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PATHOPHYSIOLOGIC AND PROGNOSTIC ROLE OF IFN-GAMMA AS A PROINFLAMMATORY AND REGULATORY CYTOKINE IN DENGUE FEVER

Microbiology

Dr. Saishruti — PG Student, Department of Microbiology, Tirunelveli Medical College, Tirunelveli - 627 011, Tamil Nadu, India

Dr. G. Sucila Thangam* — M.D., Associate Professor, Department of Microbiology, Government Theni Medical College, Theni - 625 512, Tamil Nadu, India *Corresponding Author

Dr.C. Revathy — M.D., Professor and Head, Department of Microbiology, Tirunelveli Medical College, Tirunelveli - 627 011, Tamil Nadu, India

ABSTRACT

Antibody response against Dengue virus – infection with dengue virus induces the production of both neutralizing and non neutralizing antibodies. The neutralizing antibodies are protective in nature. Such antibodies are produced against the infective serotype as well as against other serotype. Hence, protection to infective serotype stays lifelong but cross protection to other serotypes diminishes over few months. The non neutralizing antibodies last lifelong and are heterotypic in nature. They are produced against other serotypes but not against the infective serotype. Such antibodies produced following the first serotype infection can bind to a second serotype, but instead of neutralizing the second serotype, it protects it from host immune system by inhibiting the bystander B cell activation antigen against the second serotype. The above phenomenon is called Antibody Dependent Enhancement which explains the reason behind the severity of secondary dengue infection. This prospective study was conducted to assess the pathophysiologic and prognostic role of proinflammatory and regulatory cytokines in the inpatient population tested positive for Dengue fever by using commercially prepared ELISA kits for IFN-gamma. A total of 100 blood samples for this study were collected from pediatric patients and adult patients. The patients were tested for NS1 antigen of DENV and IgM antibodies against DENV. The levels of Positive cases were then selected for the study as per their platelet count. IFN-gamma was obtained using ELISA and statistically analyzed and results were interpreted. One third of the dengue positive patients had very high levels of IFN-gamma level. IFN-gamma values and clinical features were analyzed using Pearson's Chi Square Test and the Significance was calculated. There was a significant association (p=0.025) between the IFN-gamma categorization and the clinical features i.e. patients with Severe Dengue or Dengue with danger signs had significantly higher number of IFN-gamma levels. As increased IFN-gamma has been related to diminished viral clearance from the host, disease severity might also be related to its serum level.

KEYWORDS

ADE(Antibody Dependent Enhancement , Dengue with warning signs, Cytokine storm,IFN-gamma

INTRODUCTION

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. During the last fifty years, incidence has risen to 30-fold with increasing geographic expansion to new countries and, in the present scenario, from urban to rural settings. An estimated 50 million dengue infections occur every year and approximately 2.5 billion people are living in dengue endemic countries. Dengue shock syndrome is a severe complication of Dengue Hemorrhagic Fever (DHF), characterized by a massive increase in vascular permeability.[1,2]The 2002 World Health Assembly resolution 55.17 urged greater commitment to dengue by WHO and its Member States. Of particular significance is the 2005 World Health Assembly resolution 58.3 on the revision of the International Health Regulations (IHR), which includes dengue as an example of a disease that may cause a public health emergency of international concern with implications on health security due to disruption and rapid epidemic spread beyond national borders.[3]

Some 1.8 billion (more than 70%) of the population at risk for dengue worldwide live in member states of the WHO South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to Dengue. The Asia Pacific Dengue Strategic Plan for both regions was been prepared in consultation with member countries and development partners in response to the increasing threat from Dengue, which is spreading to new geographical areas and causing high mortality during the early phase of outbreaks. The strategic plan aims to aid countries to reverse the rising trend of dengue by enhancing their preparedness to detect, characterize and contain outbreaks rapidly and to stop the spread to new areas. Since 2000, epidemic Dengue has spread to new areas and has increased in the already affected areas of the region. In 2003, eight countries -- Bangladesh, India, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste -- reported Dengue cases. In India, changes in genotypes/lineages have been associated with increasing severity.[3]irunelveli district (TamilNadu) faced an epidemic in 2012 and 2013 at Kadayanallur and Ambasamudram blocks. Tirunelveli Medical College Hospital (TVMCH) is the only tertiary care centre in this district.

Dengue virus (DENV) is a positive-sense, single-stranded RNA virus in the family *Flaviviridae* that causes disease in human beings. DENV infection results in different clinical manifestations ranking from benign disease [Dengue fever (DF)] to severe disease [Dengue Hemorrhagic fever (DHF)] (Monath, 1994). In situations where the onset of hypovolaemic shock or dengue shock syndrome (DSS) persists, DHF can result in a life-threatening infection. DHF is characterized by increased vascular permeability resulting in plasma leakage and coagulation derangements. DHF can be classified further into four degrees of severity, and degrees III and IV are considered to be DSS (WHO, 2009). Due to the lack of efficient biomarkers that define endothelial damage for determining the degree of severity, there is an urgent need to find relevant biological markers of disease, as well as to determine more coherent definitions of the degree of severity in patients.[4]

Severe Dengue is defined as Dengue confirmed cases plus severe thrombocytopenia (<50, 000 platelets/mm3) and/or hypotension (postural hypotension with decrease in systolic arterial pressure in 20 mm Hg in supine position or systolic arterial pressure < 90 mm Hg) and/or plasma leakage (either haemoconcentration fluctuation of packed cell volume ≥ 20% during illness course and recovery or clinical signs of plasma leakage, such as pleural effusion) and/or severe hemorrhagic manifestations. Mild dengue – Dengue confirmed cases in absence of severe thrombocytopenia, hypotension, plasma leakage signs or hemorrhagic manifestations. Viral and host factors can both contribute to disease severity.

Epidemiologic data suggests that there is a greater risk of DHF/DSS during secondary infections, and immunopathologic mechanisms, such as immune-enhancement phenomenon, have been proposed to contribute to DHF risk. This contribution occurs when non neutralizing antibodies resulting from the primary infection favour dissemination of the second infecting Dengue virus, a phenomenon known as antibody dependent enhancement, and cross-reactive memory T cells from a primary infection recognize antigen from the secondary infection, resulting in increased T cell activation and cytokine production. Antibody enhancement, improper T cell and cytokine response and host genetic factors are amongst the postulated

immunopathogenesis leading to severe dengue.[5,6]

Cytokines are important immunomodulators and improper T-cell activation can lead to cytokine storms which are believed to result in endothelial permeability and leakage, a typical feature of Dengue disease progressing into a more severe stage. Soluble circulating immunological mediators are associated with disease severe outcomes as shock and hemorrhages. Among them inflammatory cytokines such as IFN-γ have been emphasized . Regulatory activities of the cytokines are probably crucial in controlling the inflammatory response during Dengue disease, which is necessary for the control of viral infection, and the balance among inflammatory/regulatory mediators could be related to recovery of most patients with a dengue secondary infection.[7]

MATERIALS AND METHODS

This prospective study was conducted at Tirunelveli Medical College, Tamilnadu from March to June 2017 to assess the pathophysiologic and prognostic role of proinflammatory and regulatory cytokines in the inpatient population tested positive for Dengue fever by using commercially prepared ELISA kits for IFN-gamma . The levels of IFN-gamma was obtained using ELISA and statistically analyzed and results were interpreted.

Blood samples were obtained from those received at the central laboratory for Dengue serology. Children aged 4-12 years and adults 18-60 years with clinical signs suggestive of dengue admitted in Paediatric and Medicine wards were included in the study while patients with fever not admitted in the hospital or those with other known infectious illnesses (TB/ HIV/ HCV/ malaria/ Japanese encephalitis / Typhoid / Chikungunya) were excluded from the study. All the samples included were positive for Dengue IgM antibodies and NS1 protein; and with a platelet count of less than or equal to one lakh.

Blood samples were collected, transported, stored and processed using all safety precautions throughout the course of this study. The institutional ethical clearance was obtained for this study. NS1 ELISA was done using commercially available kits and Dengue IgM ELISA kits were obtained from NIV, Pune. The IFN-gamma kits were those commercially obtained from Diaclone, France. The absorbance was read on a spectrophotometer using 450nm as the primary wavelength and optimally 620 nm as the reference wave length.

STATISTICAL ANALYSIS

The demographic parameters, clinical features and platelet counts were correlated using Chi- Square test. Subsequently the cytokines and their correlation with the clinical features was estimated individually and then a cumulative correlation of all factors, i.e. clinical features, platelet counts and the cytokines was estimated. All data were analyzed using the statistical package for social science (SPSS) 10.0 for Windows program on the computer.

RESULTS

A total of 100 blood samples for this study were collected from 34 (42.5%) pediatric patients and 46 (57.5%) adult patients. Out of these 50 were male and 50 females. The patients were tested for NS1 antigen of DENV and IgM antibodies against DENV. Positive cases were then selected for the study as per their platelet count. All the patients included in the study had a platelet count of less than 1, 00, 000 / cu.mm minimum count being 16000 and a maximum of 1, 00, 000 with a mean value of 63, 000.

Clinical features of the study population were scored (based on the WHO classification of dengue with and without warning signs an arbitrary scoring was done) as depicted in **Table 1**.It was found that the maximum number of pediatric patients did not present with significant clinical complications whereas the adult population showed bleeding manifestations in about one third of cases. The platelet counts of patients were compared to their clinical features by ANOVA test. There was a significant correlation found between the values and the clinical feature scores. The F value computed was 2.909 with a significance of.040 (<0.05) and thus there was a positive correlation between the platelet counts and the clinical features.

The IFN gamma values were graded (based on the study by ozar et al. there was an arbitrary score given for grading Interferon gamma values).**(Table-2)** The frequency of IFN-gamma values in patient population is depicted in **Table 3**. One third of the dengue positive patients had very high levels of IFN-gamma level. IFN-gamma values and clinical features were analyzed using Pearson's Chi Square Test and the Significance was calculated. There was a significant association (p=0.025) between the IFN-gamma categorization and the clinical features i.e. patients with Severe Dengue or Dengue with danger signs had significantly higher number of IFN-gamma levels.

DISCUSSION

Immunity to Dengue virus (DENV) is serotype-specific and long term. However, secondary infection by a heterologous serotype is a pre-disposing factor for severe disease. This was attributed to the phenomenon of 'original antigenic sin'.Primary dengue infection occurs when a person is infected with dengue virus for the first time with any one serotype. Months to years later, a more severe form of dengue illness may appear (called secondary dengue infection) due to infection with another serotype which is different from the first serotype causing primary infection.[8]

Antibody response against Dengue virus – infection with dengue virus induces the production of both neutralizing and non neutralizing antibodies. The neutralizing antibodies are protective in nature. Such antibodies are produced against the infective serotype (which last lifelong) as well as against other serotype (which last for some time). Hence, protection to infective serotype stays lifelong but cross protection to other serotypes diminishes over few months. The non neutralizing antibodies last lifelong and are heterotypic in nature i.e. they are produced against other serotypes but not against the infective serotype. Such antibodies produced following the first serotype infection can bind to a second serotype, but instead of neutralizing the second serotype, it protects it from host immune system by inhibiting the bystander B cell activation antigen against the second serotype. The above phenomenon is called Antibody Dependent Enhancement which explains the reason behind the severity of secondary dengue infection. Being suboptimal in specificity and function, they fail to control infection and, instead, contribute greatly to a 'cytokine storm' [8]

There were 34 pediatric and 45 adult patients included in the study. The male and female patients included in the study were equal. As stated by Deen et al and WHO definitions for Dengue 2009 severe dengue has been classified as dengue with warning signs and dengue without warning signs.[2]This criteria was used to score the patients under four categories respectively. Patients with a score of 1 and 2 showed signs and symptoms of dengue fever and were considered as Dengue without warning signs, however the ones with a score of 3 and 4 were the ones with danger signs and likely cases of severe dengue.

The frequency distribution of these features showed that most patients who tested positive for Dengue remained asymptomatic or had vague manifestations. This was found in around 37 percent of the cases. The common presenting symptom in patients with severe dengue was bleeding manifestations; which was found in around one third of the total cases. Patients with moderate dengue most commonly presented with typical features of pedal edema, abdominal bloating etc. which could be likely due to low platelet counts accounting to the beginning of capillary leakage and associated pathology. A few cases also progressed to shock or DSS (Dengue Shock Syndrome) and Encephalitis; these being the hallmarks of severe Dengue.

Similar results were found in a study done by Priyadarshini et al.,[9]in North India where based on clinical features, 71.9% (n=159) of the patients were classified as DF, 50% of them had thrombocytopenia without any bleeding. Presence of any two of the DHF defining criteria by WHO, categorized 62 patients as DHF cases. Seven patients were defined as severe DHF (grades III/IV) with signs of circulatory failure. One fatal patient had severe thrombocytopenia, plasma leakage and circulatory failure (Grade IV). Fever with chills, headache, myalgia and nausea/vomiting were reported equally by DF and DHF patients. Joint pain, retroorbital pain and itching were observed in a significantly larger number of DF cases (p, 0.05, x2 test). Abdominal pain, maculopapular rash/petechiae and conjunctival congestion were found to be more prevalent in DHF cases (p, 0.05, x2 test) [The correlation between age and clinical features was calculation using Pearsons's Chi Square test. There was insignificant correlation between the two parameters; hence proving that age does not play any prognostic role in pathogenesis of severe dengue. This was also in correspondence to a similar study done by Priyadarshini et al.[9]

The frequency of clinical features did not differ much in the two gender

groups and the results were comparable. Similarly, when correlation using Chi Square was calculated ($p < 0.025$) it was found to be insignificant showing there is possibly no significant correlation between gender and dengue prognosis. Similar results were depicted by Pandey et al.,[10] in their respective studies. Time and again it has been stated in various studies that the most severe complications following dengue fever are attributed due to plasma leakage occurring as a result of thrombocytopenia in patients with dengue. This is more severe in secondary dengue where non neutralizing antibodies are produced that lead to Antibody dependent Enhancement leading to antibodies against platelets leading to thrombocytopenia.[10]

The patients included in the study had platelet count ranging from 16000 to 100000 with a mean of 63000.There was a significant correlation between the platelet count and the clinical features i.e. 0.04 ($p < 0.05$). This has also been stated by Rathakrishnan et al.,[7]in is study in a similar group of patients where the platelet counts were significantly decreased in patients with warning signs and moreover there was also a fall in the platelet count during the post febrile phase indicating a role of immune related mechanisms playing a pathophysiological role during the defervescence stage of Dengue.[7]Interferon gamma were graded based on their OD values (optical density) and these were compared with the respective standards to compute the value in pg/ml. into negative, moderately high, high and very high.

It was found that around one third of the patients who tested positive for dengue were negative for interferon gamma ; but , interestingly; a vast majority of patients ie. around 52% had interferon values between 5 to 50. The moderate increase in the interferon gamma values can be attributed to the fact that interferon gamma is not activated by the virus directly rather by mitogens and vastly affects the nitric oxide synthatase pathways.

The frequency of interferon value with various clinical feature scores were compared and the correlation was computed. It was found that more than 90 percent of patients with severe dengue ie. dengue with warning signs have raised interferon values ; thus making it a reliable prognostic marker for severe dengue. Similar results were found in many other previous studies where interferon values in severe dengue patients were markedly higher than control patients.[11,12]

Also, there were only 3 patients who had higher interferon values even without symptoms of severe dengue. With a Asymptomatic Significance of .002 there was a strong correlation between interferon gamma and clinical feature score of the patients. ($p<0.025$).

Previous virological and clinical studies showed contribution of high virus replication and low viral clearance from the patient's body leading to disease severity during DENV infection. In the last few years , several attempts have been made to identify interferon gamma as a serum marker , an adaptive immune cytokine to predict severe dengue. As increased IFN-gamma has been related to diminished viral clearance from the host, disease severity might also be related to its serum level.[13]

Dengue viral replication within the patient body has to face powerful host immune responses induced by cytokines like interferon gamma. During early periods of viral replication , IFN gamma produced by stimulated CD4+ and CD8+ T cells, induces nitric oxide synthatase2 generation, which thereby enhances host immunity that plays an important role in virus replication control.

Enhanced DENV replication was found in IFN gamma deficient mice with more severe disease manifestations like hematological alterations and hepatic injury. Replication of HCV, another member of the *Flaviviridae* family, was also inhibited by IFN-gamma, which was associated with viral clearance within patient's body.

Therefore, low IFN gamma levels among patients without warning signs and high IFN levels in patients with warning signs in our study might have favored greater DENV replication – thus exhibiting warning signs like persistent vomiting, , severe abdominal pain and clinical accumulation fluid.

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Table 1 Frequency of clinical feature score

S.NO	SIGNS AND SYMPTOMS	SCORE	Percent
1	CLINICAL FEATURES- NO BLEEDING, VAGUE SYMPTOMS	1	37.5
2	ABDOMINAL PAIN, OEDEMA, SIGNS OF DEHYDRATION	2	22.5
3	BLEEDING MANIFESTATIOS WITH DANGER SIGNS	3	28.8
4	COMPENSATED SHOCK	4	11.3

Table 2 Grading of IFN gamma values

S.NO	VALUES	INTERPRETATION
1	0	Negative
2	0-50	Moderately high
4	51-100	High
5	>100	Very high

Table 3 Frequency of IFN-gamma values in patient population

S.NO	VALUES	CUMMULATIVE
1	neg	27.5
2	less than 5	1.3
3	less than 50	52.5
4	less than 100	10.0
5	more than 100	8.8

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