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Pathogenesis of Dengue: Challenges to Molecular Biology

SCOTT B. HALSTEAD

Dengue viruses occur as four antigenically related but distinct serotypes transmitted to humans by *Aedes aegypti* mosquitoes. These viruses generally cause a benign syndrome, dengue fever, in the American and African tropics, and a severe syndrome, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), in Southeast Asian children. This severe syndrome, which recently has also been identified in children infected with the virus in Puerto Rico, is characterized by increased vascular permeability and abnormal hemostasis. It occurs in infants less than 1 year of age born to dengue-immune mothers and in children 1 year and older who are immune to one serotype of dengue virus and are experiencing infection with a second serotype. Dengue viruses replicate in cells of mononuclear phagocyte lineage, and subneutralizing concentrations of dengue antibody enhance dengue virus infection in these cells. This antibody-dependent enhancement of infection regulates dengue disease in human beings, although disease severity may also be controlled genetically, possibly by permitting and restricting the growth of virus in monocytes. Monoclonal antibodies show heterogeneous distribution of antigenic epitopes on dengue viruses. These epitopes serve to regulate disease: when antibodies to shared antigens partially neutralize heterotypic virus, infection and disease are dampened; enhancing antibodies alone result in heightened disease response. Further knowledge of the structure of dengue genomes should permit rapid advances in understanding the pathogenetic mechanisms of dengue.

DENGUE VIRUSES, MEMBERS OF THE FLAVIVIRIDAE FAMily, occur as four distinct serotypes that are biologically transmitted from infected to susceptible human beings principally by *Aedes aegypti* mosquitoes, the yellow fever vector. This species, which bites during the day and breeds in freshwater collections in and around human habitations, now is almost universally distributed around the globe between 30°N and 20°S (*1*). In these tropical and subtropical regions live more than one half of the world's human population. The ecological disturbances of World War II, the rapid postwar growth of population and urbanization, the deterioration in urban living environments, and global economic downturns have contributed collectively to the spread of *Aedes aegypti* and to the epidemic and endemic dispersal of the different dengue serotypes (*2*).

Dengue is a human disease of global significance. Up to 100 million cases of dengue infection per year worldwide can be estimated from available data if one assumes there is an average annual infection rate of 10% for endemic areas, with most susceptible hosts being children (*3*). Although dengue infections in children usually result in mild disease, a 1962 study in Bangkok suggested that more than half of the cases were of sufficient severity to require medical attention (see *4*). When *Aedes aegypti* extends its range into areas previously free of this species, outbreaks of dengue fever may also involve a large portion of the adult population. Recent epidemics of dengue fever in Africa, Australia, Brazil, and Central America have caused medical and economic burdens, but few deaths (*5*).

In tropical Asia, the region of highest dengue endemicity, the disease is more severe. In that area, dengue viruses cause a serious, often rapidly fatal disease of children known as dengue hemorrhagic fever (DHF) or, in its most severe form, dengue shock syndrome (DSS). In DHF, hemostatic disorders and increased vascular permeability are accompanied frequently by internal bleeding and shock. These disturbances follow a minor febrile illness that lasts 3 to 5 days. At least 1.5 million children are reported to have been hospitalized and 33,000 have died with this syndrome since it was recognized in the 1950s (*6, 7*). Mortality rates vary from 2 to 10%. In 1981, the first recent outbreak of DHF/DSS outside Southeast Asia occurred in Cuba and resulted in 116,000 hospitalizations (1% of the total population) within a 3-month period (*8*).

Evidence for Immunological Modification of Dengue Illness

When it was discovered in Southeast Asia that dengue fever without complications occurred in nonindigenous foreigners while DHF/DSS occurred in indigenous children, explanations were sought (*9*). Studies of the pathogenic mechanisms of dengue virus

The author is in the Division of Health Sciences, Rockefeller Foundation, New York, NY 10036.

infections are important for the development of vaccines against the virus and provide insights into the pathogenic mechanisms of other human and animal viruses.

Fundamental to the immunological events in DHF/DSS is the existence in nature of the four serologically related dengue viruses that parenterally enter human hosts. After a short period of cross protection (*10*), individuals infected with one serotype are fully susceptible to infection with other types; these subsequent infections may be accompanied by disease. In contrast, there is lifelong immunity to reinfection by the homologous serotype. Primary and heterologous infections can be distinguished readily by their characteristic serological responses. In secondary infections, antibodies are largely of the immunoglobulin G (IgG) class and are directed against the antigens of the flavivirus group on the dengue virus complex or subcomplex. These antigens are expressed on the surfaces of the sequentially infecting viruses. Antibody responses after primary dengue infections are largely of the IgM class and are predominantly directed against type-specific determinants (*11*).

Patients with dengue infection who develop physiologically significant vascular permeability, hypovolemia, and abnormal hemostasis can be divided into two pathophysiologically similar but immunologically distinct groups: children 1 year and older producing a secondary antibody response and infants less than 1 year with primary antibody responses (*12*). Children over the age of 1 year comprise approximately 90% or more of patients with DHF/DSS; as many as 99% of the children with DHF/DSS have had a previous dengue infection (*3, 4*).

In four prospective population-based seroepidemiological studies, children 1 year and older were always found to have circulating dengue antibody before they acquired the infection that resulted in hospitalization (*3, 4, 13, 14*). To date, DHF/DSS has been documented only in individuals experiencing a second, but not a third or fourth, dengue infection. The Cuban outbreak of 1981 provided the classic test of the hypothesis that DHF/DSS occurs during a second and not a first dengue infection. Dengue was not transmitted in Cuba from the time of World War II until 1977–1978, when an outbreak of dengue 1 resulted in infection of approximately 40% of the total population in all age groups; the form of the disease was predominantly mild (*15*). In 1981, dengue 2 was introduced in Cuba and tests on paired sera from DHF/DSS patients during the epidemic regularly showed secondary-type antibody responses (*16*). As expected, children 1 and 2 years of age did not require hospital admission (Fig. 1). These children were born after the 1977–1978 dengue 1 epidemic, so they had been exposed only to dengue 2. Similarly, 1- and 2-year-old children were not among the fatalities associated with the epidemic (*17*).

Infants less than 1 year old who develop DHF/DSS during primary infections are born to dengue-immune mothers. Cases in this age and immunological group have been identified in Thailand, Burma, Indonesia, Cuba, and Puerto Rico (*12, 17, 18*). Characteristically, there are few such cases in the first 3 months of life; then attack rates rise, peaking at 7 or 8 months, and finally declining to the base line at the end of the 12th month (*4*).

Antibody-Dependent Enhancement of Viral Infection

Dengue virus replication in human beings appears to be restricted to cells of mononuclear phagocyte lineage (Table 1) (*19–27*). Dengue virus antigen and viral particles have not been seen in endothelial cells (*28*), putative cellular targets for a disease characterized by vascular permeability and hemorrhage. The liver of DHF/DSS patients usually shows swelling and hyalin necrosis of

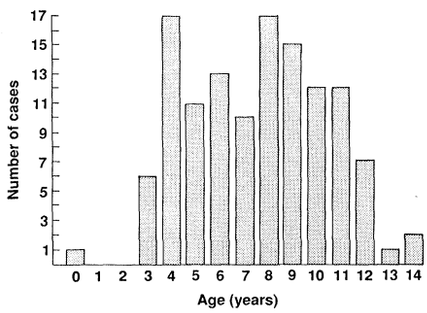

Fig. 1. Age distribution of 126 infants and children with a clinical diagnosis of dengue shock syndrome who were admitted to William Soler and Central Havana Children's hospitals in 1981 (*17*).

Kupffer cells, inflammation, and focal areas of fatty degeneration of hepatocytes (*29*). The consensus from autopsy studies is that DHF/DSS patients die from acute physiological derangements brought about by vascular permeability and inflammation (*28, 29*). The interpretation of existing data is that mononuclear phagocytes are the most important target cell for dengue infection, and although studies on this subject are not definitive, the immune enhancement hypothesis is based on the premise that the greater the number of mononuclear phagocytes infected, the more severe the illness in the individual.

Studies in vitro and in vivo show that virus-antibody complexes play an important role in the infection of mononuclear phagocytes by dengue viruses. Dengue replicates to higher titers in cultured human monocytes in the presence of heterotypic flavivirus antibody or homotypic antibody at subneutralizing concentrations than in cultures treated similarly but without flavivirus antibody (*30*). Assay systems for infection enhancement include phagocytic, Fc receptor–bearing cells of human, murine, and avian origin plus several macrophage-like cell lines (*31*). Antibody-dependent enhancement of dengue virus replication in mononuclear phagocytes provides a simple, unitary pathogenetic mechanism that explains cellular infection and disease enhancement in children with circulating antibody from an earlier heterotypic dengue infection and in infants with maternal antibody catabolized from the protective levels acquired at birth to infection-enhancing levels.

In systems in vitro, the phenomenon of antibody-dependent enhancement (ADE) of viral infection has been described for a number of flaviviruses, including dengue virus types 1 to 4, West Nile virus, yellow fever, tick-borne encephalitis, and Murray Valley encephalitis virus, as well as one or more representatives of the following families: alphaviruses, poxviruses, bunyaviruses, rhabdoviruses, coronaviruses, herpesviruses, and reoviruses (*32*). Immune complexes formed by IgG1 and IgG3 bind firmly to Fc receptors on human monocytes and macrophages, whereas complexes formed with IgG2 and IgG4 bind poorly (*33*). Mouse macrophage cell lines and mouse peritoneal macrophages bind complexes of West Nile virus and IgM antibody to West Nile virus by way of cellular receptors to the C3b fraction of complement (*34*). A similar complement-dependent ADE has been demonstrated by using rhesus monkey monocytes and complexes of dengue virus and IgM antibody to dengue virus (*30*). This phenomenon has not yet been described in human systems.

Antibody-enhanced infections have been described in some systems in vivo. Viremias were enhanced in rhesus monkeys infected initially with dengue virus types 1, 2, 3, or 4 and challenged 6 weeks to 6 months later with heterologous dengue type 2. Mean peak viremia titers were significantly higher in monkeys with secondary compared with primary virus infections (*35*). Enhanced dengue 2 viremias have also been detected in rhesus monkeys injected intravenously with dengue antibody–positive human cord blood before infection with the virus. Control animals received nonimmune

human cord blood (*35*). Other enhancement phenomena include increased growth of virus in immune compared with nonimmune hosts, shorter incubation periods from infection to onset of disease or death, and enhanced disease severity. Where the infected cells are known, macrophages frequently constitute important sites of viral replication. Examples include intranasal influenza infection of immunized mice, feline infectious peritonitis in kittens born to immune dams, St. Louis encephalitis (SLE) virus infections in birds acquiring SLE antibody transovarially (*36*), and Aleutian disease of mink (ADV) in immunized animals. Porter *et al.* (*37*) speculated that the production of nonneutralizing antibody against ADV led to the formation of infectious virus-antibody complexes, the uptake of these complexes by macrophages, and a process of chronic infection enhancement. In this respect, ADV resembles other viral infections of animals, such as lymphocytic choriomeningitis virus infections of mice, lactate dehydrogenase virus infections of mice, equine infectious anemia, and chronic African swine virus and chronic hog cholera virus infections in domestic hogs (*38*).

The question of how viruses that enter macrophages complexed to subneutralizing concentrations of antibody can escape lysosomal killing has been studied with the use of radiolabeled virus and by electron microscopy (*39*). Antibody-coated particles were found to be five to six times as infectious as nonopsonized particles, and opsonized virus was found to enter mouse macrophages through coated pits. Morphologically, the entry pathways of virus particles with or without subneutralizing antibody were identical. Penetration of flavivirus genetic material into the cytosol involves an acid-dependent fusion reaction similar to that of several other enveloped viruses (*40*). An interpretation of these experiments is that virus-antibody complexes attach to Fc receptors on the surface of mononuclear phagocytes with enhanced efficiency compared with viruses without antibody. Once attachment occurs, opsonized viruses are brought into close conjunction with coated pits, enhancing the efficiency of penetration and perhaps of fusion of the virion envelope with membranes of acidic endocytic vacuoles.

Direct confirmation of enhanced infection in DHF/DSS has been technically difficult to achieve. Viremias during secondary dengue infections are depressed by rapid and early formation of effective neutralizing antibodies. In theory, it should be possible to compare viremia titers in infants with primary infections who have been born to nonimmune mothers with titers in infants who have circulating residual maternal-enhancing antibody. No such studies have been reported, however. In practice, infants with DHF are seen as patients late in their illness when their immune responses are at the stage of eliminating extracellular virus and intracellular infections.

Several studies have shown that the quantity of dengue virus antigen circulating as immune complexes, measured directly or indirectly, varies linearly with disease severity. Further, complement consumed by the classic pathway, which provides a measure of antigen-antibody complexing, varies directly with disease severity (Table 2) (*27, 41–48*). Reductions in circulating C3, C4, and C5 proteins were also shown to be correlated with disease severity. These losses could not be attributed to transudation because reductions in plasma levels exceeded those of transferrin (*41, 45*). Normal C3 levels were observed in children older than 1 year who had chikungunya, rubella, or primary dengue infections (*49*). Catabolic rates of C1q varied between 3.8 and 8.3% per hour in seven DHF/DSS patients compared with 2.7 to 3.4% in controls (*45*). The three studies with negative results involved detection of immunoglobulin, C3, or dengue antigen on the surface of platelets or B lymphocytes (*46–48*). Since the attachment of immune complexes or complement to platelets could cause their subsequent destruction, quantitating immune complexes with this system is unlikely to be meaningful. The data cited are compatible with the infection enhancement hypothesis, but it is still necessary to quantitate antigenemia by illness severity with greater specificity.

Control of Disease Severity

Epidemiological data suggest that such host attributes as ethnicity, age, and sex influence the severity of dengue illness. In the 1981 epidemic of DHF/DSS in Cuba, among 124 Havana children clinically classified as DSS, only 14% were blacks and mulattos, although the contribution of these ethnic groups to the open population was 34% (*50*). A retrospective seroepidemiologic study in Havana showed no difference in dengue infection by ethnic group (*51*). Cases of DHF/DSS have not been reported from Africa, and sizable outbreaks have not occurred on Caribbean islands where populations are predominantly black. Sex and age are established risk factors for DSS. In a Thai study, females between the age of 4 and 14 years were hospitalized for DSS at a rate nearly twice that of males of the same age (*12*). Dengue infection rates did not differ by sex or age in the open population of Bangkok during the study period (*4*). Increased numbers of females among DSS cases and fatalities also were observed in the Cuban DHF outbreak (*17*). This

Table 1. Observations on sites of dengue virus replication in human beings.

Method	Observations	Patients (positive studied)	Reference
Virus isolation from autopsy tissues	Recovery of virus from liver, heart, lymph node, lung, and bone marrow; fatal DSS; secondary infections	7/199	(*19–22*)
Virus isolation from autopsy tissues	Recovery of virus from liver, spleen, thymus, and lung; fatal DSS; primary infections in infants	2/2	(*23*)
Virus isolation from blood leukocytes	Recovery of virus from adherent peripheral blood leukocytes; DHF/DSS; secondary infections	76/322	(*24*)
Fluorescent antibody test on skin biopsies	Dengue antigen in mononuclear phagocytes infiltrating dermal papillae; DHF/DSS; secondary infections	14/53	(*25*)
Fluorescent antibody test on autopsy tissues	Dengue antigen in large lymphoid or reticulum cell in liver sinusoid; DHF/DSS; secondary infections	1/21	(*26*)
Fluorescent antibody test on autopsy tissues	Dengue antigen in Kupffer cells, or splenic, thymic, and pulmonary macrophages; fatal DSS; primary infections in infants	2/2	(*23*)
Electron microscopy on kidney biopsies	Crystalline arrays of dengue virion-like particles in cytoplasm of monocytes in renal biopsies; DHF/DSS; secondary infections	12/20	(*27*)

outbreak provided an opportunity to observe the effect of age on disease severity independently of immunity. Cases of DSS in Cuba, stable from ages 3 to 11 years, declined sharply after age 11 with few cases of hypovolemic shock after age 14 (Fig. 1). Fatality rates due to dengue were five times as high in children as in adults (*17*). A cross-sectional seroepidemiologic study, however, has shown no difference in dengue 1 or dengue 2 infection rates in any age group among children and somewhat higher infection rates in adults remaining at home (*51*).

Geographic inhomogeneities in endemicity of multiple dengue serotypes and of DHF/DSS make it likely that viral factors also are important in regulating disease severity. Variation among dengue viruses has been studied both by means of RNA oligonucleotide fingerprinting and by hybridization with synthetic complementary DNA probes. With these methods, strains of dengue 1 and dengue 2 have been grouped into topotypes, members of which share 80% or more of their long oligonucleotides. On the basis of these analyses, Caribbean and Southeast Asian dengue 1 and 2 viruses were shown to be similar within a geographic region but distinct across regions (*52*). As further evidence of viral variation, differences in the ability of dengue viruses to multiply in human monocytes have been observed. Five dengue 2 viruses recovered from humans infected in Caribbean outbreaks during the 1970s (excluding Cuba) exhibited a reduced ability to replicate in human monocytes when compared with seven Southeast Asian strains of dengue 2 (*53*). Morens and coworkers (*54*) carried the monocyte infection marker one step further. When strains of dengue 2 recovered from 13 patients with DHF/DSS in Bangkok in 1980 were tested for human monocyte infectivity as a function of disease severity, the strains isolated from three relatively mildly ill children showed consistently lower growth than strains recovered from ten moderately or severely ill children (*54*). Whether the differences in ability of dengue 2 viruses to replicate in monocytes are related to inhomogeneities in viral receptors for attachment to monocytes or differences at other stages of replication is not known. By antigen signature analysis, dengue 2 strains recovered from Southeast Asia were found to be similar, but they differed from dengue 2 strains from the Caribbean (*55*).

Intraregional variations in disease outcomes suggest that other viral factors may be important. In Thailand, the incidence of DSS among children experiencing a secondary dengue infection with any serotype has been estimated at between 0.5 and 20 per 100 (*3, 4, 13*). Why does enhanced disease constitute such a small fraction of all secondary dengue infections? One possibility is the dampening effect of heterotypic neutralization on infection. With dengue viruses, infection with one serotype often raises low levels of cross-reacting neutralizing antibody to one or more other serotypes (*56*). In a 3-year prospective study of 1700 Bangkok schoolchildren, from 1978 to 1980, nine children had documented illnesses during secondary dengue 2 infections and seven of these were hospitalized. An additional 32 children had secondary dengue 2 infections but without sickness. When undiluted serum samples taken before the illness were tested for growth of dengue 2 in monocytes, 25 of 32 children without illness but only 2 of 7 children with severe illness had heterotypic dengue 2 neutralizing antibodies. All of the sera enhanced dengue 2 infections at dilutions above the neutralization end point and, in the case of the five symptomatic infections, undiluted sera produced dengue 2 infection enhancement (*14, 57*).

In another approach, 13 mothers of infants who acquired DHF/DSS during primary dengue 2 infections were studied for immunological risk factors (*58*). Serum samples from the mothers were used as substitutes for antibody passively transferred to the infants before birth. Each woman had previously had two or more dengue infections. Dengue 2 neutralizing antibody titers of the mothers correlated with the age of onset of DHF in the infant. For example, when sera from the mothers had low concentrations of dengue 2 neutralizing antibodies, 1:30 to 1:100, the infants acquired DHF/DSS at 4, 6, and 8 months, ages that were quite close to the predicted time of disappearance of these antibodies to titers of 1:1 or below. When sera of the mothers had high titers of dengue 2 neutralizing antibodies, 1:2000 to 1:8000, infants acquired DHF/DSS at 11 or 12 months of age, a time after birth also consistent with catabolic loss of passively acquired antibodies to titers below 1:1 (*58*).

Table 2. Studies relating quantity of circulating antigen-antibody complexes to severity of DHF. Observations made during defervescence or shock stage.

Measurement system	Patients with immune complexes detected	Reference
Positive correlation		
C1q precipitates (serum)	34	(*41*)
Raji cell assay (serum)	12	(*42*)
C1q latex agglutination inhibition (serum)	67	(*43*)
Raji cell assay (serum)	56	(*43*)
C1q inhibition test (serum)	43	(*44*)
C3 catabolism (serum)	17	(*45*)
Immune complexes observed in glomeruli by electron microscopy	10	(*27*)
Negative correlation		
B lymphocyte surfaces stained for dengue Ag and C3	55	(*46*)
Platelets stained for dengue Ag, IgM, and C3	51	(*47*)
Platelets stained for surface C3	13	(*48*)

Laboratory studies show that infection enhancement can be controlled by matching epitopes located on sequential infecting virus pairs. Such epitopes are heterogeneously distributed. This phenomenon was discovered when seven geographically diverse strains of dengue 2 were tested for ADE against a panel of monoclonal antibodies (Mab) to dengue 2, New Guinea strain. Type-specific and group-reactive Mab enhanced infection with most but not all dengue 2 strains (*59*). A larger number of enhancing epitopes were detected when the same experiment was repeated with a battery of 19 Mab to dengue 4, 4328-S strain. When 17 strains of dengue 2 isolated in 1980 from DHF/DSS patients were tested for ADE by using eight Mab to dengue 4, all four strains isolated from patients with milder syndromes showed little growth in monocytes, while nearly all 13 strains from patients with severe disease were enhanced. Both dengue 4–specific as well as flavivirus group-reactive Mab produced ADE with dengue 2 strains (*60*). These studies provide evidence that type-specific epitopes are shared among different dengue serotypes.

Future Research

Many data implicate the infection of mononuclear phagocytes as the central pathogenic phenomenon in human dengue infections. If these data are substantiated, it will be possible to integrate a number of seemingly disconnected clinical and epidemiological observations into an orderly and predictive model. In this model the most important factor in determining disease severity is the success of viral invasion of mononuclear phagocytes. Afferent events may add to or subtract from this success. Of those factors controlled by the human genome the most exciting is the protective effect of black ethnicity. Whether a genetic mechanism operates to restrict viral replication in monocytes, as suggested by a preliminary report (*61*), or whether restriction occurs at some other level in the pathogenic

cascade is not known. Other afferent events are controlled by the viral genome. These include the sharing of "enhancing epitopes" between sequential pairs of infective viruses, and the sharing of "neutralizing epitopes." The latter elicit the antibodies during an initial infection that are partially cross-protective to a successive infection with a different serotype.

The recent success in obtaining the gene sequences and inferred protein structure for three of four dengue serotypes and the gene sequences for related flaviviruses should make it possible to relate protein structures to biologic function and to specified surface epitopes (*62*). These advances should thus facilitate the development of vaccines against dengue viruses.

Immunization against dengue without complete protection against all four serotypes presumably may sensitize individuals to DHF/DSS. Using the empirical method of selecting mutants by serial passage in tissue culture, a research group in Thailand is attempting to produce a live-attenuated vaccine that will incorporate all four dengue serotypes (*63*). However, less expensive and safer methods of vaccine production will have to be found. One avenue for exploration is immunization with nonstructural proteins. This seemingly contradictory method is protective in experimental yellow fever. In mice and rhesus monkeys the passive transfer of Mab to the yellow fever nonstructural protein NS-1, or active immunization with this protein, resulted in resistance to challenge with wild-type virus (*64*). Similarly, mice immunized with NS-1 from dengue 2 showed significant resistance to intracerebral challenge with dengue 2 virus (*65*). While E and pre-M proteins usually are not detected in plasma membranes of dengue-infected cells, NS-1 is relatively abundant on the cell surface (*66*). In flavivirus infections this protein may contribute to the identification of infected cells as non-self, thus targeting them for immunological destruction.

The recent increases in dengue transmission in many parts of the world provide a challenge to investigators seeking means to prevent, cure, or control the disease (*67*). Mosquito control programs have been inadequate to prevent virus transmission in many areas, and new approaches to vector control are needed. The hazards posed by vaccination against dengue infection have not been encountered in vaccine development for other diseases, and when overcome may reveal new features of viral pathogenesis. The key role of dengue antibody in mediating infection of phagocytic target cells presents an unprecedented system in which to study biologic function and molecular structure in the context of causation of disease.

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