Review Paper

The Pathogenesis and Immune-response in Dengue haemorrhagic fever

Abstract

Introduction

Dengue fever has spread globally to be endemic in more than 100 countries to a total estimate incidence of 50 – 100 million cases annually globally. About 0.7% of these cases become the complication that is dengue hemorrhagic fever which is severe and lead to about 22,000 deaths annually.

The pathogenesis of benign dengue fever becoming dengue hemorrhagic fever, and aspects of the immune-response behind it, have remained relatively unknown.

Method

Existing literature on the Topic was retrieved through Google Scholar and PubMed searches, and the literature reviewed.

Results

Dengue hemorrhagic fever appears commoner in females and those with co-morbids such as diabetes-mellitus and obesity. Also, the case-fatality rate in severe dengue appears much higher in females.The reasons for this is largely unknown but the more robust immune response in females, resulting in females to be more

prone to develop greater inflammatory response or higher susceptibility to capillary permeability could be the reason..

It has been shown that viremic-load, including the initial viremic-load at the bite of the Aedes-mosquito may be a factor leading to dengue hemorrhagic fever.

Yet different factors felt to be causative in the pathogenesis of dengue hemorrhagic fever include the role of the viral-protein, and then that which is termed the original antigenic-sin, the antibody-dependent enhancement, autoimmunity, inhibition of interferon-alpha and cytokine-storm within the memory-cells.

Regionally, certain different strains of the DENV also seems to be associated with dengue hemorrhagic fever.

Newer-vaccines, based on the immunology of the disease, offer much hope in the near future.

Conclusion

Much knowledge has been forthcoming in realizing the pathogenesis of dengue hemorrhagic fever. But, more needs to be done.

Key-words:Dengue fever; dengue hemorrhagic fever; pathogenesis; immune-response; gender; co-morbids; inflammatory response; capillary permeability; bleeding diathesis; disseminated intravascular coagulation (DIVC); viremic-load;

role of viral-protein; original antigenic-sin; antibody-dependent enhancement; autoimmunity; inhibition of interferon-alpha; cytokine-storm

INTRODUCTION

Dengue is considered the most problematic mosquito-transmitted viral disease in terms of morbidity and mortality. It is the most prevalent viral mosquito-borne disease, with over 2.5 billion humans at risk of exposure given its endemicity in more than 100 countries, compared to nine countries in 1970 [1–11].

Dengue fever is a benign, acute febrile syndrome found mainly in tropical regions. In a small proportion of cases, the virus causes increased vascular permeability that leads to a bleeding diathesis or disseminated intravascular coagulation (DIVC) known as dengue haemorrhagic fever (DHF) [3].

The WHO estimates that 50-100 million cases of dengue are incident annually, with approximately 500,000 of those cases (0.7%) resulting in dengue haemorrhagic-fever (DHF), with an estimated 22,000 deaths per year - mostly in children. In 20-30% of DHF cases, the patient develops shock, known as the dengue shock syndrome (DSS). [1, 4, 5 – 8]

The vectors are *Aedes aegypti*, which breeds in and around houses and buildings, and the smaller *Aedes albopictus* which breeds outdoors. They are day-biting

mosquitoes, whose peak biting-hours are dawn, early morning and dusk [1–11]. Besides various different factors, the bite of the *A. aegypti* is more likely to cause severe dengue (DHF and dengue shock syndrome, DSS).This appears related to the initial viral-load from the bite of the vector – the bite of the *A. aegypti* appears to cause a larger initial viral-load [12– 14].

Major sources of Aedes-breeding are at construction sites, solid-waste dumps, open-spaces and in factories [1–11].

METHODOLOGY

Existing literature on the Topic was retrieved through Google Scholar and PubMed searches, and the literature reviewed.

DISCUSSION

There are four (4) distinct, but closely-related, serotypes of the virus that cause dengue (DEN-1, DEN-2, DEN-3 and DEN-4). Recovery from infection by one provides lifelong-immunity against that specific-serotype. Still, cross-immunity to the remaining serotypes after recovery is only partial and temporary. Subsequent infections by the remaining serotypes increase the risk of developing severe dengue [1–11].

Clinically, dengue fever can manifest as uncomplicated dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The mechanism

behind the severe forms of dengue is the capillary leakage and increased vascular permeability of endothelial cells[1 – 11].

Severe dengue (DHF and DSS) may be commoner in females and those with co-morbids such as diabetes-mellitus and obesity. The case-fatality rate in severe dengue appears much higher in females [15 – 16].

The phases of dengue fever are febrile-phase, critical-phase and convalescence-phase.

Febrile-phase: The febrile-phase occurs 4-7 days after being bitten by an infected-mosquito and it lasts between 2 and 7 days. In this phase, there is high-fever (described as saddle-back fever), headache, retro-orbital pain, myalgia, typical macular-rash and facial-flushing [17 – 18]. There may be petechiae and minor gum bleeding, leucopaenia and thrombocytopenia. After about 3 to 5 days of fever, the patient enters into the critical-phase.

Critical-phase: Critical-phase occurs when the temperature settles (defer-vescence) typically during day 3 to 7 of dengue [17 – 19]. Plasma-leakage may be manifest during this period. In a patient who is not improving clinically despite the defervescence, besides a rising haematocrit and the presence of pleural effusion and ascites, it suggests plasma-leakage. Plasma-leakage must be detected early and treated to prevent it from progressing to severe decompensated and intractable

shock - ultimately leading to multi-organ failure and death. Patients with coexisting conditions such as diabetes mellitus, pregnancy, obesity, renal-failure, advanced age and chronic haemolytic-anaemia have a higher risk of dengue complications.

Whitehorn J et al (2000) showed that there was a dose-response relationship between the plasma viremia level and the proportion of mosquitoes with infectious saliva 14 days after blood-feeding. But, the likelihood of detecting infectious-saliva differed by mosquito-species and DENV-serotype. The detection of infectious-saliva was less likely in blood-fed *A. albopictus*, compared with blood-fed *A. aegypti* [20].

By serotype, the odds of *A. albopictus* having infectious-saliva were significantly lower for blood-meals involving uptake of DENV-2 and DENV-4, compared with those involving uptake of DENV-1 or DENV-3. The authors' data identified the odds of *A. albopictus* becoming infectious as lower than the odds of *A. aegypti* becoming infectious after feeding on the blood of viremic patients [20].

Further, it was seen that plasma viremia-level as a risk factor for infectiousness among *A. aegypti* and *A. albopictus* [20].

Vaughn DW et al (2000) showed that higher peak-titers were associated with increased disease-severity. Increased dengue disease-severity correlated with high viremia-titer, secondary dengue virus-infection, and DEN-2 virus-type [21].

Sam SS (2013) showed that nine out of ten fatal-cases in a Malaysian hospital involved adult females. The mean age was 32, and not commoner in children as used to be thought. All had secondary dengue infection. The mean duration of illness prior to hospitalization was 4.7 days and deaths took place at an average of 2.4 days post-admission. Gastrointestinal pain, vomiting, diarrhea, intravascular leakages and bleeding was seen in the majority of cases. DSS complicated with severe bleeding, multi-organ failure and coagulopathy were the primary causes of deaths [16].

Seven patients presented with thrombocytopenia and hypoalbuminemia, five of which had hemoconcentration and increased ALT and AST indicative of liver-damage. Co-morbidities, particularly diabetes mellitus, was common in the cohort. Prominent unusual presentations included acute renal failure, acute respiratory distress syndrome, myocarditis with pericarditis, and hemorrhages over the brain and heart.The majority of the patients presented with common clinical and laboratory warning-signs of severe dengue. Underlying co-morbidities appeared to contribute to the rapid clinical-deterioration in severe dengue [16].

Thus, there is a higher preponderance seen of fatal DHF/DSS amongst females. This is despite >55% of dengue cases seen at that hospital involved males. The observation was similar to that reported earlier where there was higher tendency of females to develop DHF/DSS [22 - 23] with higher mortality rate in females [24] even though males consistently comprised the larger proportion of both DF and DHF, especially in the ≥15 years age group [24 - 25]. More deaths among girls, especially those among the pediatric group, was also reported in Vietnam in 1996–2009, despite the predominance of boys in dengue cases [26 - 27]. Currently, there is no satisfactory explanation for this phenomenon but there are suggestions that this may be due to the more robust immune response in females, resulting in females to be more prone to develop greater inflammatory response or higher susceptibility to capillary permeability [28 - 29]. An explanation need to be sought.

Thrombocytopenia, endothelial-activation, liver-dysfunction and disseminated intravascular coagulation (DIVC) associated with severe dengue, all contribute to bleeding in dengue. Minor bleeding like gum-bleeding and skin-petechiae are relatively common in dengue fever. These are usually self-limiting and insignificant. Gastrointestinal (GI) bleeding, menorrhagia, epistaxis and haemoptysis may be severe and require blood-transfusion. Frequently, the bleeding is occult.

Prolonged-shock and metabolic-acidosis further leads to severe bleeding and disseminated intravascular coagulation (DIVC).

Besides plasma-leakage and bleeding, dengue virus also has pathogenic-involvement of the liver. Elevations of liver-enzymes are usually mild and are common in dengue. Aspartate aminotransferase (AST) is usually more severely elevated compared to alanine aminotransferase (ALT). The relatively frequent isolation of dengue virus from liver-tissues of fatal-cases of dengue-hepatitis suggests that liver-injury is directly mediated by dengue virus infection of hepatocytes and Kupffer-cells [30]. Thus, liver-damage may also affect clotting-factors, in turn contributing to the bleeding.

Related to DENV infection of the liver, haemophagocytic lymphohistiocytosis (HLH) is rare but is a potentially fatal medical-condition that can happen after a patient has had severe dengue-infection. Haemophagocytic lymphohistiocytosis is characterized by hyperinflammation, uncontrolled-proliferation of activated-lymphocytes, prolonged fever, pancytopenia, jaundice and hepatosplenomegaly. But, dengue-associated HLH may be under-recognized due to overlapping signs and symptoms of HLH and dengue (e.g., fever, hepato-splenomegaly and pancytopenia). Identification of dengue-patients with, or at risk of developing, HLH is by detection of markedly-high serum-ferritin levels (≥ 500 mg/l) or serum IL2-receptor (≥ 2400 units/L). Signs and symptoms associated with HLH include

hepatomegaly, splenomegaly and lymphadenopathy. Dengue with HLH usually has more prolonged -ever and hospitalization and is associated with warning-signs. Laboratory findings include anemia, besides raised aspartate transaminase and alanine transaminase. Treatment includes high-dose corticosteroids and IVIG with or without etoposide (Meer Ahmad AM et al 2018).

Just recently, we have also seen quite a number of patient who present with progressive liver impairment accompanied by AST higher then ALT without plasma leakage. We thought that cytokine storm could have caused this even before full-blown haemophagocytosis, and we are still debating concerning when is the best time to initiate steroids in these patients (Leong CL, *ibid* Dec 2018)

The heart, kidneys, lungs and central nervous system may also be involved both by direct action by the virus besides plasma-leakage and bleeding into and around these organs.

One hypothesis concerning virus-virulence and the immune-enhancement hypothesis has been debated. Although dengue disease-severity has been associated with evidence of genetic-differences in dengue-strains, virus-virulence has been difficult to measure because of the lack of in-vivo and in-vitro models of the disease [31]. In addition to the outcome from virus-load and virus-variation, abnormal immune-responses of the host after dengue-virusinfection may also

account for the progression to DHF/DSS. Viral-autoimmunity is also involved in the pathogenesis of numerous similar viral-infections, such as human immunodeficiency virus, human hepatitis C virus, human cytomegalovirus, herpes simplex virus and Epstein- Barr virus. Antibodies directed against dengue-virus non-structural protein 1 (NS1) shows cross-reactivity with human-platelets and endothelial-cells, which lead to platelet and endothelial-cell damage and inflammatory-activation. The hypothesis is that anti-DV (dengue virus) NS1 is involved in the pathogenesis of DHF/DSS [32]. There is also evidence that the presence of certain serotypes, including primary-infection with DENV-3 in the South-east Asia region and secondary-infection with DENV-2, DENV-3, and DENV-4 also in the South-east Asia region, besides DENV-2 and DENV-3 from non-South-east Asia regions, increased the risk of severe dengue [33].

Immune-responses in dengue fever

Immune-responses to dengue-virus induces the production of all classes of antibodies, primarily targeting the virus envelope-proteins. The level of antibodies primarily depends on whether the individual has a primary or a secondary dengue-infection [34 - 35].

Fig. 1 shows the Timelines of Laboratory Changes viz-a-viz Clinical-features. In a primary-infection, low antibody-titres are observed - while in a secondary-

infection, high IgG is detectable in the acute-phase which sometimes masks the detection of secondary-infection [36].

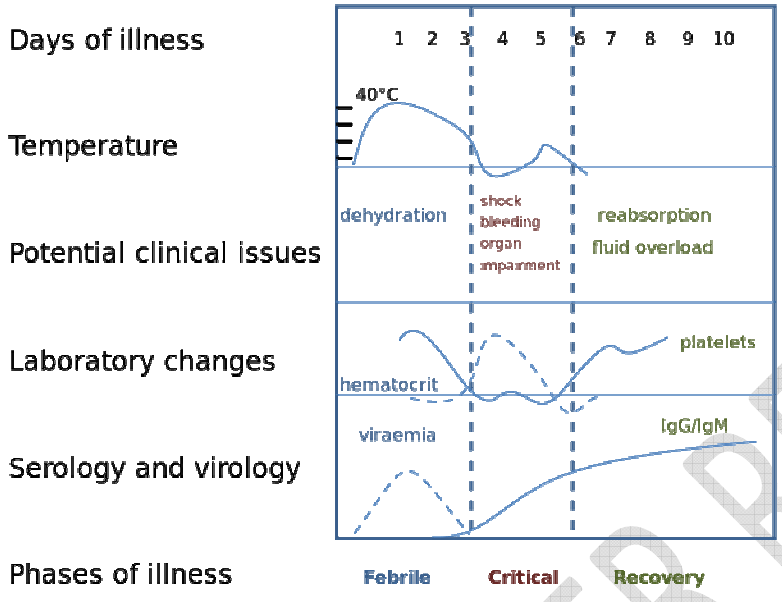

Fig 1. Timelines of Laboratory Changes viz-a-viz Clinical-features [36]

Antigen-detection is detectable through the incubation-period till the 12th Day (convalescent) of the illness. IgG and IgM start rising from the 2nd Day. IgM starts falling after the 10th to 11th Day – detectable window-period is found from 6th Day to about 30th Day. IgG becomes detectable from about 10th to 11th Day, and remains detectable, although peaking on about the 15th Day only. In secondary-infection, IgG is markedly elevated and remains elevated. IgM is not as markedly elevated as in primary-infection [36].

Primary Prevention of diseases classically comprises of Health Promotion and Specific Protection [37 - 41]. The best form of Specific Protection and which has been very successful in the Prevention and Control of previous vaccine-preventable infectious-diseases comprises of an appropriate Mass-vaccination Program in the Endemic Areas. Vaccines are very much based on immunology, once again.

In late 2015 and early 2016, the first dengue-vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, was registered in several countries for use in individuals 9-45 years of age living in endemic areas. But overall, the much waited-for dengue-vaccine has been a disappointment both in its efficacy and its safety [42– 46]. If a sufficiently effective and safe vaccine can be found, it will transform dengue fever into a vaccine-preventable disease, and the disease can be quickly brought to near-eradication levels just like all of the previous vaccine-preventable diseases.

Takeda Pharmaceutical Company Limited, ("Takeda") in November 2017 announced the data from an 18-month interim analysis of the ongoing Phase 2 DEN-204 trial of its live, attenuated tetravalent dengue vaccine-candidate, TAK-003 (also referred to as TDV). This interim-analysis showed that children and adolescents who received TAK-003 had a relative-risk of symptomatic-dengue of 0.29 (95% CI: 0.13–0.72) compared to children and adolescents in the placebo control-group [47].

TAK-003 was found to be safe and well-tolerated in terms of solicited local-reactions and systemic adverse-events, relative to the placebo control-group. In participants who were sero-negative at baseline, a second-dose given at Month 3 improved the tetravalent sero-positivity rate at Month 6 to 86%, compared to 69% in the one-dose group. A booster dose at Month 12 resulted in a 100% tetravalent sero-positivity rate at Month 13 in participants who were sero-negative at baseline [47].

TAK-003 is currently under evaluation in the Tetravalent Immunization against Dengue Efficacy Study (TIDES), a large-scale Phase 3 efficacy-trial being conducted in eight dengue-endemic countries. Data from TIDES will be available in late 2018 [47].

The US National Institute of Allergy and Infectious Diseases (NIAID) has developed the LATV dengue vaccines TV003/TV005. A single-dose of either TV003 or TV005 induced sero-conversion to four DENV-serotypes in 74-92% (TV003) and 90% (TV005) of flavivirus-seronegative adults and elicited near-sterilizing immunity to a second dose of vaccine administered 6-12 months later [48–50].

The Phase III clinical-trial of the TV003 commenced in February 2016 among 17,000 volunteers in multiple locations in Brazil with the aim of determining its

efficacy and safety. The estimated primary-completion date is June 2018, and the estimated study-completion date is December 2022 [48–50].

When vaccines are available which afford greater than 90% protection against all four strains, the risk of antibody-directed enhancement (ADE) in subsequent natural-infections, causing severe dengue, becomes remote because secondary infections would be rare. Dengue fever very likely will become reduced to sporadic-outbreaks of mostly the Sylvan-type, just like yellow-fever, once a successful mass-vaccination program of a safe and highly-effective vaccine becomes feasible and affordable (Meer Ahmad et al 2018).

Acquiring deeper insights into the different pathogenic mechanisms in the causation of dengue hemorrhagic fever will enable us in improving the different treatment strategies.

Several potential mechanisms have been identified which will be discussed here.

Role of viral proteins

The DENV is transmitted from the infected *Aedes egypti* mosquito which inoculates the virus into the capillaries and the first targets of the virus are the dendritic cells of the skin [51].The DENV is composed of an electron-dense core made of the nucleo-capsid and the single stranded RNA. The core is surrounded by a lipid-bilayer with two transmembrane-glycoproteins. The virus enters the cells by

receptor mediated endocytosis[52 - 53]. After fusion of the viral and vesicular membranes the nucleo-capsid is released and translation of viral proteins begins in the endoplasmic reticulum[54]. The process releases 10 proteins- 3 structural proteins - capsid, prM, and E and seven nonstructural-proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The NS-proteins are multifunctional ranging from suppressing the host immune-response, creating appropriate environment for viral-replication, enzymatic-activities and remodeling cellular-membranes[55]. The CD 8+ T cells epitopes are located mainly in the non-structural proteins NS3 and NS5. Weiskopf et al demonstrated that live-attenuated tetravalent-vaccine opposed dengue-initiated CD8+ T cell responses against NS3 and NS 5 protein[57].

In contrast to CD8+ T cells, the CD4+T lymphocytes-epitopes are present in the structural-proteins, the envelope and the capsid.

Original antigenic sin

The human-body's immune-system is the primary-defense against the dengue-virus. When someone is infected with dengue, the body's innate and adaptive immune-responses work together to fight the virus. B-cells from the immune-system produce antibodies that recognize and neutralize dengue viral-particles, and cytotoxic T-cells recognize and kill cells that are infected with the virus. The mechanism of severe form of dengue-infection is not clearly understood yet. One

of the mechanisms that is thought to contribute to DHF is original antigenic sin (Rothman et al)[56]. It implies that the antibody-response to the secondary-infection is influenced by the proliferation of cross-reacting memory-cells induced by the primary-infection. These memory-cells have a lower threshold for activation of the immune-system than the naive-cells and because of this feature, there is a risk of lower-affinity of binding with the infected-macrophages – and, hence are not as effective at clearing the secondary-infection. In addition to this, it may recall a memory-response to other flavi-viruses, like Japanese encephalitis and yellow fever virus augmenting the immunopathology and the damage caused to the cells[56 - 57].

Antibody-dependent enhancement

People who are infected a subsequent time with a different-type of the dengue-virus may experience something called "antibody-dependent enhancement" in which the body's immune-response actually makes the clinical-symptoms of dengue worse and increases a person's risk of developing severe dengue[57]. There is a correlation between high viral-load and the disease-severity in dengue infection which has been attributed to antibody-dependent enhancement. During primary-infection by one DENV-serotype, the primary immunoglobulin-antibodies are produced which give life-long immunity. When a different DENV-serotype infects the person subsequently, the disease tends to be more severe with a high

viremia. The antibody binds to the virus but does not neutralize it, or only partially neutralizes the virus because of small antigenic-differences between the serotypes. The immune-system is tricked, and the virus continues to infect the macrophages, multiplying andleading to a more acute viral-infection with loads of cytokines being released, resulting in the severe-forms of DHF[58 - 59].

Autoimmunity in dengue infection

Molecular-mimicry exists between the platelets, endothelial-cells, coagulatory-proteins and the viral-NS1, prM and E-proteins which leads to cross-reactivity of the anti-NS1, anti-prM and anti-E with the host-proteins[60]. The level of the antiplatelet and the anti-endothelial cell antibodies are higher in patients with DHF/DSS than DF-patients. These antibodies may be associated with thrombocytopenia and plasma-leakage due to action on endothelial-cells of (micro)-vasculature. Abnormal coagulopathy has also been observed in DHF which could be explained by the anti-NS1 & anti-E antibodies which cross-react with human-blood coagulation-factors, fibrinogen, and plasminogen[60 - 61].

Inhibition of interferon-alpha

After the entry of the DENV and infection of the cells, IFNα/β is secreted by the T-cells to destroy the virus-infected cells. In case of dengue infection, the IFNα/β binds to the infected-cells and activates the JAK/STAT pathway resulting in

transcription and induction of numerous genes creating an antiviral-state[62]. Experimental-studies in mice have shown that pre-treatment with IFNα/β has protected from DENV-infection. But, IFNα/β does not inhibit replication after DENV-infection, meaning that IFN may not be active in secondary-infection. Jorge et al studied the role of the tenproteins coded by DEN-2especially NS4B& NS4A in the inhibition of the IFNα/β response. Also, Jorge et al showed that the DENV-pathogenicity was higher in patients with a high-titres of IFNα/β[63].

A different studyyet described the action of NS2B on cGAS degradation which prevented sensing of mitochondrial-DNA of the virus-infected cells and subsequent inhibition of IFN[64]. Hence, it is proven that the viral NS4B & NS4A inhibit the IFN and result in a high viremia in secondary-infection.

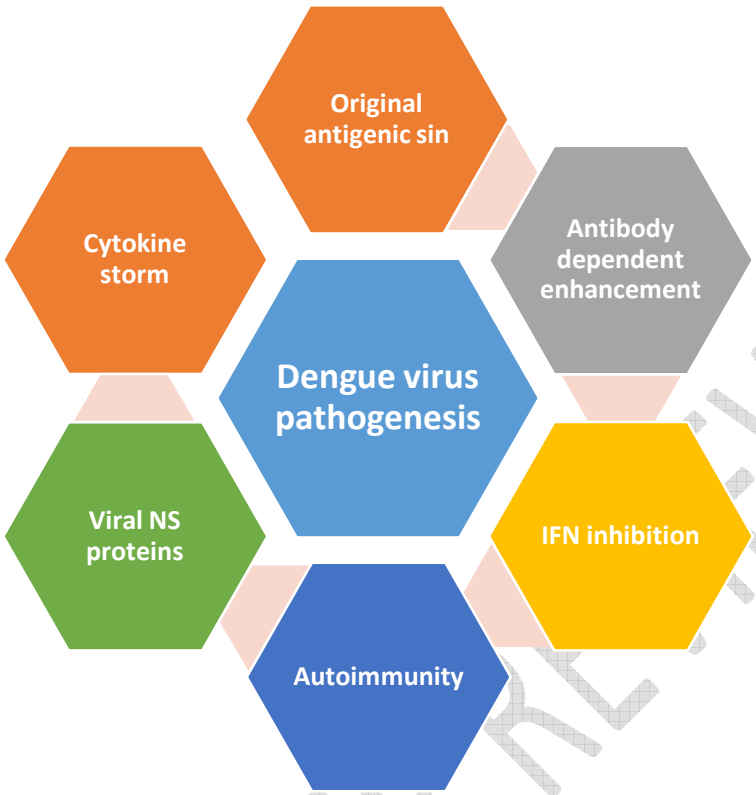

Fig 2. Potential mechanisms of DHF and DSS pathogenesis

Cytokine storm

Following secondary-infection with a dengue-virus of different serotype, severe disease is also linked to high-levels of antibody□enhanced viral-replication early in illness which is followed by a cascade of memory T□cell activation and a 'storm' of inflammatory-cytokines and other chemical mediators. These compounds are released mainly from T-cells, monocytes/macrophages and endothelial cells, and ultimately cause an increase in vascular-permeability [65]. St John et al demonstrated in a mouse-model that mast-cells are activated during DENV-infection and mast-cell deficient mice had greatly reduced capillary-

leakage[66]. In humans, mast-cell derived mediators like chymase are elevated in the blood of dengue-patients[67]. More clinical-studies are needed involving drug-probe studies against mast-cells to better fathom their contribution to disease.

The pathogenesis of severe dengue is a complex interplay between the viral-factors and proteins and the most intriguing immune-pathogenesis of the dengue infection. There is still lots to learn about inducing immunity to DENV and developing targeted therapy. Focused research efforts will help to improve disease prevention and management in future.

Mangada MM and Rothman AL (2005) studied the interplay of different inflammatory-cytokines induced during a dengue-virus infection which appear to play a role in either protection or increased disease severity. The researchers measured the frequencies and characterized the cytokine-responses of DEN virus-specific memory CD4+ T cells in six volunteers who received experimental live-attenuated monovalent dengue-vaccines. IFN-gamma and TNF-alpha responses to inactivated dengue-virus antigens were detected in up to 0.54 and 1.17% of total circulating CD4+ T cells, respectively. Antigens from the homologous-serotype elicited the highest IFN-gamma response. The ratio of TNF-alpha- to IFN-gamma-producing CD4+ T cells was higher after stimulation with antigens from heterologous DENV-serotypes. Peptide-specific CD4+ T cell frequencies of up to 0.089% was detected by direct-staining using HLA class-II tetramers. IFN-gamma

and TNF-alpha responses to individual HLA class II-restricted peptide-epitopes were detected in up to 0.05 and 0.27% of CD4+ T cells, respectively. Peptide-sequences from the homologous-serotype elicited a variety of cytokine-response patterns. TNF-alpha- to IFN-gamma-positive CD4+ T cell-ratios varied between peptides, but the ratio of the sum of responses was highest against heterologous-serotypes. The researchers conclude that the results demonstrate epitope sequence-specific differences in T-cell effector-function. These patterns of effector-responses may play a role in the immune-pathogenesis of dengue hemorrhagic fever [57].

CONCLUSION

Dengue fever has spread globally to be endemic in more than 100 countries to a total estimated incidence of 50 – 100 million cases annually globally. About 0.7% of these cases become the complication that is dengue hemorrhagic fever which is severe and lead to about 22,000 deaths annually.

The pathogenesis of benign dengue fever becoming dengue hemorrhagic fever, and aspects of the immunologic-response behind it, have remained relatively unknown.

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It has been shown that viremic-load, including the initial viremic-load at the bite of the Aedes-mosquito may be a factor leading to dengue hemorrhagic fever.

Additional factors felt to be involved in the pathogenesis of dengue hemorrhagic fever include the role of the viral-protein, and then that which is termed the original antigenic-sin, besides antibody-dependent enhancement, autoimmunity, inhibition of interferon-alpha and cytokine-storm in the memory-cells.

Regionally, certain different strains of the DENV also seems to be associated with dengue hemorrhagic fever.

Vaccines against the disease are based on the immunologic-response. In this aspect, one recent vaccine has been a disappointment from the point of its efficacy and safety. Two more vaccines, conversely, offer much hope in the near future in both their efficacy and safety.

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