογένειες του δάγκειου. Η θεωρία αυτή υποστηρίζει ότι κατά την διάρκεια δευτερογενούς λοίμωξης με δάγκειο διαφορετικού ορότυπου από αυτόν της πρωτογενούς λοίμωξης, ο ασθενής παρουσιάζει αυξημένο κίνδυνο ανάπτυξης αιμορραγικού πυρετού. Εξαιτίας διασταυρούμενων αντιδράσεων οι ανοσοαπαντήσεις (κυρίως αντισώματα) κατά την διάρκεια δευτερογενών λοιμώξεων συντελούν στην ενίσχυση της λοίμωξης και όχι στον περιορισμό της (antibody dependent enhancement of infection [ADE]).

Οι χυμώδεις ανοσοαπαντήσεις θεωρούνται ότι διαδραματίζουν σημαντικό ρόλο κατά τη διάρκεια λοιμώξεων του δάγκειου και θα μπορούσαν να συνδεθούν άμεσα ή έμμεσα με την παθογένεια της σοβαρής ασθένειας (DHF/DSS). Στο κεφάλαιο 3 περιγράφονται οι χυμώδεις ανοσοαπαντήσεις ασθενών με λοιμώξεις δάγκειου ποικίλης σοβαρότητας (π.χ. DF & DHF/DSS).

Στο κεφάλαιο 3.1 περιγράφονται οι χυμώδεις ανοσοαπαντήσεις των τάξεων IgM, IgA & IgG, καθώς επίσης και των υποτάξεων IgG 1, 2, 3 & 4, σε ασθενείς με DF & DHF/DSS. Αντισώματα των τάξεων IgM, IgG1 & IgG3 αξιολογήθηκαν ως τα κυρίαρχα αντισώματα κατά την διάρκεια οξέως επεισοδίου δάγκειου. Παράλληλα, IgA, IgG4 & IgG1 αντισώματα βρέθηκαν σημαντικά αυξημένα σε ασθενείς με σοβαρή ασθένεια (DSS) έναντι ασθενών με ήπια μορφή δάγκειου, προτείνοντας την πιθανότητα αυτά τα αντισώματα να χρησιμοποιηθούν ως δείκτες κινδύνου για την ανάπτυξη αιμορραγικού πυρετού.

Στο κεφάλαιο 3.2 περιγράφονται οι ανοσοπαντήσεις της τάξης αντισωμάτων IgE (ολικών και ειδικών για τον δάγκειο αντισωμάτων), σε ασθενείς με πρωτογενείς λοιμώξεις ή ύστερα από πολλαπλές λοιμώξεις δάγκειου. IgE αντισώματα (ειδικά για τον δάγκειο) βρέθηκαν σημαντικά αυξημένα σε ασθενείς με DHF/DSS έναντι ασθενών με DF ανεξάρτητα από το ιστορικό τους (εάν έπασχαν από πρωτογενή ή δευτερογενή λοίμωξη δάγκειου).

Στο κεφάλαιο 3.3 δείγματα από το ίδιο σετ ασθενών χρησιμοποιήθηκαν για την μέτρηση επιπέδων του διαλυτού αγγειακού μορίου προσκόλλησης -1 (soluble-vascular cell adhesion molecule-1 [s-VCAM-1]). Ο παράγοντας s-VCAM 1 βρέθηκε σημαντικά αυξημένος κατά την διάρκεια οξέως επεισοδίου δάγκειου αιμορραγικού πυρετού, αλλά όχι κατά την διάρκεια της ανάρρωσης ασθενών με δάγκειο αιμορραγικό πυρετό, ή σε ασθενείς με ήπια μορφή δάγκειου.

Συνολικά στο κεφάλαιο 3 προσδιορίστηκαν δείκτες κινδύνου για την ανάπτυξη σοβαρών επιπλοκών κατά την διάρκεια λοιμώξεων δάγκειου και αξιολογήθηκε η προγνωστική τους αξία. Αυτοί οι δείκτες είναι σημαντικοί όχι μόνο για την πρόγνωση της ασθένειας του δάγκειου αλλά μπορεί να διαδραματίσουν σημαντικό ρόλο στην παθογένεση των λοιμώξεων δάγκειου.

Μέχρι σήμερα δεν υπάρχει ζωικό μοντέλο για την μελέτη των λοιμώξεων δάγκειου, καθώς πειραματόζωα που έχουν χρησιμοποιηθεί ως τώρα δεν αναπτύσσουν συμπτώματα της ασθένειας, παρόλο που είναι δυνατόν να μολυνθούν με τον ιό. Εντούτοις, πρωτεύοντα θηλαστικά (πλην του ανθρώπου), θα ήταν τα πιο κατάλληλα πειραματόζωα για την μελέτη λοιμώξεων δάγκειου με σκοπό την διεύρυνση των γνώσεών μας για την παθογένεια του ιού. Στο κεφάλαιο 4 περιγράφεται η ανάπτυξη ενός ζωικού μοντέλου για μελέτη των λοιμώξεων δάγκειου και την χρησιμοποίηση αυτού του μοντέλου για την αξιολόγηση υποψήφιων εμβολίων κατά του δάγκειου.

Στο κεφάλαιο 4.1 πίθηκοι *Macaca fascicularis* χρησιμοποιήθηκαν για πρόκληση πειραματικών λοιμώξεων με τους ορότυπους 1 & 4 του δάγκειου ιού. Την πρωτογενή λοίμωξη ακολούθησε δευτερογενής λοίμωξη με τον ορότυπο 3 του δάγκειου ιού και τριτογενής λοίμωξη με ορότυπο 4. Σκοπός της έρευνας αυτής ήταν η παρακολούθηση των κυτταρικών και χυμωδών ανοσοαπαντήσεων, καθώς επίσης και η μέτρηση του επιπέδου ιαιμίας κατά την διάρκεια οξέως επεισοδίου δάγκειου. Οι παραπάνω μελέτες οδήγησαν στα εξής συμπεράσματα:

(i)   η παρουσία του ιού στον ορό πειραματικά μολυσμένων πιθήκων είναι ανάλογη με την παρουσία του ιού στον ορό ασθενών με δάγκειο.

(ii)   οι ανοσοαπαντήσεις τόσο των B όσο και των T λεμφοκυττάρων είναι παρόμοιες σε πιθήκους και ανθρώπους, ύστερα από πρωτογενή, δευτερογενή ή τριτογενή λοίμωξη δάγκειου με ομόλογο ή ετερόλογο ορότυπο.

(iii)   οι ανοσοαπαντήσεις των B & T λεμφοκυττάρων είναι διασταυρούμενες ύστερα από διαδοχικές ετερόλογες λοιμώξεις δάγκειου.

(iv)   οι ανοσοαπαντήσεις B & T λεμφοκυττάρων κατά την διάρκεια δευτερογενούς ή τριτογενούς λοίμωξης δάγκειου παρουσιάζονται αυξημένες ως προς τον ορότυπο της πρωτογενούς λοίμωξης.

Στο κεφάλαιο 4.2 το ζωικό μοντέλο *M. fascicularis* χρησιμοποιήθηκε για την αξιολόγηση ενός υποψήφιου εμβολίου κατά του δάγκειου. Το εμβόλιο αυτό αποτελείται από μείγμα και των τεσσάρων ορότυπων του δάγκειου, των οποίων η δραστικότητα έχει εξασθενήσει ύστερα από συνεχείς καλλιέργειες σε κυτταρικές σειρές. Το εμβόλιο χορηγήθηκε σε δύο δόσεις σε

πειραματόζωα, τα οποία δεν είχαν έρθει σε επαφή με άλλο Φλαβιϊό, καθώς επίσης και σε πειραματόζωα, τα οποία είχαν πρωτύτερα ανοσοποιηθεί με τον ορότυπο 3 του δάγκειου ιού.

Σε όλα τα πειραματόζωα μετρήθηκαν ομοιόμορφες ανοσοαπαντήσεις (B & T λεμφοκυττάρων) προς τους 4 ορότυπους του δάγκειου ιού ύστερα από την πρώτη ή την δεύτερη δόση του υποψήφιου εμβολίου. Το εμβόλιο πρόσφερε προστασία από λοίμωξη με δάγκειο ιό, αφού κανένας πίθηκος δεν είχε ανιχνεύσιμο ιό κατά την πειραματική πρόκληση με ορότυπο 4 του δάγκειου. Επιπλέον παρατηρήθηκε ότι ανοσία προς τον δάγκειο ιό πριν τον εμβολιασμό δεν επηρέασε τις ανοσοαπαντήσεις των πειραματόζωων, ούτε παρεμπόδισε την προστασία αυτών των πιθήκων κατά την διάρκεια πρόκλησης με τον ορότυπο 4 του δάγκειου ιού.

Τέλος στο κεφάλαιο 4 περιγράφεται για πρώτη φορά μια αντιγονική περιοχή 100 περίπου αμινοξέων στην πρωτεΐνη NA2A του δάγκειου. Αυτή η περιοχή είναι πιθανόν να συμπεριλαμβάνει ειδικούς αλλά και διασταυρωμένους αντιδρώντες επίτοπους T λεμφοκυττάρων.

Τα αποτελέσματα αυτής της διατριβής ελπίζουμε να συμβάλλουν στην βαθύτερη κατανόηση των λοιμώξεων του δάγκειου ιού, κυρίως όσων αφορά τις χυμώδεις ανοσολογικές αντιδράσεις. Συνέχιση των ερευνών αυτών θα συμβάλλει σημαντικά στον σχεδιασμό νέων μεθόδων και πειραματικών προσεγγίσεων ώστε να αναπτύξουμε καλύτερες διαγνωστικές μεθόδους, κατάλληλα εμβόλια και περισσότερη γνώση σχετική με την παθογένεια του δάγκειου ιού.

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it's party time☺

Miss P

Curriculum Vitae

The author of this thesis was born on September 18th, 1973 in Athens, Greece. She obtained her bacherlor's degree as Technician of Medical Laboratory from the Technological Educational Institute of Athens, Greece, in 1997. From 1998 until 2001 she worked as a research technician in the Laboratory of Exotic Virus Infections, at the Academic Hospital (Erasmus Medical Centre) of Rotterdam, the Netherlands under the supervision of Dr. J Groen. In 2001 she obtained her Master of Science degree on Immunology of Infectious Diseases at De Montfort University, Leicester, United Kingdom. In the same year she started her Ph.D research at the Department of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands under the supervision of Prof. Dr. A.D.M.E OSterhaus. During her Ph.D study she followed courses on Medical Virology, Immunology, Molecular Medicine and Laboratory Animal Science. Since July 2006 she works as a research scientist at Viroscope BV, Rotterdam, the Netherlands.

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