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Dengue in India

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Dengue virus belongs to family *Flaviviridae*, having four serotypes that spread by the bite of infected *Aedes* mosquitoes. It causes a wide spectrum of illness from mild asymptomatic illness to severe fatal dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Approximately 2.5 billion people live in dengue-risk regions with about 100 million new cases each year worldwide. The cumulative dengue diseases burden has attained an unprecedented proportion in recent times with sharp increase in the size of human population at risk. Dengue disease presents highly complex pathophysiological, economic and ecologic problems. In India, the first epidemic of clinical dengue-like illness was recorded in Madras (now Chennai) in 1780 and the first virologically proved epidemic of dengue fever (DF) occurred in Calcutta (now Kolkata) and Eastern Coast of India in 1963-1964. During the last 50 years a large number of physicians have treated and described dengue disease in India, but the scientific studies addressing various problems of dengue disease have been carried out at limited number of centres. Achievements of Indian scientists are considerable; however, a lot remain to be achieved for creating an impact. This paper briefly reviews the extent of work done by various groups of scientists in this country.

Key words *Aedes* mosquitoes - dengue - DF/DHF - dengue vaccine - DV - *Flaviviridae* - pathogenesis

Introduction

Dengue is an acute viral infection with potential fatal complications. Dengue fever was first referred as "water poison" associated with flying insects in a Chinese medical encyclopedia in 992 from the Jin Dynasty (265-420 AD). The word "dengue" is derived from the Swahili phrase Ka-dinga pepo, meaning "cramp-like seizure". The first clinically recognized dengue epidemics occurred almost simultaneously in Asia, Africa, and North America in the 1780s. The first clinical case report dates from 1789 of 1780 epidemic in Philadelphia is by Benjamin Rush, who coined the term "break bone fever" because of the symptoms of myalgia and arthralgia (quoted from *www.globalmedicine.nl/index.php/dengue-fever)*. The term dengue fever came into general use only after 1828. Dengue viruses (DV) belong to family *Flaviviridae* and there are four serotypes of the virus referred to as DV-1, DV-2, DV-3 and DV-4. DV is a positive-stranded encapsulated RNA virus and is composed of three structural protein genes, which encode the nucleocapsid or core (C) protein, a membrane-associated (M) protein, an enveloped (E) glycoprotein and seven non-structural (NS) proteins. It is transmitted mainly by *Aedes aegypti* mosquito and also by *Ae. albopictus*. All four serotypes can cause the full spectrum of disease from a subclinical infection to a mild self limiting disease, the dengue fever (DF) and a severe disease that may be fatal, the dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). The WHO 2009 classification divides dengue fever into two groups: uncomplicated and severe[1], though the 1997 WHO classification is still widely used[2]. The 1997 classification divided dengue into undifferentiated fever, dengue fever (DF), and dengue haemorrhagic fever (DHF)[1]. Four main characteristic manifestations of dengue illness are *(i)* continuous high fever lasting 2-7 days; *(ii)* haemorrhagic tendency as shown by a positive tourniquet test, petechiae or epistaxis; *(iii)* thrombocytopoenia (platelet count $<100x10^9/l$); and *(iv)* evidence of plasma leakage manifested by haemoconcentration (an increase in haematocrit 20% above average for age, sex and population), pleural effusion and ascites, *etc*. Excellent work has been done at some of the centres in India on molecular epidemiology of dengue immunopathology and vaccine development. This paper reviews the work done in this country. The key words "dengue/India" reflected 784 papers in PubMed. Only some of the representative papers could be cited here due to constraint of space.

History

Dengue virus was isolated in Japan in 1943 by inoculation of serum of patients in suckling mice[3] and at Calcutta (now Kolkata) in 1944 from serum samples of US soldiers[4]. The first epidemic of clinical dengue-like illness was recorded in Madras (now Chennai) in 1780 and the first virologically proved epidemic of DF in India occurred in Calcutta and Eastern Coast of India in 1963-1964[5-7]. The first major epidemic of the DHF occurred in 1953-1954 in Philippines followed by a quick global spread of epidemics of DF/DHF[8]. DHF was occurring in the adjoining countries but it was absent in India for unknown reasons as all the risk factors were present. The DHF started simmering in various parts of India since 1988[9-11]. The first major wide spread epidemics of DHF/DSS occurred in India in 1996 involving areas around Delhi[12] and Lucknow[13] and then it spread to all over the country[14].

Epidemiology of dengue

The epidemiology of dengue fevers in the Indian subcontinent has been very complex and has substantially changed over almost past six decades in terms of prevalent strains, affected geographical locations and severity of disease. The very first report of existence of dengue fevers in India was way back in 1946[15]. Thereafter, for the next 18 years, there was no significant dengue activity reported anywhere in the country. In 1963-1964, an initial epidemic of dengue fever was reported on the Eastern Coast of India[7,16-20], it spread northwards and reached Delhi in 1967[21] and Kanpur in 1968[22,23]. Simultaneously it also involved the southern part of the country[24, 25] and gradually the whole country was involved with wide spread epidemics followed by endemic/hyperendemic prevalence of all the four serotypes of DV. The epidemiology of dengue virus and its prevalent serotypes has been ever changing. The epidemic at Kanpur during 1968 was due to DV-4[22] and during 1969 epidemic, both DV-2 and DV-4 were isolated[26]. It was completely replaced by DV-2 during 1970 epidemic in the adjoining city of Hardoi[27]. Myers *et al*[24, 28] had reported the presence of DV-3 in patients and *Ae. aegypti* at Vellore during the epidemic of 1966 while during the epidemic of 1968, all the four types of DV were isolated from patients and mosquitoes[29]. In another study Myers & Varkey[30] reported an instance of a third attack of DV in one individual. DV-2 was isolated during the epidemics of dengue in urban and rural areas of Gujarat State during 1988 and 1989[31]. Outbreaks of dengue occurred in Rajasthan by DV-1 and DV-3[32], DV-3[33], Madhya Pradesh by DV-3[34], Gujarat by DV-2[31] and in Haryana by DV-2[35]. DV-2 was the predominant serotype circulating in northern India, including Delhi, Lucknow and Gwalior[12,13,36] while DV-1 was isolated during the 1997 epidemic at Delhi[37]. The phylogenetic analysis by the Molecular Evolutionary Genetics Analysis programme suggests that the 1996 Delhi isolates of DV-2 were genotype IV. The 1967 isolate was similar to a 1957 isolate of DV-2, from India, and was classified as genotype V. This study indicates that earlier DV-2 strains of genotype V have been replaced by genotype IV[38]. The Gwalior DV-2 viruses were classified into genotype-IV, and were most closely related to Delhi 1996 DV-2 viruses and FJ 10/11 strains prevalent in the Fujian State of China. However, two earlier Indian isolates of DV-2 were classified into genotype-V. Genotype V of DV-2 has been replaced by genotype IV during the past decade, which continues to circulate silently in north India, and has the potential to re-emerge and cause major epidemics of DF and DHF[39]. DV-2 has also been reported from southern India - in Kerala alongwith DV-3[40].

DV-3 has been isolated during the epidemics at Vellore in 1966[24,28], at Calcutta in 1983[41] and in

1990[10], at Jalore city, Rajasthan in 1985[33] at Gwalior in 2003 and 2004[42,43] and at Tirupur, Tamil Nadu in 2010[44]. Phylogenetic analysis showed that the Madurai isolates were closely related to Gwalior and Delhi isolates. The emergence of DV-4 has been reported in Andhra Pradesh[45] and Pune, Maharashtra[46], which was also implicated in increased severity of disease. At Delhi, till 2003, the predominant serotype was DV-2 (genotype IV) but in 2003 for the first time all four dengue virus subtypes were found to co-circulate in Delhi thus changing it to a hyperendemic state[47] followed by complete predominance of DV serotype 3 in 2005[48]. During the 2004 epidemic of DHF/DSS in northern India a sudden shift and dominance of the DV serotype-3 (subtype III) occurred replacing the earlier circulating serotype-2 (subtype IV)[43]. Co-circulation of DV serotypes in Delhi in 2003-2004 has also been reported[43], which may have implications for increased DHF/DSS. Emergence of a distinct lineage of DV-1, having similarity with the Comoros/Singapore 1993 and Delhi 1982 strains, but quite different from the Delhi 2005 lineage and microevolution of the pre-circulating DV-3 has been reported[49]. Co-circulation of several serotypes of dengue viruses has resulted in concurrent infection in some patients with multiple serotypes of DV[50]. Further, replacement of DV-2 and 3 with DV-1 as the predominant serotype in Delhi over a period of three years (2007-2009) has been reported[51]. Concurrent infection by Chikungunya and DV-2 was reported from Vellore[52] and Delhi[53] (Table I).

Dengue virus and its serotypes

DV-1 was isolated in 1956 at Vellore. All the Indian DV-1 isolates belong to the American African (AMAF) genotype. The Indian DV-1 isolates are distributed into four lineages, India I, II, III and the Africa lineage. Of these, India III is the oldest and extinct lineage; the Afro-India is a transient lineage while India I is imported from Singapore and India II, evolving *in situ*, are the circulating lineages[75]. The American genotype of DV-2 which circulated predominantly in India during the pre-1971 period, was subsequently replaced by the Cosmopolitan genotype. Post-1971 Indian isolates formed a separate subclade within the Cosmopolitan genotype. DV-2 strains were isolated in India over a time span of more than 50 years (1956-2011). The re-emergence of an epidemic strain of DV type-3 in Delhi in 2003 and its persistence in subsequent years marked a changing trend in DV circulation in this part of India[49]. Occasional reports of circulation of DV-4 are also seen, though it is not the predominant type in India[76,47].

Clinical presentation

The classical clinical presentation of dengue virus infection has been observed in the country, however, several atypical clinical presentations have also been reported (Table II).

Experimental studies on immune response and pathogenesis in DV infection

Extensive studies have been carried out in mouse to understand the immune response and the mechanisms of immunosuppression and pathogenesis of severe dengue disease. DV induces mainly humoral immune response while the delayed type hypersensitivity response is poor. DV-infected sick mice develop immunosuppression, both to homologous and heterologous antigens[104-106]. Macrophages process DV antigen by serine proteases and present it to B cells *in vitro* and *in vivo*, leading to their clonal expansion[107-110].

DV-specific follicular helper T cells (ThF)

DV induces generation of helper T cells (ThF) in mouse spleen which enhance clonal expansion of DV-specific B cells[111] (Table III). DV-specific ThF secrete a cytokine, the helper factor (HF)[112] which is composed of two chains, one has antigen and the other has I-A determinants; both chains are essential for helper activity[113]. The helper signal is transmitted only by a close physical contact of the plasma membranes of B cells and ThF or HF-adsorbed macrophage. ThF and HF help in production of DV-specific antibodies[115,116].

T cells producing cytotoxic factor (TCF)

Cytotoxic factor (CF), a unique cytokine, is produced by $CD4^+$ T cells in DV infected mice and man. CF has no homology with any of the known proteins in their amino-terminal sequence[134]. Most of the patients with dengue virus infection have CF in their sera, with peak amounts in the most severe cases of DHF[136,139]. $CD4^+$ T cells and $H\text{-}2A^-$ macrophages are killed by CF while it induces $H\text{-}2A^+$ macrophage to produce another cytokine, the macrophage cytotoxin (CF2) which amplifies the effect of CF, thus producing immunosuppression to heterologous antigens[106]. CF appears in the serum before the clinical illness and is present in 100 per cent patients with DF/DHF up to day 4 of illness, detectable up to day 20 of illness[139]. CF is produced in *ex vivo* cultures of $CD4^+$ T cells obtained from peripheral blood of the patients with severe

Table I. Epidemiological studies where dengue virus was identified

Year	Region where study was conducted	Type of dengue virus detected	Reference
1964	Vellore, Tamil Nadu	DV-2	52
NA	South India	DV-3	24
1966	Vellore, Tamil Nadu	DV-3	28
1968	Vellore, Tamil Nadu	DV- 1,2,3 & 4	29
1968	Kanpur, Uttar Pradesh	DV-4	22
1969	Kanpur, Uttar Pradesh	DV-4 and DV-2	26
1970	Hardoi, Uttar Pradesh	DV-2	27
NA	NA	DV- 1,2,3 & 4	30
1983	Kolkata, West Bengal	DV-3	41
1985	Jalore town, South-West Rajasthan	DV-3	33
NA	Chikalthana, Pimpalgaon and Waloor villages in Parbhani district of Maharashtra.	DV-1 & 2	54
1988	Delhi	DV-2	9
1990	Calcutta, West Bengal	DV-3	10
1988	Rural and urban areas of Gujarat	DV-2	31
1993	Mangalore, Karnataka	DV-2	55
NA	Assam and Nagaland	DV-2	56
1996	Ludhiana, Punjab	DV- 1,2,3 & 4	57
1996	Lucknow	DV-2	13
1996	Delhi	DV-2	58, 59
1996	Delhi	DV-2	12
1997	Delhi	DV-1	60
1996	Delhi	DV-2 (Genotype IV)	38
NA	Ahmedabad, Gujarat	DV-2	61
1997	Delhi	DV-1	37
NA	Delhi	DV-2 (Genotype IV)	62
1996	Rural areas of Haryana	DV-2	35
2001	Dharmapuri district, Tamil Nadu	DV-2	63
NA	Andaman and Nicobar Islands	DV-2	64
2001	Gwalior, Madhya Pradesh	DV-2	36
NA	Northern India	DV-2 (Genotype IV)	39
2001	Chennai, Tamil Nadu	DV-3	65
2003	Northern India (Delhi & Gwalior)	DV-3	42
2005	Kolkata, West Bengal	DV-3	66
2003	Kanyakumari district, Tamil Nadu	DV-3	67
2003-04	Delhi	DV-3 (subtype III)	43
2003-05	Delhi	2003 - DV - 1,2,3 & 4 2005 - D - 3	48
2006	Delhi	DV-3	50
2006	Delhi	DV-1 & 3	49
2001-07	North India (Delhi and Gwalior region)	DV-1 (Genotype III)	68
2006	Delhi	DV-1,3 & 4	53
2008	Delhi region	DV-1,2 & 3	51
1956-2005	Entire country	DV-2	69
2002-06	Delhi	DV-1, 2, 3 & 4	70
2003	Delhi	DV-3 (Genotype III)	71
2008	Ernakulam, Kerala	DV-2 & 3	40
2007	Rural areas of Madurai, Tamil Nadu	DV-3 (Genotype III)	72
2007	Andhra Pradesh	DV-1 & 4 (Genotype I)	45
2003-08	Different parts of the country	DV-3 (Genotype III)	73
2007-09	Delhi	DV 1, 2, 3 & 4	74
2009-10	Pune, Maharashtra	DV-4 (Genotype I)	46

Table II. Atypical clinical presentations of dengue virus infection

System/Organ	Clinical presentation	References
Neurological manifestations	Encephalopathy, acute motor weakness, seizures, neuritis, Guillain Barre syndrome, hypokalemic paralysis acute viral myositis, acute encephalitis	77-80
Hepatic involvement	Acute liver failure, significant mortality, hepatic encephalopathy, hepatomegaly epistaxis, jaundice and petechial rashes	81-84
Myositis	Acute myositis, pure motor quadriplegia	85- 87
Cardiac involvement	Acute reversible cardiac insult, sinoatrial block and atrioventricular dissociation	88, 89
Lupus erythematosus (systemic)	Abnormal immune response leading to systemic lupus erythematosus	90, 91
Occlusion complications & uveitis	Unilateral blurring of inferior visual field	92
Oxidative stress	Increase in oxidative stress, significantly elevated PCOs and low PBSH group levels	93, 94
Acute renal dysfunction	Renal dysfunction, acute kidney injury	95, 96
Acute inflammatory colitis	Lower gastrointestinal bleeding and acute inflammatory colitis	97
Cutaneous manifestations	Maculopapular/morbilliform eruption followed by ecchymotic, petechial, and macular/ scarlatiniform eruption	98
	Confluent erythema, morbilliform eruptions, and haemorrhagic lesions	99
Kawasaki disease	Young child developed Kawasaki disease later in disease	100
Haemophagocytic syndrome	Bone marrow haemophagocytosis associated with nasal bleeding and pancytopenia	101-103

PCO, protein carbonyls; PBSH, protein bound sulfhydral group

Table III. T cell functions in dengue virus infection in mice

T-Cells	Cell subtype	Cytokine	Functions	References
T Helper	Follicular	Helper factor	Clonal amplification of DV-specific B cells	112-116
T Suppressor (Regulatory T cells)	TS1	Suppressor factor 1	Induction of TS2	117-124
	TS2	Suppressor factor 2	Induction of TS3	
	TS3	Suppressor factor 3	Suppression of DV-specific B cell response	123, 125
T Cytotoxic factor	TCF mouse	Mouse cytotoxic factor	Increase capillary permeability, kill a subpopulation of macrophage and CD4 T cells	112, 126-135
	TCF human	Human cytotoxic factor		135, 136, 138

dengue disease[137]. Human peripheral blood leucocyte cultures inoculated with DV, produce CF[139,140]. CF is DV-specific, therefore, can be used for developing a diagnostic kit.

Suppressor T cells

For the first time microbe-induced suppressor cells or T cells cascade was shown in DV-infected mice[117,118,120,123] which was subsequently confirmed in a large number of viruses[138]. DV-specific suppressor T cell (TS) cascade has three sequential subpopulations of TS1, TS2, TS3 cells (Table III) and their secretary soluble suppressor cytokines (SF1, SF2, SF3). DV-infected macrophage transmits the signal to recruit TS1 cells, which secrete a suppressor cytokine, SF1. SF1 is composed of two polypeptide chains[141], the alpha-chain binds to the beta-chain of the SF receptor (SF-R) present on macrophage[142] while the beta-chain of SF1 binds to H-2A determinants on macrophage[143, 144]. SF1 is internalized by live syngeneic macrophage, processed and binds to H-2K antigen and is transported to a site other than SF-R on macrophage membrane for recruitment of TS2 cells[145] (Table IV). TS2 produce a prostaglandin-like suppressor cytokine

Table IV. Macrophage functions in dengue virus infection in mice

Function	Mechanisms	References
DV-antigen presentation	Induction and clonal expansion of DV-specific B cells	107-110
Transmission of suppressor signals	Live macrophage internalize, process and externalize SF1 to present to naïve T cell by cell-cell contact to recruit TS2 cells	119, 120, 141-143, 145, 146
	Live macrophage internalize, process and externalize SF2 to present to naïve T cell by cell-cell contact to recruit TS3 cells	118, 147, 148
Transmission of T helper factor signals	Live macrophage internalize, process and externalize HF to present help to naïve B cell by cell-cell contact to produce DV-antibody	112, 116, 149
Production of cytotoxin	Amplification of the functions of CF. Increase capillary permeability, kill a subpopulation of macrophage and CD4 T cells	150-152
Production of nitric oxide	Increases vascular leakage, cell apoptosis, inhibits virus replication, role in pathogenesis	153-157

(SF2). Live syngeneic macrophage transmits the SF2 signal to recruit a third subpopulation of TS3, which suppresses humoral immune response in an antigen-specific and genetically restricted manner[118-120,123]. DV-induced suppressor pathway suppresses antigen-specific antibody production (immunosuppression to homologous antigen). Thus, suppression of neutralizing antibody would delay elimination of DV from the body causing pathological lesions[123,125]. In another study also suppressor T cell activity in dengue type 3 virus infected mice has been shown[124].

Macrophage & macrophage-like cells

Macrophages are the primary component of the host innate immune system and provide first line of defence against viral infections. But in dengue viral infection, the macrophages play multiple paradoxical roles (Table IV), sometimes these help in eradicating the virus, while sometimes these actually increase its replication within the host[158]. On one hand, macrophages are the main cells which replicate dengue virus in man, mouse and monkey[128] and the presence of macrophages is obligatory for the transmission of DV-specific suppressor signal from the first subpopulation of suppressor T cells (TSl) to the second subpopulation, TS2[123]. On the other hand, macrophages are responsible for processing and presentation of DV antigen to B lymphocytes leading to their clonal expansion and immune response[107,108]. Further, it has been observed that DV induces generation of follicular helper T cells (ThF) in mice which secrete a helper cytokine (HF) which enhances clonal expansion of B lymphocytes in an antigen-specific and H-2 restricted manner[112,149]. The DV- induced CF kills H2-A negative macrophages[111] by causing calcium influx, whereas it induces H2-A positive macrophages to produce a cytotoxin - CF2 which acts synergistically with CF[150]. CF-2 is a biologically active protein and causes various immunopathological effects including increased vascular permeability and damage to the blood brain barrier[132,152,159,160]. It has also been demonstrated that CF-2 induces production of NO_2^- in the spleen cells of mice thus mediating its cytotoxic effect on target cells[155]. This might be also one possible important trigger for switch from DF to DHF/DSS. In addition, in response to variety of stimuli, including viral infections, macrophages release migration inhibitory factor (MIF), which is a hormone released by different cells in many tissues in response to a variety of stimuli[161]. Macrophages also secrete a number of cytokines in viral infections, including DV infection[158].

Cerebral oedema/encephalopathy during DV infection

Earliest reference to involvement of brain in dengue disease was encephalopathy or cerebral oedema, which was rare[162]. Therefore, the mechanism of cerebral oedema was studied in mouse model. A breakdown of the blood-brain barrier occurs in mice inoculated intracerebrally or intraperitoneally with DV 2 resulting in leakage of protein-bound Evans blue dye and ^{51}Cr-labelled erythrocytes into the brain tissue. Similar breakdown of the blood-brain barrier also occurred in mice inoculated intravenously with CF and CF2; the damage is dose-dependent and the vascular integrity is restored during the 3 h period after inoculation. Treatment of mice with antihistamine drugs, blocking H1 or H2 receptors, decreases the DV2-induced protein leakage. Pretreatment with CF-specific or DV2-specific antiserum inhibits protein leakage. Thus CF/CF2-mediated breakdown of the blood-brain barrier leads to cerebral oedema during DV infection[112,132,136,163].

Capillary leakage in DV infection

One of the cardinal features of severe dengue is capillary leakage resulting into accumulation of fluids in various body cavities. Therefore, experiments were conducted to find out the mechanism of this phenomenon. It was observed that intraperitoneal inoculation of CF or CF2 in mice results in increased capillary permeability in a dose-dependent manner, as shown by leakage of intravenously injected radiolabelled iodine or Evans blue dye. Peak leakage occurs 30 min after inoculation of CF and the vascular integrity is restored by 2 h. The increase in capillary permeability is abrogated by pretreatment of mice with anti-CF antibodies, avil (H1 receptor blocker) or ranitidine (H2 receptor blocker)[132,152]. CF purified from the pooled sera of the DHF patients on intravenous inoculation into mice increased capillary permeability and damaged the blood-brain barrier[136].

CF and CF2 appear to be pathogenesis-related proteins, that can produce DHF-like pathological lesions in mice, such as capillary leakage, cerebral oedema, and blood leukocyte changes[112,132,137,152,163]. Pretreatment of mice with the anti-CF antibodies prevents pathological lesions produced by CF/CF2. Immunization of mice with CF protects them against subsequent challenge with CF, while challenge of such mice with a lethal intracerebral dose of DV prevents only the clinical symptoms not the death[135]. With the availability of endothelial cell monolayer models, extensive work has been done in recent times to understand the pathophysiology of vascular endothelium during dengue virus infection leading to plasma leakage as seen in severe dengue[164-167].

Cardiac damage during DV infection

During the 1968 epidemic of DF at Kanpur, a few cases were suspected to have myocarditis[22,23]. Therefore, an effort was made to develop a mouse model to study it. Infant mice inoculated with DV show minimal histological cardiac injury in the form of cytoplasmic vacuolation of myocardium and foci of infiltration by mononuclear cells[27]. Subsequently, cardiac involvement in dengue disease was reported during 1996 epidemic of DHF at Delhi (Table I).

Effect of DV infection on megakaryocytes and platelets

DV-2 inhibits *in vitro* megakaryopoiesis and induces apoptotic cell death in a subpopulation of early megakaryocytic progenitors which may contribute to thrombocytopoenia in dengue disease[166]. In another study it was shown that DV-2 may directly interact with and activate platelets and thus may be responsible for thrombocytopenia[168]. Significant ultrastructural changes in DV infected cells specially endomembrane re-organization and formation of autophagososmes have been shown using whole mount transmission electron microscopy[169]. These changes, taken together with a later study, that showed marked elongation of endothelial cell processes after transfection with the DV-E protein, provided early insights that the replication biology of the virus is coupled closely with the host cell physiology[167].

Pathogenesis of DF/DHF

Understanding the factors that are involved in the pathogenesis of DHF continues to be one of the most active areas of dengue research. It has been established that DHF is caused by a "Cytokine Tsunami" but despite extensive studies for over four decades, its genesis is still not fully understood. The mechanisms that have been considered to cause DHF include antibody-dependent enhancement (ADE)[170], T cell response[122,123,171], and a shift from Th-1 to Th-2 response[172]. The combined effect of all of these is cytokine tsunami[125] resulting in movement of body fluids in extravascular space. Various cytokines have been implicated in the immuno-pathogenesis of DF/DHF as summarized in Table V. It has been suggested that in dengue a Th1 response is linked to recovery from infection while a Th2 type response leads to severe pathology and exacerbation of the disease[172,182]. The role of Th17 cells in dengue pathogenesis has been examined and warrants serious consideration by researchers[183]. CF/CF2 induces macrophage to produce free radicals, nitrite, reactive oxygen and peroxynitrite[153,154,158,182,184,185]. The free radicals, besides killing the target cells by apoptosis also directly upregulate production of pro-inflammatory cytokines; interleukin (IL-1), tumour necrosis factor (TNF)-alpha, IL-8, and hydrogen peroxide in macrophage. Oxidative stress develops from the onset of dengue infection. Plasma protein carbonylation, protein carbonylation to protein-bound sulphydryl group ratio are reported to predict DHF/DSS[93,94]. The change in relative levels of IL-12 and transforming growth factor (TGF)-beta shifts a Th1-dominant response to a Th2 biased response resulting in an exacerbation of dengue disease. The vascular permeability is increased due to combined effect cytokine tsunami, release of histamine, free radicals and the products of the complement pathway, *etc*. Thus the key player appears to be CF/CF2, but the activity is regulated by CF-autoantibodies generated in patients with dengue disease[186].

The accompanying factors that have been discussed from time to time are dengue non-structural protein of virus type 1 (NS1)-antibodies cross-reacting with vascular endothelium (a type of autoimmune phenomenon), immune complex disease, complement and its products, memory T cells, various soluble mediators including cytokines selection of virulent strains and virus virulence, *etc*.[125,157,172,182,187]. Further, DV has been shown to evade the innate immune mechanisms of the host by inhibiting both type I interferon (IFN) production and signaling in susceptible human cells, including dendritic cells (DCs). DV also encodes proteins that antagonize type I IFN signaling, including NS2A, NS4A, NS4B and NS5 by targeting different components of this signaling pathway, such as STATs. This contributes to the pathogenesis and host tropism of this virus[188]. Further, a critical role for invariant natural killer (iNK) T cells in mice[189]; altered plasma concentrations of vitamin D and mannose binding lectin[190]; shift from Th1 cytokine to Th2 cytokine expression; role of saliva of *Ae. aegypti*[191]; and intracellular changes in host proteins[192] have been reported. Two loci on chromosomes 6 and 10 have been identified that are associated with susceptibility to DSS[193]. Classical and non-classical HLA alleles have been attributed to be related with disease severity in the host[158,194,195]. Other mechanisms that have been suggested are that DV utilizes calcium modulating cyclophilin-binding ligand to subvert the apoptotic process which in turn favoured efficient virus production[196]. A correlation of elevated lipopolysaccharide levels with disease severity has also been reported[197].

Table V. Serum cytokine levels in the pathogenesis of dengue

Cytokine	Levels in DF	Levels in DHF	References
IL-1β	-	-	173
IL-2	↑↑	↑	139
IL-4	↓	↑↑	139, 174, 175
IL-6	↑	↑↑	139, 173, 176
IL-8	↑↑	↑↑	173, 176, 177
IL-10	↓	↑↑	139, 173
IL-12	↑↑	↓	173, 178
IL-13	↓	↑↑	179
IL-18	↑	↑↑	179
TNF-α	↑↑	↑↑	139, 173, 175, 176, 180
IFN-γ	↑↑	↑	139, 174, 175, 176, 180
TGF- β	↓	↑↑	181
Human CF	↑	↑↑	136, 139

IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; TGF, transforming growth factor

Still the exact cascade of mechanisms involved in dengue disease pathogenesis remains unexplained and lot more needs to be done.

Establishment of mosquito cell line

A forerunner of mosquito cell line C6/C36 was established at Pune[198] for the isolation of dengue viruses. This was the first time when mosquito cells were used as cell culture.

Diagnosis of dengue virus infection

Diagnosis of DV infection is routinely done by demonstration of anti DV IgM antibodies or by NS-1 antigen in patients' serum depending upon day of illness using ELISA kits (prepared by National Institute of Virology, Pune) and commercial kits[199]. Molecular methods (reverse transcriptase PCR) are being increasingly used in diagnosis of DV infection. A single tube nested PCR for detection and serotyping of DV was developed and used for detection of co-infection by two viruses[200]. DV isolation in tissue culture cells and its sequencing is also being done[175].

Treatment of dengue virus infection

The management of dengue virus infection is essentially supportive and symptomatic. No specific treatment is available. However, there are Indian studies which have contributed in terms of better management of DHF/DSS. A rapid response to platelet and fresh frozen plasma (FFP) transfusion is reported in a study[201]. Anti-D has been used in children with DHF and severe refractory thrombocytopenia[202]. In experimental study pre-feeding mice with trivalent chromium picolinate (CrP) in drinking water could abolish the adverse effects of DV infection on most of the haematological parameters[203]. *Hippophae rhamnoides* (Seabuckthorn, SBT) leaf extract has been shown to have a significant anti-dengue activity[204].

Vaccine for dengue virus

Dengue vaccines have been under development since the 1940s, but a tetravalent vaccine which simultaneously provides long-term protection against all DV serotypes is round the corner[205].

Table VI*. Dengue antigens developed with potential for vaccine purposes

Expression	Antigen	Antigen design/ salient findings	Ref
Escherichia coli	DV 4 envelope domain III	Overexpressed in the form of insoluble inclusion bodies	207
	DV 4 envelope domain III	Molecular interaction with heparan sulphate, refolded and purified to homogeneity	208
	rDen 4 EDIII	Highly immunogenic with compatible adjuvants	209
	r-D2EIII	Purified from inclusion bodies; protected cells against DV-2 challenge	210
	r-DME-G	Multiepitope antigen containing IgG-specific epitopes	211
	r-DME-M	Multiepitope antigen containing IgM-specific epitopes; used to develop a rapid strip assay	212
	r-HD	Domain II of *M. tuberculosis* Hsp70 fused to r-DME-G; enhanced immunogenicity of r-DME-G did not elicit DENV neutralizing antibodies	213
	r-EDIII-4/2	Fusion of envelope domain IIIs of DENV-4 and DENV-2; elicit neutralizing antibodies to DENV-4 and DENV-2	214
	r-EDIII-T	Envelope domain IIIs of the four types linked in a tandem array; detects anti-DV IgM & IgG antibodies, sensitivity is enhanced by coating biotinylated r-EDIII-T on streptavidin plates	215, 216
	b-EDIII-T	*In vivo* biotinylated version of r-EDIII-T antigen	217
Pichia pastoris	Den2E-HBsAg	A hybrid antigen containing the ectodomain of DV-2 E (aa 1-395) fused to hepatitis B surface antigen	218
	Den2E-HBsAg	Exist as virus like particles and acts as a bivalent immunogen	219
	EDIII-2	Antigen corresponding to DV-2 envelope domain III; expressed in methanol-induced *Pichia* cells; elicit DV-2-specific neutralizing antibodies	220
	sEDIII-2	Secrets recombinant DV-2 envelope domain III	221
	r-EDIII-T	A tetravalent envelope antigen domain IIIs linked in a tandem array; unlike its *E. coli*-expressed counterpart, the *Pichia*-expressed tetravalent antigen elicited neutralizing antibodies specific to all four DENV serotypes	206
Adenovirus	DENV-2 E	Last 31 aa of DV-2 prM + the first 395 aa of E encoded by an adenovirus vector; elicit DV-2 specific neutralizing antibodies	222
	DENV-2 EDIII	Monovalent DV-2 EDIII gene expressed using plasmid and adenoviral vectors; elicit DV-2-specific neutralizing antibodies and T cell responses	223
	EDIII-4/2	Fusion of envelope domain IIIs of DV-4 and DV-2, expressed using plasmid and adenoviral vectors elicit neutralizing and T cell responses DV-2 and DV-4	224
	EDIII-T	The EDIII-based tetravalent antigen expressed using plasmid and adenoviral vectors; elicit neutralizing antibodies and T cell responses specific to four DV serotypes	225

*Modified from the Table provided by Dr S. Swaminathan and Dr Navin Khanna (personal communication)

r-D2EII, envelope domain III encoded by dengue virus type-2 (DENV-2); r-DME-G, dengue multiepitope antigen specific to IgG class of anti-dengue antibodies; r-DME-M, dengue multiepitope antigen specific to IgM class of anti-dengue antibodies; r-HD, fusion antigen comprising mycobacterial Hsp70 domain II fused in-frame to the r-DME-G antigen; r-EDIII-4/2, bivalent fusion antigen comprising envelope domain III of dengue virus type 4 linked in-frame to envelope domain III of DENV-2; r-EDIII-T, tetravalent fusion antigen comprising envelope domain IIIs corresponding to the four dengue virus types, linked in-frame in a tandem array; b-EDIII-T, r-EDIII-T antigen fused to a biotin acceptor peptide at its N-terminus (to permit *in vivo* biotinylation; Den2E-HBsAg, bivalent fusion antigen comprised of the first 395 amino acid (aa) residues of DENV-2 envelope linked to the 224 aa residue Hepatitis B virus surface antigen; EDIII-2 (or DENV-2 EDIII), envelope domain III encoded by DENV-2; sEDIII-2, secreted form of EDIII-2; DENV-2 E, envelope antigen (aa 1-395) encoded by DENV-2

A tetravalent antigen was designed by splicing the EDIIIs of DV-1, DV-2, DV-3 and DV-4 using flexible pentaglycyl linkers. A synthetic gene encoding this tetravalent antigen was expressed in *Pichia pastoris* and purified to near homogeneity. This tetravalent antigen when injected into inbred BALB/c mice, elicited neutralizing antibodies specific to each of the four DVs in plaque reduction neutralization tests[206]. Efforts are underway to present the tetravalent antigen on a chimeric VLP platform. Some promising dengue antigens have been developed using different systems (Table VI).

Recombinant dengue virus antigens

Several studies have contributed in terms of developing new reagents or technology for diagnostic purposes (Table VI). A recombinant DV3 envelope domain III (rDen 3 EDIII) protein has been produced in *Escherichia coli* for potential use in diagnosis[210,226]. A biotinylated chimeric dengue antigen to exploit the high affinity of biotin-streptavidin interaction to detect anti-dengue antibodies has been developed which incorporates the envelope domain III of all four DV serotypes[216,217]. Immunosensor has been established for label free and real time assay for the serological diagnosis of DV infection. Scope for development of biosensors for diagnosis was demonstrated[227]. The recombinant dengue multiepitope (rDME-M) protein specific to IgM in *E. coli* was produced in a 5-L fermentor for use in diagnostic purpose[228].

Vector control

Aedes aegypti is the commonest vector of DV in India, followed by *Ae. albopictus*. Larval indices indicate that *Ae. aegypti* is well established in peri-urban areas and is beginning to displace *Ae. albopictus*. Water-holding containers, *viz.* plastic, metal drums and cement tanks facilitate breeding of *Ae. aegypti*[229,230]. Expansion in the risk area of diseases borne by it in the context of urbanization, transport development and changing habitats is a major concern[231].

Vector control is known to be a good method for prevention of vector borne diseases. There are several reports from India which have demonstrated resistance of mosquito vector with anti larval substances like DDT and dieldrin but susceptibility to malathione is reported[232]. Temephos is relatively more effective in controlling *Ae. aegypti*, followed by fenthion, malathion and DDT[233]. Peridomestic thermal fogging reduced the resting and biting for the 3 days after treatment, whereas indoor fogging suppressed adult populations for 5 days[234].

Plant based repellent against mosquito borne diseases[235] have also been described. Flavonoid compounds derived from *Poncirus trifoliata* compounds have various activities against different life stages of *Ae. aegypti*[239.] Larvicidal and ovicidal activities of benzene, hexane, ethyl acetate, methanol and chloroform leaf extract of *Eclipta alba* have shown potential for controlling *Ae. aegypti* mosquito[237]. Hydrophobic nanosilica at 112.5 ppm is effective against mosquito species[238].

Assessment of public awareness on dengue virus infection

Dengue is one of the major public health problems which can be controlled with active participation of the community. Need is to organize health education programmes about dengue disease to increase community knowledge and sensitize the community to participate in integrated vector control programmes[229,239].

Conclusions

Dengue disease continues to involve newer areas, newer populations and is increasing in magnitude, epidemic after epidemic. Every aspect of dengue viral infection continues to be a challenge; the pathogenesis of severe dengue disease is not known, no vaccine is yet available for protection and the vector control measures are inadequate. Dengue virus was isolated in India in 1944, but the scientific studies addressing various problems of dengue disease have been carried out at limited number of centres. Though clinical studies have reported on dengue disease in India, but these are largely based on diagnosis made by kits of doubtful specificity and sensitivity. A lot more remains to be achieved for creating an impact.

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