

DENGUE HAEMORRHAGIC FEVER: RECENT ADVANCES

Tikki PANG Ph.D.*

INTRODUCTION

Diseases caused by the dengue viruses comprise of the classical form of dengue fever (DF) and the more severe dengue haemorrhagic fever (DHF) sometimes accompanied by the dengue shock syndrome (DSS). These diseases continue to pose an important public health threat in many parts of the tropical world^{1,2}. In recent years, DF outbreaks have occurred in India (1982) and Northern Australia (1981) and DF/DHF outbreaks in the Pacific Islands (1979), Vietnam and China (1979-80), Thailand (1980) and Indonesia (1982). In 1982, Malaysia suffered what was probably the biggest outbreak in the country's history with 3005 cases of DF/DHF and 35 deaths. A very significant event also occurred in Cuba in 1981 where a very large epidemic of DF/DHF was recorded with 116,143 cases (of which 10,000 were DHF) and 158 deaths. This event represents the first occurrence of DHF outside the Southeast Asian region and illustrates the potential threat of severe dengue disease in parts of the world where dengue viruses circulate but which, for the time being, do not have cases of DHF among affected individuals.

Despite the obvious importance of the problem, many unknowns remain in the various aspects of DHF and much more intensive research needs to be done.

EPIDEMIOLOGY OF DHF

A recent conference held in Kuala Lumpur discussed the latest advances in the various aspects of DF/DHF research.* Important features of recent outbreaks of DF/DHF were discussed. It includes a description of the 1982 DF/DHF outbreak in Malaysia, the largest in the country's history. The final figures were 3,005 notified cases (of which 860 were DHF) with 35 deaths (case fatality rate of 1.2%). Of these 3,005 cases, there were 1,001 laboratory confirmed cases. All states in Peninsular and East Malaysia were affected during this epidemic with the majority of cases reported in Perak, Selangor, Penang, Kelantan, Johore and Federal Territory. Two important

features were noted during this outbreak. Firstly, there appeared to be a shift in the main age group affected; about 56% of the cases were over 15 years of age (compared with 35% in 1974). More severe manifestations of disease (e.g. haemorrhages) were also observed in adult cases. Secondly, there also appeared to be a marked increase in the number of Malays affected, probably a reflection of increased migration into urban areas in recent years. From a virological point of view, an interesting feature was the isolation of dengue-3 virus from severe cases of DHF/DSS. This is in contrast to the observation in Thailand where dengue-2 virus is more frequently associated with severe disease. It was also noted that a large majority of DHF cases were undergoing a secondary response.

An unofficial report was presented on the 1981 DHF outbreak in Cuba, a very important event as it represents the first time that DHF was detected outside of Southeast Asia. This outbreak involved 116,143 cases (of which 10,000 were DHF) and 158 deaths, a remarkably low mortality rate of 0.02%. The large majority of DHF cases were undergoing secondary infections and it seemed likely that dengue-2 virus was involved (with dengue-1 virus being responsible for the first infection). Another interesting feature of this outbreak was the fact that the majority of DHF cases were seen in Caucasian children with a marked under-representation of black children in the population.

Reports were also given of recent dengue activity in other parts of the world including Thailand, Indonesia, Northern Australia, Sri Lanka and the Pacific Islands. An elegant long-term epidemiological study in the city of Rayong, Thailand suggested that risk factors of DHF/DSS were secondary infections with dengue-2 virus which followed primary infections with dengue-1, dengue-3 and dengue-4, in descending order of risk. All cases which developed DHF/DSS showed secondary antibody responses and only dengue-2 viruses were recovered from these patients. These conclusions were supported by data obtained

*Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur.

*Proceedings from the Conference has been published by and is available from the Conference Secretariat.

from the capital, Bangkok. In contrast, the Indonesian data, in agreement with the data from the 1982 outbreak in Malaysia, indicated that dengue-3 can also be associated with **DHF/DSS**. Northern Australia suffered a severe dengue fever outbreak in 1981-82, the first time dengue disease was seen since 1955. Although no **DHF/DSS** was documented, quite severe bleeding was noted in some of the cases.

A thorough description was given for the existence of a jungle cycle for dengue viruses in Malaysia. Extensive studies over a 10-year period demonstrated that all four types of dengue viruses circulate between monkeys and mosquitoes in the high canopy of primary undisturbed Malaysian forests, isolated from human activity and in the absence of the principal known vector, *Aedes aegypti*. The most likely canopy vectors appear to be *Aedes niveus* spp. It was concluded from this study that dengue virus activity includes a silent enzootic jungle phase (monkeys and *Aedes niveus* group mosquitoes at canopy level); rural endemicity (man and *Aedes albopictus*); and urban endemicity with occasional epidemics, involving both *Aedes aegypti* and *Aedes albopictus*.

VECTORS OF DENGUE AND VECTOR CONTROL

The most significant development was the submission of a paper from Burma which reported the transovarial transmission of dengue-2 virus by *Aedes aegypti* in nature. Dengue-2 virus was recovered from 3 of 123 pools of naturally infected *Aedes aegypti* larvae (total of 6,200 insects) collected from water containers in Rangoon and was also isolated from male *Aedes aegypti*, collected as larvae and reared in the laboratory to adults. This is a very important discovery as it may explain how dengue viruses persist and are transmitted in nature and also has important implications with regard to vector control measures. Reports were also presented on the ovipositional preferences of the main dengue vector, *Aedes aegypti*, in relation to that of its predator, *Toxorhynchites splendens* which indicated that *Toxorhynchites* mosquitoes could be used as a method to control *Aedes aegypti*.

CLINICAL ASPECTS OF DHF

The most interesting discussions with regard to the clinical aspects of **DF/DHF** were the increasing observations of unusual manifestations of **DF/DHF** noted in various parts of the world. It seems likely, for example, that

neurologic disorders associated with dengue infection are more common than previously thought. Common encephalitic signs associated with dengue infection have been convulsion, delirium, somnolence, lethargy, restlessness, stiff neck and paresis. Encephalopathy associated with marked liver dysfunction were also reported in some **DHF** cases from Thailand and, in the fatal cases, autopsy findings revealed intracranial haemorrhage and congestion and oedema of the brain. Unusual liver involvement as manifested by marked liver dysfunction or massive centrilobular congestion and necrosis of the liver appeared to be constant findings in these cases. Evidence for the involvement of the central nervous system have been reported from many endemic areas and include both adult and paediatric cases. It is not known, however, whether these signs and symptoms are due to the invasion of the central nervous system by the virus or to some other pathologic mechanism (~) and no dengue virus has been isolated from either cerebrospinal fluid or brain tissues. However, it seems prudent to consider that neurologic manifestations may be a part of the spectrum of illness associated with dengue disease.

PATHOGENESIS AND IMMUNOLOGY

The long-standing controversy on the pathogenesis of **DHF/DSS** was by no means resolved but some significant new data were discussed. It was shown that dengue infections exhibited the phenomenon of 'original antigenic sin' which has enabled the identification of the initial dengue infection in cases of secondary infections. This observation was used in the Rayong study described above to delineate that a second infection with dengue-2 virus (after a first infection with dengue-1, dengue-3 and dengue-4) represents a significant risk factor for **DHF/DSS**.

Current thinking on the pathogenetic basis of **DHF/DSS** revolves around the role of 'enhancing' antibodies present in a person undergoing a second infection. These antibodies are thought to promote virus entry (and subsequent replication) within monocytes/macrophages. Such infected monocytes/macrophages then become targets for an immune-elimination response and release various chemical mediators which in turn produces the symptoms of shock and haemorrhage seen in **DHF/DSS** cases. Recent immunological studies provided additional insights into pathogenetic mechanisms at the cellular level.

Investigations into the enhancing antibody phenomenon suggested that complement

receptors on the surface of monocytes, in addition to Fc receptors, may play a role in promoting entry of virus into susceptible target cells. Other studies also reported preliminary results on the use of monoclonal antibodies as reagents to study the mechanisms of enhancement and the distribution of enhancing epitopes. Seven dengue-2 strains were studied for antibody-dependent enhancement in a mouse macrophage line (**P388-D1**), using a panel of five monoclonal antibodies to dengue-2. The results indicate a heterogeneity of infection enhancement in that two dengue-2 strains were enhanced by only three monoclonal antibodies, two by four antibodies and three by all five antibodies. It was also shown that macrophages activated by a variety of agents effectively supported dengue virus replication. In an important study of fatal cases of **DHF/DSS** it was noted that dengue viral antigen could be localized in the mononuclear phagocytic cells of several **reticuloendothelial** organs at the time of death. These cells are the Kupffer cells of the liver, **sinusoidal** lining cells in the spleen and lymph nodes, in the thymic cortex and in alveolar macrophages of the lung. In studies of experimental dengue infection of mice, various parameters of the T cell response to dengue virus infection were described; the response (as measured by a DTH assay) was mediated by both **Ly-1⁺** and **Ly-2⁺** T cells, was **enhanced** by T cell adjuvants, was influenced by route of immunization and was genetically restricted by the H-2 complex. Other studies in mice demonstrated the fact that dengue virus infection induces splenic T lymphocytes to produce a cytotoxic factor which induces macrophages to produce a cytotoxin (**CF2**). These two factors are then thought to result in depressed and altered macrophage functions in the infected animal. However, the relevance of studies carried out in mice to the human disease is unclear although they may serve as a means to standardize new investigative techniques which may ultimately be applied to studies in man.

An interesting alternative hypothesis relating to the pathogenesis of DHF was also postulated. This hypothesis proposes that the various dengue virus strains which infect humans differ in the content of sialic acid in their envelopes and that this plays a central role in pathogenesis of DHF; viruses which replicate in mosquitoes (in contrast to those which replicate in vertebrate cells) are thought to contain sialic acid, and persons infected with this virus are at greater risk of getting **DHF/DSS** as a result of

the virus directly activating the alternative complement pathway. The renewed interest in the probable role of complement in the pathogenesis of DHF was also reflected in another study using the technique of crossed immunoelectrophoresis to measure C3 and C3 split products (**C3SP**) in **DHF/DSS** cases. It was reported that in severe (DSS) and in fatal cases, the native C3 level was lowest and the **C3SP** level highest. In an *in vitro* study, it was also shown that dengue viral antigens activates complement through the alternative pathway while dengue antigen-antibody complexes seemed to depend mainly on the classical pathway.

VIROLOGY AND MOLECULAR BIOLOGY

In addition to the obviously important role played by the host immune response in disease production, the role of the virus should also be considered. Monoclonal antibodies have been prepared against all four dengue serotypes and have been utilized in an attempt to define more clearly the structure and biological function of antigenic determinants on the surface of dengue virions. Most studies were performed with dengue-2 virus and the **specificities** and spatial arrangements of the monoclonal antibodies (**MAB**) determined by **haemagglutination** inhibition, plaque-reduction neutralization, immunofluorescence and also competitive binding assays.

Biological activities of the **MAB's** were determined by antibody-enhancement assays in macrophage cell lines and mouse protection assays. Using this approach, four 'linkage groups' or domains were defined, in which the MAB binding sites or epitopes are organised on the surface of the dengue virion. These specific antigenic regions were shown to play functional roles in viral pathogenesis and immune protection. Further studies examining the interaction of antibodies directed against unique regions on dengue antigens will hopefully lead to a greater understanding of the molecular basis of DHF pathogenesis.

What of the other side of the coin? The controversy in the pathogenesis of DHF has always involved an alternative hypothesis proposed by Rosen³ which postulates that severe disease is due to a variant virus with increased pathogenic potential. Techniques to test this hypothesis have been lacking for a long time. However, efforts have been made recently to document genetic variation among the dengue viruses using RNA fingerprinting techniques. This powerful technique has been able to detail differences between the

four dengue serotypes and has been invaluable in detecting differences between strains of the **same** serotype (e.g. dengue-1) isolated in various geographical regions at various time periods and have thus allowed the identification of genetically related strains. However, and in relation to the virus variant hypothesis, no correlation between virulence and the genetic maps of specific strains of dengue viruses have been found to date. More significantly, other workers have reported success in cloning **cDNA** copies of dengue RNA **which** may ultimately lead to the elucidation of the nucleotide sequence of the entire dengue genome and the identification of specific regions coding for structural and non-structural proteins. The production of relevant viral **subunits** or **peptides** for vaccine use by gene cloning methods will be possible once these important genes have been cloned and will, ultimately, contribute to the control of dengue disease.

LABORATORY DIAGNOSIS

Some significant recent advances have also been made in the techniques for the laboratory diagnosis of dengue infections. A mosquito cell line from *Toxorhynchites amboinensis* (TRA-284) appeared, in one study, to be the most sensitive system for isolation of dengue viruses. Additionally, these cells can be grown in the absence of foetal bovine serum. A new isolation method, using the **larvae** of *Toxorhynchites*, suggested that a result can be obtained in 2-3 days, a significant improvement over the 8-10 day period previously required. This technique **involved** the intracerebral inoculation of larvae with blood specimens and detection of viral antigens by **immunofluorescence**. A rapid test to detect dengue antibodies, using the single radial haemolysis technique was also described, in which results can be obtained in 4-5 hours. The exciting possibility of using the **cDNA** probes described above for diagnostic purposes (ie. detection of complementary dengue RNA) was also discussed.

VACCINE DEVELOPMENT

The development of vaccines against the dengue viruses was also discussed at the Conference. The Thai group at **Mahidol** University described the development of a live attenuated vaccine against dengue-2 virus using two approaches. The first approach used clonal

selection of **small** plaque, temperature sensitive viruses, while the other attempts the attenuation of dengue by serial passages of parental strains in primary dog **kidney** cell cultures. Biological markers for attenuation include small plaque size, temperature restricted growth, inability to grow in human monocytes, reduced suckling mouse neurovirulence and reduced virulence in monkeys. Several candidate vaccine strains have been prepared and human trials in Thailand are expected to commence soon. Data was also presented from the Walter Reed Army Research Institute regarding the development of dengue vaccines using a similar approach with multiple passage in primary dog kidney but with final passages in diploid foetal rhesus lung cells. A dengue4 candidate vaccine was found to have low infectivity and unacceptable clinical responses and further testing was suspended. **A dengue-1** vaccine developed at Fort Detrick will undergo initial human safety testing early next year. Work on the dengue3 vaccine is in progress and will require safety testing before initial human trials can begin; this vaccine has been the most difficult strain to grow to **high** enough titres to use for vaccine preparation. An attempt to use the **C6/36** mosquito cell line as an alternative vaccine substrate failed because of unacceptable human hypersensitivity to mosquito antigens. On the basis of the **immunoepidemiological** data described above, some felt that immunization with dengue-2 vaccine alone would be sufficient to prevent the large majority of **DHF/DSS** cases. Although this may possibly be so in Thailand, other data would suggest that caution is still required and that, perhaps, a polyvalent vaccine composed of **all** four types of dengue would still be the prudent choice.

REFERENCES

1. Halstead SB. Dengue haemorrhagic fever-A public health problem and a field for research. Bull WHO 1980; 58:1-21.
2. Halstead SB. The pathogenesis of **dengue**-Molecular epidemiology in infectious disease. **Am J Epidemiol** 1981; 144:632-48.
3. Rosen L. The emperor's new clothes revisited, or reflections on the **pathogenesis** of dengue haemorrhagic fever. **Am J Trop Med Hyg** 1977; 26:337-43.