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# The Dengue Viruses

ERIK A. HENCHAL* AND J. ROBERT PUTNAK

*Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100*

INTRODUCTION ....376
EPIDEMIC PATTERNS ....376
Discovery of the Agent and Identification of Vectors ....377
RELATIONSHIP OF THE DENGUE VIRUSES TO OTHER FLAVIVIRUSES ....377
THE DENGUE VIRUS COMPLEX ....378
VIRUS STRUCTURE AND COMPOSITION ....378
Physiochemical Properties of the Virion ....378
The Viral RNA Genome ....378
Virus Structural Proteins ....379
Composition of the Viral Envelope ....381
Virus Nonstructural Proteins ....381
VIRUS REPLICATION SCHEME ....382
Attachment, Penetration, and Uncoating ....382
Primary Translation and Early RNA Replication ....382
Synthesis and Proteolytic Processing of Viral Proteins ....382
RNA Replication ....383
Virus Assembly and Release ....383
BIOLOGICAL CHARACTERISTICS ....383
Infection in Experimental Animals and Host Range ....383
Propagation in Cell Cultures ....384
Virus Variants and Mutants ....385
CLINICAL FEATURES, PATHOGENESIS, PATHOLOGY, AND DIAGNOSIS ....385
Clinical Features ....385
Pathology ....386
Laboratory Diagnosis ....387
PREVENTION AND CONTROL ....388
CONCLUSIONS ....389
ACKNOWLEDGMENT ....389
LITERATURE CITED ....389

## INTRODUCTION

Dengue is an acute infectious disease of viral etiology characterized by biphasic fever, headache, pain in various parts of the body, prostration, rash, lymphadenopathy, and leukopenia (98, 227). Dengue hemorrhagic fever (DHF) is a severe febrile disease characterized by abnormalities of hemostasis and increased vascular permeability, which in some instances results in a hypovolemic shock syndrome, dengue shock syndrome (DSS) (98, 292). A major public healthy problem throughout subtropical and tropical regions, dengue is probably the most important arthropod-borne viral disease in terms of human morbidity and mortality (99). For the last two centuries, dengue has been known by any of the following pseudonyms: break-bone fever, dandy fever, denguero, bouquet fever, giraffe fever, polka fever, or the 5-day or 7-day fever (227). Four distinct serotypes of the dengue virus (dengue-1, dengue-2, dengue-3, and dengue-4) exist, with numerous virus strains found worldwide.

## EPIDEMIC PATTERNS

The first recorded epidemics of denguelike disease ("joint fever") occurred in 1779 in simultaneous outbreaks in Batavia (Jakarta) and Cairo as reviewed by Siler et al. (250). Subsequently, dengue outbreaks were reported in Philadelphia (1780), Zanzibar (1823 and 1870), Calcutta (1824, 1853, 1871, 1905), the West Indies (1827), and Hong Kong (1901) (238). Whether all of these epidemics can be attributed to dengue virus rather than to clinically similar chikungunya virus has been disputed (40, 170). Nevertheless, major dengue epidemics have occurred in this century at irregular intervals wherever the mosquito vector can be found. Some of the largest outbreaks have occurred in the United States (1922), Australia (1925–1926; 1942), Greece (1927–1928), and Japan (1942–1945) (227). The 1922 dengue fever epidemic in the southern United States may have affected 1 to 2 million people (227). Epidemic dengue fever is responsible for hundreds of thousands of cases each year in southeast Asia, where all four serotypes of the virus can be found. Similarly, the introduction of single virus serotypes into Central America and the Caribbean basin resulted in large epidemics of dengue fever in 1952, 1963–1964, 1977, and 1981 (31, 44, 82). Since then, multiple dengue virus serotypes have become endemic in most countries of tropical and subtropical America and are regularly associated with disease outbreaks (82).

After World War II, new endemic patterns of disease, accompanied by an increased incidence of complicated dengue (DHF and DSS), emerged in southeast Asia (96). Hammon et al. (W. M. Hammon, A. Rudnick, G. E. Sather, K. D. Rogers, V. Chan, J. J. Dizon, and V. Basaca-Sevilla,

* Corresponding author.

Proc. 9th Pacific Sci. Congr., Bangkok, Thailand, 1957, p. 67–72) were the first to describe epidemic DHF in the Philippine Islands in 1954. Halstead (95) has suggested that DHF was not a new disease and that dengue epidemics in which patients had symptoms of severe hemorrhage and shock were described in Queensland (1897), the southern United States (1922), Durban, South Africa (1927), Greece (1928), and Formosa (1931). While hemorrhagic manifestations were rarely reported before 1944, DHF and DSS have become endemic in many southeast Asian countries. Between 1982 and 1987, more than 403,405 cases (with 2,395 fatalities) of DHF were reported in Thailand (293). Similarly, increasing numbers of denguelike illness with hemorrhagic manifestations have been reported every year in the Caribbean basin since a major epidemic in Cuba (82, 92). During that outbreak, over 116,243 hospitalized patients, 24,000 DHF cases, and 158 deaths were reported during a 3-month period (92). Three factors have been implicated as responsible for increased dengue virus transmission in the Americas: the failure to control the *Aedes aegypti* populations, the introduction and rapid dissemination of new virus strains within the region by increased airplane travel, and the creation of ecological conditions in tropical American cities that have allowed coexistence of multiple serotypes of dengue viruses (82, 83).

Although dengue fever was eliminated from the southern United States in the 1920s after populations of the vector mosquito were controlled, the potential for reintroduction of the disease into the region still exists. Between 1977 and 1987, 1,655 suspected cases of imported dengue, of which 345 cases were serologically confirmed, were reported to the Centers for Disease Control (46). Because the principal vector mosquito, *A. aegypti*, is found throughout the southern United States, indigenous transmission of dengue in these areas is possible. In 1980, 63 cases of dengue fever were identified in Texas. Twenty-seven (43%) of these cases were in patients who had not traveled outside of the United States before becoming ill (93). In 1981, investigators identified a cluster of imported dengue fever cases in Florida, where significant vector populations exist (163). In 1982, a total of 45 serologically confirmed cases were recorded by the Centers for Disease Control; 8 of these cases were in states that have populations of the vector mosquito (81). Recently, the Asian dengue vector *A. albopictus* has become established in focal areas of the eastern United States as far north as latitude 42° N (45). While dengue virus transmission by this mosquito has not been documented in the United States, the potential exists for more serious outbreaks. Continued, aggressive dengue virus surveillance is required to monitor transmission in states with significant vector populations as epidemic outbreaks increase in contiguous subtropical and tropical areas.

### Discovery of the Agent and Identification of Vectors

Dengue fever was the second human disease (after yellow fever) whose etiology was critically identified as a "filterable virus" (7, 238). Early investigators had suggested that dengue virus was transmitted by mosquitoes (78), but actual transmission by a true vector, *A. aegypti*, was not demonstrated until 1906 (9). In the following year, Ashburn and Craig provided the first data demonstrating the filterable, ultramicroscopic character of the etiological agent (7). Confirming studies were performed by Cleland et al. (56) and Siler et al. (250). Simmons et al. (251) established the role of *A. albopictus* in the transmission of dengue virus and showed that certain species of monkeys have clinically inapparent dengue virus infections. These results suggested that dengue virus may be transmitted in a fashion similar to the "jungle cycle" of yellow fever virus. Evidence for a forest maintenance cycle in Malaysia, Vietnam, and Africa has been provided in additional work by Rudnik (212) and Yuwono et al. (295). Other *Aedes* species have since been implicated in Asia and Africa (61, 84; A. Rudnick, Proc. Int. Conf. Dengue/Dengue Haemorrhagic Fever, 1984, p. 7–10).

Dengue virus strains were generally unavailable for extensive laboratory study until after 1944–1945. In comprehensive studies stimulated by the demands of a South Pacific war, Sabin and his co-workers isolated a number of dengue virus strains by inoculating infectious sera into human volunteers (223). At least two different serotypes (Hawaii and New Guinea B, C, and D strains) were recognized, based on the susceptibility of human volunteers to heterologous challenge after convalescence from an initial dengue virus infection. Using infectious sera and mosquito transmission, these investigators made several remarkable discoveries with regard to the spectrum of clinical symptoms observed after experimental infection, the particle size of the virus, routes of infection, vector competence, stability, the sensitivity of the virus to denaturation agents, the susceptibility of certain host animals to infection, induction and duration of protective immunity after infection, and interference by heterologous viruses (223–228).

The beginning of the modern era of dengue virology can be attributed to the development of the suckling mouse infection model. Kimura and Hotta were the first to report the adaptation of dengue virus strains to the Swiss albino mouse (124, 136). These investigators established three mouse-adapted strains (all dengue-1 strains), using isolates from the 1943 and 1944 dengue epidemics in Japan. Independently, Sabin and Schlesinger (223, 228) adapted the Hawaiian (dengue-1) strain to 2-week-old mice and demonstrated that adapted strains retained the serological characteristics of nonadapted virus strains. Both groups of investigators found that some mouse-adapted virus strains were attenuated with regard to their ability to cause human disease. Meiklejohn et al. (173) were the first to suggest the use of new-born mice for the cultivation of the dengue viruses, leading to the adaptation of dengue-2 virus (New Guinea C and D strains) for growth in mice. Similarly, Schlesinger and Frankel (239) adapted the dengue-2 (New Guinea B) virus. Later, mouse-adapted dengue-3 and dengue-4 virus strains were developed by using virus strains isolated during epidemics of hemorrhagic fever in the Philippine Islands (109).

## RELATIONSHIP OF THE DENGUE VIRUSES TO OTHER FLAVIVIRUSES

The four serotypes of dengue viruses are members of the family *Flaviviridae*, which consists of over 60 arthropod-borne viruses; yellow fever virus is the family prototype (282). In addition to sharing a common morphology and genomic structure, all members share common antigenic determinants, which make identification of individual family members difficult by classical serological techniques. In 1828, Osgood first suggested that the agent causing dengue was the same as that causing yellow fever based on epidemiological characteristics (191). Other early investigators, including Craig (62, 63) and Siler et al. (250), also regarded dengue and yellow fever viruses as belonging to the same group. The serological relationship of the dengue viruses to other flaviviruses was demonstrated in 1950 by Sabin, who

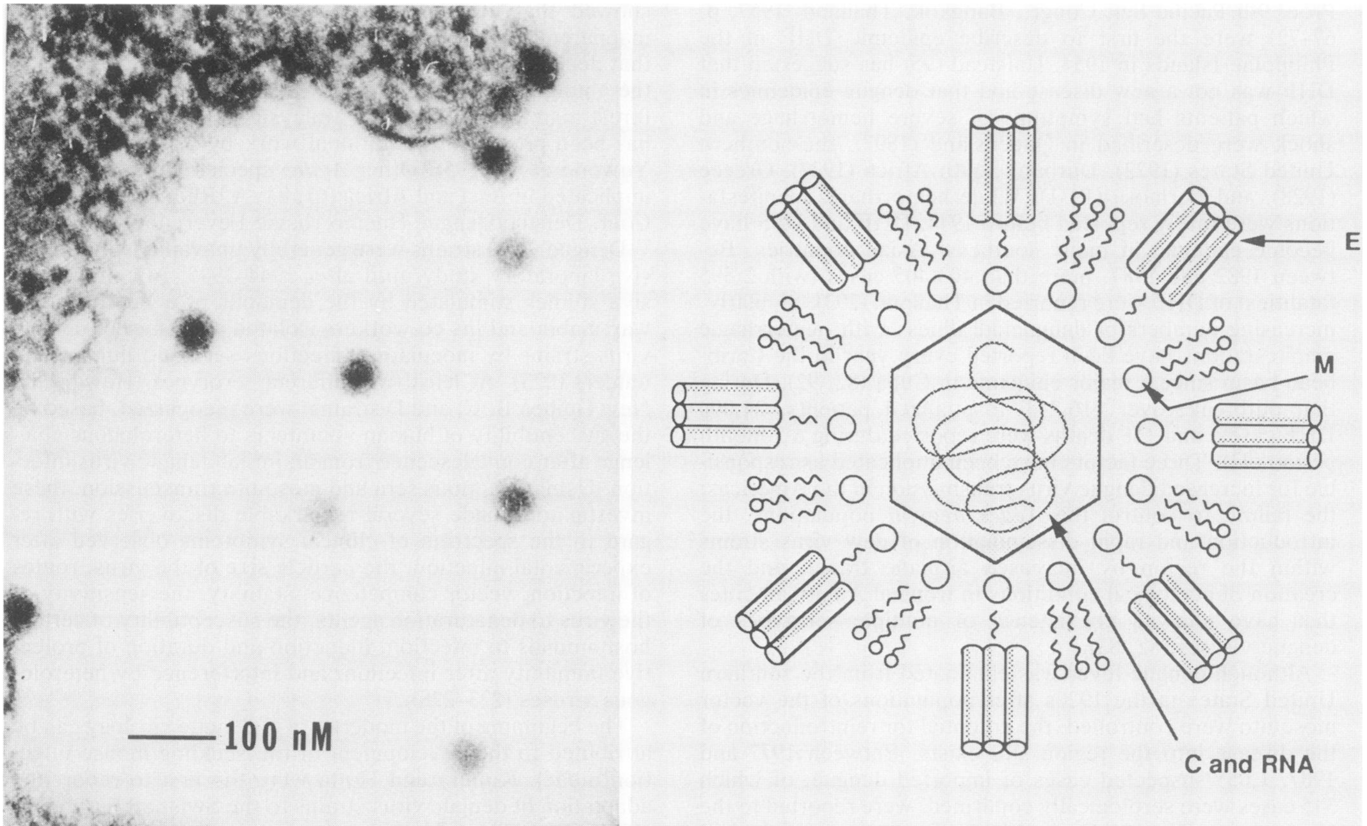



FIG. 1. Release of dengue-2 virions from infected cells (left) and schematic representation of mature virions (right). E, Envelope protein; M, membrane-associated protein; C, core protein.

reported serological cross-reactions of human anti-dengue convalescent sera with yellow fever virus, Japanese encephalitis virus, and West Nile virus antigens (222). Also, it has been shown that yellow-fever-immunized individuals later infected with dengue viruses or immunized with dengue antigens had anamnestic immune responses (240, 250). Casals and Brown used carefully standardized hemagglutination inhibition assays to formalize serological relationships among many arboviruses (41). Later, Henchal and co-workers defined a unique antigenic epitope shared by the flaviviruses on the envelope glycoprotein (118).

## THE DENGUE VIRUS COMPLEX

Recognition that the dengue viruses belong to a distinct virus complex was originally based on clinical, biological, and immunological criteria. The first report suggesting the existence of complex-specific antigenic determinants was made in 1954 by Smithburn, using neutralization assays (253). Russell et al. (216) demonstrated the presence of a dengue complex-specific antigenic determinant on the NS1 nonstructural protein (soluble complement-fixing antigen) by complement fixation assays and immunoprecipitation analysis. Using an improved plaque reduction neutralization assay, De Madrid and Porterfield (68) conclusively demonstrated in a comprehensive study that dengue virus serotypes constituted a single unique complex of viruses. This work was significantly extended when Henchal et al. (119, 120) specifically identified complex-reactive epitopes on structural and nonstructural antigens by using mouse monoclonal antibodies. Since DHF and DSS have been associated only with immune reactions within the dengue complex (98), these antigenic determinants may play a role in the immunopathology of the disease (see below).

## VIRUS STRUCTURE AND COMPOSITION

### Physiochemical Properties of the Virion

Similar to other flaviviruses, mature dengue virions consist of a single-stranded RNA genome surrounded by an approximately icosahedral or isometric nucleocapsid about 30 nm in diameter. This nucleocapsid is covered by a lipid envelope about 10 nm deep. The complete virion is about 50 nm in diameter (Fig. 1). The virion has a density of about 1.23 g/cm$^3$ as measured by equilibrium centrifugation in deuterium oxide-sucrose gradients and a sedimentation coefficient of around 210 $s_{20,w}$ (214).

### The Viral RNA Genome

The single-stranded RNA of dengue and other flaviviruses has a sedimentation coefficient of around 42 $s_{20,w}$ and a molecular weight of around $3.3 \times 10^6$ (259). Flavivirus RNA is infectious, has a messengerlike positive polarity, and can be translated in vitro (2, 186, 258, 264). The 5′ end of the RNA is capped (54, 277), but it lacks a poly(A) tail at the 3′ end (94, 130, 202).

Sequence analysis of cDNA derived from a flavivirus virion RNA by using reverse transcriptase was first com-

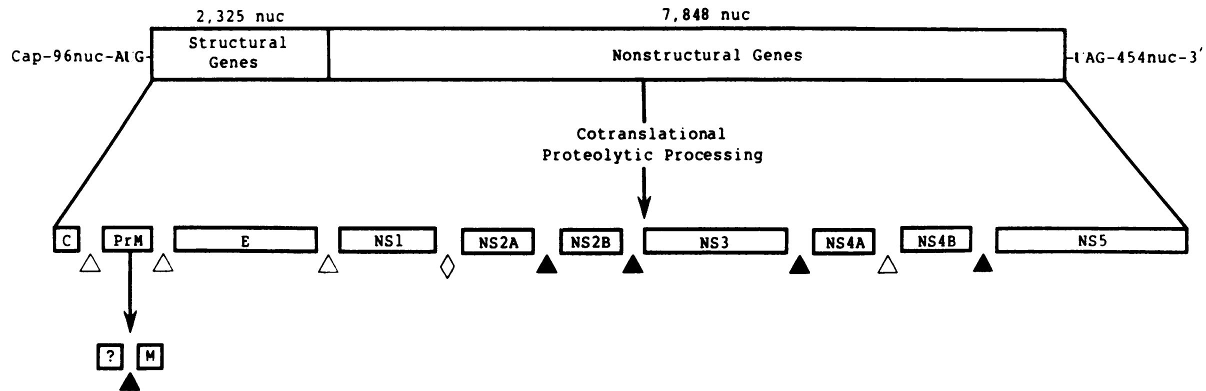



FIG. 2. Dengue virus RNA protein-coding regions and genome organization. Open triangles indicate possible cleavage sites by signalase enzyme. Closed triangles point to possible cleavage by proteases which act after two basic amino acids. The open diamond shows a cleavage by an unidentified protease.

pleted for yellow fever virus (202). The RNA genome of yellow fever virus is 10,862 nucleotides long and has an open reading frame of 10,233 nucleotides, which could encode a polypeptide of 3,411 amino acids. The noncoding region at the 5′ end is only 118 nucleotides long followed by the first AUG codon at which translation is initiated. This is considerably shorter than the noncoding region of picornaviruses, for example, where translation does not start at the first AUG codon. The first termination codon is encountered at nucleotide 10,352. The complete or nearly complete sequences of many other flaviviruses, including dengue-1 (168), dengue-2 (69, 94, 130), dengue-3 (190), dengue-4 (159, 297), Japanese encephalitis (171, 262), West Nile (42, 43), St. Louis encephalitis (272), Murray Valley encephalitis (64), Kunjin (58), and tick-borne encephalitis (164), have been obtained.

Little is known about the secondary structure of the flaviviral RNA, within either the virion or the infected cell. From sequence information, regions of secondary structure in the noncoding 5′ (33) and 3′ (94, 202, 262) ends have been predicted. Their roles in processes such as transcription, RNA replication, and encapsidation are unknown at present.

Now that many of the flaviviruses have been cloned and sequenced, the opportunity arises for development of infectious cDNA clones. An infectious cDNA clone of yellow fever virus has been constructed (201). The availability of flavivirus infectious clones should allow us to explore the function of viral genes and develop better attenuated vaccines through site-specific mutagenesis.

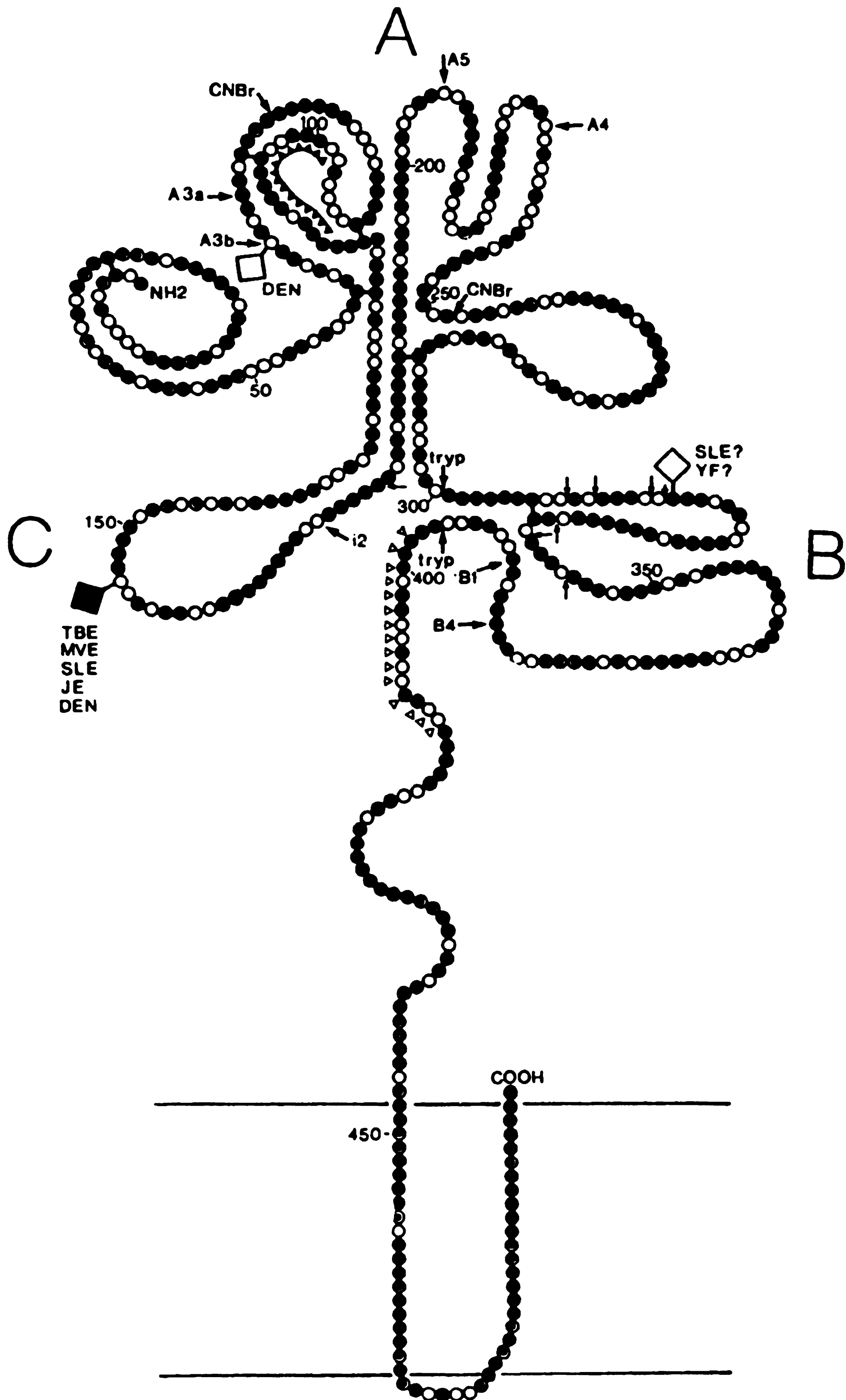
### Virus Structural Proteins

Currently accepted nomenclature for flavivirus structural and nonstructural virus proteins was first proposed by Rice and co-workers (202, 203). The mature virion contains three structural proteins: C, the nucleocapsid or core protein; M, a membrane-associated protein; and E, the envelope protein (202, 257). In addition, immature mainly intracellular virus contains a protein known as prM (sometimes, preM), a precursor of M (247, 276). The genes that encode the dengue virus structural proteins are located at the 5′ end of the genome and comprise slightly more than one-fourth of the coding capacity of the viral RNA (15, 69, 94, 168, 190, 202, 297). The gene order for the structural proteins from the 5′ terminus is C-prM(M)-E (Fig. 2). The proteins are derived from a single, long, precursor polypeptide or polyprotein (15, 53, 69, 94, 168, 190, 202, 297).

The C protein is the first viral polypeptide synthesized during translation, has a molecular weight of about 13,500, and is rich in lysine and arginine residues (about 25%). This highly basic character probably enables it to interact with the virion RNA (69, 94, 168, 190, 202, 297). C protein lacks an N-terminal, hydrophobic signal sequence, which suggests that its synthesis is on non-membrane-bound ribosomes. A hydrophobic stretch of amino acids at the C protein carboxy terminus probably acts as the transmembrane signal for the adjacent M-protein precursor, prM. This hydrophobic domain may serve to transiently anchor the C protein to a membrane at the replication site after cleavage, probably by a host cell "signalase" (166, 187), at the N terminus of prM. Later, the hydrophobic domain is cleaved from C, perhaps by a virus-encoded protease, before virus maturation is complete (187). Hydropathy plots of C protein indicate that it is conserved structurally among the flaviviruses, but less than other structural proteins (69, 94, 155, 168, 190, 202, 297).

Specific proteolytic cleavage of a glycosylated prM precursor (22,000) during virus maturation results in the formation of the 8,000-dalton M protein (69, 94, 168, 190, 199, 202, 247, 297). This cleavage, which may occur in the acidic post-Golgi vesicles, appears to precede virus release from the cell, as the amount of prM associated with extracellular virus is low. The formation of M from prM appears to be the crucial, terminal event in virion morphogenesis (199). It results in a large increase in virus infectivity and a reorganization of the virus surface structure, which is composed of E-prM heterodimers in immature virions (276). The role that M plays in the mature virion is not known.

The E glycoprotein, the major virion envelope glycoprotein (51,000 to 60,000), appears as a homotrimer on the surface of mature virions (278) and may be found intracellularly in E-prM heterodimers (276). Comparison of the nucleic acid sequences of several flavivirus E genes has shown perfect conservation of 12 cysteine residues which form six disulfide bridges. Nowak and Wengler (188) deduced the location of these disulfide linkages and provided a structural model for the envelope protein. This model has been further refined by Mandl et al. (165), who correlated structural properties of different epitopes with disulfide bridge assignments. The current model of the flavivirus E protein (Fig. 3)


A
A5
CNBr
100
A4
200
A3a
A3b
DEN
NH2
250
CNBr
50
tryp
SLE?
YF?
300
150
C
i2
tryp
400
B1
350
B
B4
TBE
MVE
SLE
JE
DEN
COOH
450

consists of three nonoverlapping antigenic domains (composed of at least 16 distinct epitopes): A, B, and C. Domains A and B contain discontinuous epitopes whose integrity is dependent on intact disulfide bridges. Since the highly variable domain C lacks disulfide bridges, epitopes in this region are not destroyed by reduction, carboxymethylation, or sodium dodecyl sulfate denaturation. (Denaturation resistance may be lost after removal of the carbohydrate side chain.)

Functional domains responsible for neutralization of the virus (198), hemagglutination of goose erythrocytes (67, 246), fusion, and interaction with specific cell surface virus receptors (67, 76, 77, 90, 138, 263) are associated with the E protein. Correlation of these domains with structural regions of the protein are just beginning to be made. Antigenic epitopes associated with neutralization of the virus have been mapped by using mouse monoclonal antibodies in competitive binding assays (90, 117, 120, 165, 188, 285). The epitope maps that have emerged show three or four major antigenic sites. However, epitopes immunodominant in mice may not be immunogenic in humans and vice versa. Significant efforts are being made to localize neutralizing antigenic epitopes to specific regions of the E protein by using synthetic peptides, expressed proteins, or monoclonal antibody neutralization escape variants (1; J. T. Roehrig, A. R. Hung, A. J. Johnson, and R. A. Bolin, W.H.O. Programme for Vaccine Development, Dengue Steering Committee Meeting, 1989; V. Deubel and M. Bordier, Second Int. Symp. Positive Strand Viruses, 1989, P.103, p. 44.; T. Trirawatanapong and R. Padmanabhan, Second Int. Symp. Positive Strand Viruses, 1989, P.108, p. 45).

### Composition of the Viral Envelope

What little is known about the lipid composition of the flavivirion envelope was last reviewed by Russell and coworkers in 1980 (214). The lipid composition of the virion envelope reflects, with minor exceptions, that of the host cell membrane from which virus budding presumably occurs. For flaviviruses, these are probably intracellular membranes of the endoplasmic reticulum (28, 32, 131). Upon examination of St. Louis encephalitis virions, lipid was found to account for 17% of the dry weight: 90% of that is phospholipid, 7% is sphingomyelin, and the remainder consists of cholesterol and neutral lipids (269).

Since the nucleocapsid of these viruses is a tenuous structure which is permeable to RNases, an important role of the lipid envelope is to protect the genomic RNA. The envelope also fuses with host cell membranes during penetration and uncoating, presumably promoted by a unique fusion domain of E protein (77, 137, 138, 263, 283).

### Virus Nonstructural Proteins

Seven, nonoverlapping, virus nonstructural (NS) proteins have been identified and mapped to the viral RNA by limited amino- and carboxy-terminal amino acid sequencing. Encoded 3′ to the structural protein-coding region (Fig. 2) and following the E protein are NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5.

NS1, the first nonstructural protein, is a ~48,000-molecular-weight glycoprotein containing two signals of the type, Asn-X-Ser/Thr, used for addition of N-linked carbohydrate. These sites appear to be conserved among all flaviviruses. NS1 is synthesized in the rough endoplasmic reticulum as a hydrophilic, water-soluble, monomeric glycoprotein. Shortly thereafter, it forms a noncovalently linked homodimer, which is more hydrophobic than the monomer (286, 287). Whether this increase in hydrophobicity is a result of dimerization or some other posttranslational modification is not known. After formation of the NS1 dimer, this glycoprotein is transported to the Golgi apparatus, where two of its four N-linked glycans are modified from a high mannose to a complex type (286). NS1 may remain intracellular, be transported to the plasma membrane, or secreted from the cell. Secretion of large amounts of NS1 may be restricted to infected mammalian, not mosquito, cells (167). N-glycosylation does not appear to be required for either dimerization or secretion of NS1 (286). The role of NS1 in virus replication is not known, but it has been speculated that NS1 assists virus morphogenesis (203). NS1 may have immunological importance since infected cells expressing the protein on the surface become targets for immune cytolysis (234, 235).

The NS2 coding region consists of two proteins, NS2a and NS2b, which were only tentatively mapped when the new nomenclature was proposed. NS2a has been identified as an ~20,000-molecular-weight hydrophobic protein with several putative transmembrane domains. It is required for proper proteolytic processing of the C terminus of NS1 (74). NS2b is a ~14,500-molecular-weight hydrophobic protein without any known function in virus replication (47, 254).

NS3 is a ~70,000-molecular-weight hydrophilic protein. It has been suggested that NS3 may be a viral protease active in the posttranslational processing of the polyprotein, a component of the viral RNA polymerase, or a virus protein with both enzymatic activities (13, 32). While the N-terminal region of NS3 shares sequence homology with trypsinlike serine proteases, the C-terminal resembles the sequence of nucleoside triphosphate-binding proteins involved in nucleic acid replication (13).

Similar to proteins encoded in the NS2 region, NS4a and NS4b proteins have been mapped only recently (47, 254, 255). These hydrophobic proteins have molecular weights of about 16,000 and 27,000, respectively. While their roles in virus replication are unknown, they might be RNA replication complex cofactors along with the putative viral RNA-dependent RNA polymerase, NS5.

Based on its large size of ~105,000 and the presence of a Gly-Asp-Asp sequence common with other viral RNA

FIG. 3. Model of flavivirus E protein. Open circles represent hydrophilic amino acid residues (Arg, Lys, Asn, Asp, Gln, Glu, His), dotted circles show intermediate amino acid residues (Pro, Tyr, Ser, Trp, Thr, Gly), and solid circles show hydrophobic amino acid residues (Ileu, Val, Leu, Phe, Cys, Met, Ala). Amino acids were classified by the scale of Kyte and Doolittle (134). Position numbers are shown every 50 amino acids. Cysteine residues forming disulfide bridges are connected by solid lines. Arrows indicate potential cleavage sites. Two solid lines stand for the lipid membrane that is spanded by two transmembrane regions of protein E. The polypeptide chain is folded to indicate the locations of the mutations identified in the respective antigenic variations of the tick-borne encephalitis (TBE) virus by sequence analysis. A line of solid triangles indicates the most perfectly conserved sequence within domain A. A line of open triangles marks the region of a potential T-cell determinant. A solid diamond represents the carbohydrate side chain of the TBE virus. Dengue viruses have potential N-glycosylation sites at the homologous position and in domain A. The homologous positions of TBE are shown by open diamonds. MVE, Murray Valley encephalitis virus; SLE, St. Louis encephalitis virus; JE, Japanese encephalitis virus; YF, yellow fever virus; CNBr, cyanogen bromide cleavage site; tryp, trypsin cleavage site. Adapted from Mandl et al. (165) with permission of the publisher.

polymerases, the NS5 protein is thought to be an RNA-dependent RNA polymerase (80, 203, 262).

## VIRUS REPLICATION SCHEME

### Attachment, Penetration, and Uncoating

Dengue viruses attach to susceptible cells by either of two known mechanisms. In one case, dengue viruses complexed to non-neutralizing, but antivirion, immunoglobulin G (IgG) antibodies may attach to macrophages or monocytes via Fc receptors found at cell surfaces. When Fc receptor-bearing cells are infected in the presence of serologically cross-reactive antisera diluted beyond the neutralization endpoint, a greater number of infected cells and higher virus titers are obtained (65, 103). This immunological contradiction, known as immune infection enhancement, may contribute to the pathogenesis of DHF or DSS (103). Alternatively, dengue viruses may attach to cells, including monocytes, via a trypsin-sensitive virus receptor (65). The composition and structure of this host cell receptor, which has been hypothesized to bind to distinct regions of the E glycoprotein, are unknown.

There are two methods by which attached infectious virus may penetrate host cells. The virion envelope may fuse with the plasma membrane with immediate deposition of the nucleocapsid into the cytoplasm, or the plasma membrane may invaginate, forming an endocytotic vesicle (endosome) around the still enveloped virus. Hase et al. (113), using electron microscopy, reported that dengue and Japanese encephalitis virions penetrate the plasma membranes of mosquito cells through membrane disruptions created at adsorption sites. In the same study, these authors reported that dengue viruses entered human peripheral blood monocytes through plasma or macropinocytic vacuolar membranes in the same manner. Gollins and Porterfield (76) reported that West Nile virus, a related flavivirus, entered host cells via receptor-mediated endocytosis and fusion from within acidic endosomes. These data suggest that flaviviruses have evolved different mechanisms for entering cells. However, interpretation of electron micrographs, upon which most conclusions are based, is complicated by the high particle/PFU ratio for dengue viruses.

Similar to other enveloped viruses, fusion of the dengue virus envelope with host cell membranes appears to be pH dependent (76, 77, 137, 138). Acidic conditions have been shown to activate a fusion protein (90, 283), which leads in an unknown way to the deposition of the nucleocapsid within the cytoplasm. Evidence that the E glycoprotein is the fusion protein comes from experiments that show that (i) anti-E monoclonal antibodies inhibit fusion (90, 263) and (ii) the E protein undergoes an irreversible conformational change at acidic pH (90).

### Primary Translation and Early RNA Replication

Very early processes in dengue virus replication are unknown. Since the dengue virus RNA genome has a positive sense, it must first be translated to make the RNA polymerase required for its replication. The polymerase must transcribe the positive-strand RNA to negative-strand RNA, which then serves as template for additional positive strands. During the long eclipse period (12 to 16 h) leading to the formation of the first progeny virus, the RNA must serve primarily as a template for replication and translation but not encapsidation. Also, positive and negative strands should be made at approximately equal rates to allow RNA amplification to occur exponentially. However, positive-strand RNA must be diverted to virus assembly during late eclipse.

TABLE 1. Dengue-2 proteins: formation and function

| Name | Glycosylated | N-terminal cleavage[a] | No. of amino acids in mature proteins | Function |
|---|---|---|---|---|
| C | No | M ¦NNQ (aminopeptidase) | 99 | Nucleocapsid |
| prM | Yes | VMA¦FHL (signalase) | 166 | M precursor |
| M | No | EKR¦SVA (dibasic) | 75 | Membrane protein |
| E | Yes[b] | SMT¦MRC (signalase) | 495 | Envelope protein |
| NS1 | Yes | VQA¦DSG (signalase) | 352 | Virus assembly? |
| NS2a | No | VTA¦GHG (unknown) | 218 | NS1 processing? |
| NS2b | No | KKR¦SWP (dibasic) | 130 | Unknown |
| NS3 | No | KQR¦AGV (dibasic)[c] | 618 | Protease/NTPase? |
| NS4a | No | GKR¦SLT (dibasic) | 150 | Unknown |
| NS4b | No | TMA¦NEM (signalase) | 248 | Unknown |
| NS5 | No | TRR¦GTG (dibasic) | 900 | RNA polymerase? |

[a] ¦, Amino acid cleavage site at the amino terminus. The possible protease responsible is given in parentheses.

[b] E protein is glycosylated in dengue virus but not in all flaviviruses.

[c] Motif similar to that of the dibasic amino acid-specific protease.

### Synthesis and Proteolytic Processing of Viral Proteins

Viral structural and nonstructural proteins are derived from a large precursor polyprotein, encoded by a long, open reading frame. This precursor protein is not usually seen in infected cells. Translation begins at the first AUG codon of the RNA genome (69, 94, 168, 190, 202, 297), and individual viral proteins are formed by cotranslational proteolytic processing of the precuror peptide (202). Since the C protein, the first protein synthesized, does not have an N-terminal hydrophobic "signal sequence," translation must occur initially on non-membrane-bound ribosomes. After encountering the hydrophobic signal sequence at the carboxy terminus of C, RNA-ribosome complexes probably become membrane associated before translation and concomitant membrane translocation of the remaining proteins. Proteases associated with polyprotein processing appear to be host and virus encoded.

Host "signalases" (23, 195) in the lumen of the endoplasmic reticulum are probably responsible for proteolytic reactions at the N termini of prM, E, NS1, and NS4b (47, 159, 166, 187, 254). Possible cleavage sites, preceded by hydrophobic signal-like sequences, have been identified. Also, cellular membranes have been shown to be required for cotranslational proteolytic processing of structural protein precursors, using in vitro translation systems (166, 187, 264). Mutations which abrogate translocation also affect cleavage (166).

Other protease activities associated with polyprotein processing have been identified. One type of activity has been identified after a short-side-chain amino acid, such as occurs at the N terminus of NS2a (254). Another type occurs after two basic amino acids such as Arg-Arg at the N termini of M, NS2b, NS4a (255), and NS5 (22, 203, 254). While cleavage at the N terminus of NS3 usually occurs after a pair of basic amino acids with other flaviviruses, the dengue virus NS3 is cleaved after Gln-Arg (22). The nature of the nonsignalase proteases and whether they are host cell or virus encoded are not yet clear. Table 1 shows the deduced amino acid sequence and the putative cleavage sites used in processing the polyprotein of dengue-2 virus, New Guinea C strain (130).

Few clear precursor-product relationships have been identified for dengue virus proteins with the exception of the processing of prM to M. Since proteolytic cleavages occur so rapidly, precursors are difficult to identify. This observation may have led to earlier reports of multiple internal initiation of translation of flavivirus proteins (280). The cleavage between NS1 and NS2a is interesting in that it requires the presence of a major portion of the NS2a protein (74). NS1 fused to a truncated NS2a is not processed in vitro, resulting in a protein longer than mature NS1. Whether NS2a is a protease or is merely required for the correct conformation of the cleavage site is not known; however, it lacks homology with known proteases. If NS2a is a protease, it would appear to be *cis* acting since a truncated NS1-NS2a construct is not cleaved by free NS2a (74).

### RNA Replication

Flavivirus RNA replication has been extensively reviewed by Brinton (32). RNA replication can be detected as early as 3 h postinfection and appears to occur in the perinuclear region of the infected cell in association with smooth membranes (265). Membrane-containing replication complexes, which incorporate radiolabeled nucleoside triphosphate precursors, have been isolated (37). Three forms of RNA can be extracted from dengue virus-infected cells and isolated by sedimentation through sucrose gradients: RNase-resistant 20S to 22S RNA called replicative form; partially RNase-resistant, heterodisperse, 20S to 28S RNA called replicative intermediate; and RNase-sensitive 42S RNA (50, 258, 277). Replicative form can be converted to 42S RNA by heat or other denaturants, whereas replicative intermediate is converted to 42S RNA and many smaller fragments (55). These studies suggest that replicative form is a full-length, double-stranded RNA containing one positive strand completely annealed to one negative strand. Replicative intermediate is only partially double stranded due to strand displacement by elongation of nascent chains occurring during RNA synthesis. Both replicative form and replicative intermediate RNA may serve as precursors to plus-strand 42S viral RNA (50, 55).

It is not known how RNA replication is regulated, but it is possible that early and late RNA polymerase complexes have different affinities for positive- and negative-strand templates. Late replication processes favor synthesis of the infectious positive strand (258, 281). Increasing concentrations of C protein late in infection may begin the assembly of nucleocapsids, removing positive strands as a substrate for replication. It has been proposed that binding of C protein to a site at the 3′ end of positive-strand RNA prevents it from being recognized by RNA polymerase, but not by ribosomes which bind at the 5′ end (281). This would allow for its continued translation and explain the predominance of positive-strand RNA later in infection (258, 281).

### Virus Assembly and Release

Assembly of dengue viruses has the following phases: (i) assembly of nucleocapsids from C protein and RNA; (ii) "budding" of nucleocapsids through membrane containing integral E and prM proteins to acquire an envelope; (iii) exit from the cell, either as a result of the budding process or, afterwards, in exocytic vesicles, and (iv) cleavage of the prM protein, resulting in a reorganization of the virion surface and virion maturation.

In contrast to alphaviruses which acquire their envelope as nucleocapsids in the cytoplasm and bud through the plasma membrane, flaviviruses appear to mature in a different manner. With few exceptions (see below), flavivirus nucleocapsids are not seen free in the cytoplasm but as enveloped, viruslike particles associated with intracytoplasmic vacuoles and Golgi vesicles (75, 157, 169) and within the cisternae of the rough endoplasmic reticulum (115, 116, 185). The enveloped particles, which appear to be derived from intracytoplasmic membranes, are sometimes larger than mature virions and may represent precursors (157).

Flavivirus nucleocapsids assemble from C protein, two forms of which have been demonstrated in West Nile virus-infected cells (187). One form of C protein contains a hydrophobic stretch of amino acids at the carboxy terminus which may anchor it to the membrane of the rough endoplasmic reticulum and is removed by proteolytic cleavage during the maturation of virions. These data led to the speculation that membrane-bound core protein assembles into nucleocapsids which then simultaneously bud so that free, unenveloped nucleocapsids are not seen. Here it should be mentioned that some apparently contradictory evidence exists. Ota shows electron micrographs interpreted as showing nucleocapsids in the process of budding in Japanese encephalitis virus-infected porcine kidney cells (192). Hase et al. reported that the PR 159 strain of dengue-2 virus matures in mosquito cells by budding at both intracytoplasmic and plasma membranes (114). However, these investigators (114, 115) do not see budding in Japanese encephalitis or dengue-2 (New Guinea C strain) virus-infected mosquito cells. Therefore, morphogenetic pathways may differ depending on the host cell or virus strain. Regardless of how envelopment occurs, its source appears to be intracytoplasmic rather than at the plasma membrane. The lipid composition of the virion envelope more closely resembles that of cytoplasmic membrane (214).

Release of virus from the infected cell presumably occurs via secretory exocytosis as virus-containing secretory vesicles fuse with the plasma membrane (115, 157). Released virus contains little, if any, prM; therefore, cleavage of prM must occur before or during exit from the cell. Cleavage of prM is accompanied by reorganization of the virion envelope from one containing prM-E heterodimers to one containing E-protein trimers (199, 276). Immature, prM-containing flavivirions are about 60-fold less infectious than mature virus (276). prM may maintain the virion in a highly stable but relatively inert state. The final cleavage step makes the virus competent for infection but more labile.

## BIOLOGICAL CHARACTERISTICS

### Infection in Experimental Animals and Host Range

Humans, lower primates, and mosquitoes represent the only natural hosts for dengue virus infections. The first attempts to infect nonhuman hosts were made by Simmons et al. (251), who demonstrated that the virus can be transmitted from monkey to monkey and from monkey to humans. Several species of lower primates (chimpanzees, rhesus, gibbons, and macaques) develop viremias of a magnitude sufficient to infect mosquitoes and mount an immune response without any detectable clinical signs after infection (106, 143, 207, 233, 284). Although lower primates do not manifest clinically apparent disease, they have often been used as models for studying the immune response to flavivirus infections and as subjects of test vaccines since the

onset of viremia in these animals is similar to that of humans (3–6, 142, 144). However, viremias in lower primates generally last only 1 or 2 days and rarely reach maximum titers of $10^6$ 50% mosquito infectious doses (106, 207, 233, 284). Viremias in human hosts last 2 to 12 days and reach as high as $10^8$ 50% mosquito infectious doses (86, 88, 89).

The only natural mosquito hosts for the dengue viruses are members of the genus *Aedes* (9, 210, 250, 251). In spite of several reports in the literature which incriminate *Culex* species (48, 78, 109, 294), members of this genus are known to resist dengue virus infections (209, 250, 251). *A. aegypti* and *A. albopictus* have long been recognized hosts. Other species of the widely distributed subgenus *Stegomyia* which can transmit the dengue viruses include *A. scutellaris*, *A. africanus*, and *A. leuteocephalus*. *A. niveus* (subgenus *Finlaya*), and *A. taylori* or *A. furcifer* (subgenus *Diceromyia*) appear to be part of the forest maintenance cycle in Asia and Africa (61, 84; Rudnick, Proc. Int. Conf. Dengue/Dengue Haemorrhagic Fever, 1984, Kuala Lumpur, p. 7–10). Dengue viruses have been propagated in *A.* (*Gymnometopa*) *mediovittatus* and *A.* (*Protomacleaya*) *triseriatus*. Transovarial transmission of dengue viruses, which assists in the maintenance of the virus reservoir in nature, has been demonstrated in several studies (135, 206, 211, 268).

There have been many attempts to adapt the dengue viruses to other nonprimate animal models. Shortt et al. (249) reported the growth of dengue virus strains on the chorioallantoic membrane of chickens, but sustained passage (over 90 passages) of the virus in chicken embryos was not achieved until 1950 by Schlesinger (236–238). A comprehensive search for alternative animal models was performed by Sabin and his co-workers from 1944–1945 (223). Infant mice and hamsters, newborn and adult guinea pigs, cotton rats, rabbits, and rhesus monkeys were inoculated intracerebrally, intra-abdominally, or both. Monkey sera collected 6 days after infection were the only samples which, after inoculation into human volunteers, produced typical dengue fever symptoms. Virus replication was demonstrated by passage of recipient sera into other volunteers, who developed the same disease symptoms (223, 228).

The development of a mouse infection model ushered in the modern era for study of the dengue viruses (124, 136, 173, 223, 227, 228). Dengue viruses may infect mice by a number of routes, but the intracranial route is the most sensitive, especially in 1- or 2-day-old suckling mice (173, 223, 238). Suckling mice challenged intracranially with well-adapted virus usually die of encephalitis after 1 week, dependent on the initial virus dose. Since dengue viruses are not usually neurotropic, the adaptation process usually requires several serial blind passages. Schlesinger and Frankel (239), and later Cole and Wiseman (59, 60), showed that the 50% lethal dose for both suckling and adult mice increased after repeated intracranial passage of dengue viruses. However, some strains of virus resist isolation with this method (79, 215, 256). Peak virus titers in the range $10^8$ to $10^9$ PFU/g of brain tissue are not uncommon. Mice generally become less susceptible to dengue virus encephalitis as they become older (173, 239).

Factors other than age of the mice have also been found to influence dengue virus pathogenesis. Dengue virus strains which differ in their pathogenicity for mice have been identified (59, 60, 79, 215, 256). Also, different strains of mice have different degrees of susceptibility to dengue viruses (32, 223, 228, 238). Sabin elegantly showed that the resistance of Princeton Rockefeller Institute mice to flavivirus infection, as reflected in a depression of viral multiplication, is controlled by a single, dominant, autosomal gene, inherited in accordance with Mendelian laws (224, 225, 238). Brinton suggested that the survival of mice resistant to flavivirus infection is associated with a functioning immune system and the increased generation of defective interfering genomes and particles (32).

### Propagation in Cell Cultures

The dengue viruses can be propagated in a wide range of host cells in culture, including those of mammalian and insect origin. Cytopathic effects as a result of virus infection range from severe to inapparent dependent on the cell line and the virus strain used. As with other flaviviruses, dengue viruses do not shut off host cell protein biosynthesis (279). Cytopathic effects are usually seen as increased cellular refractility, cell rounding, and, sometimes, cell fusion (124, 194, 273). There seems to be less cytopathic effect in mosquito cells than in mammalian cells, but this depends in large part on the virus serotype, strain, and passage history. After infection of cells in culture with dengue viruses, there is a long latent period, typically 12 to 16 h, before progeny virus can be detected in the extracellular medium (175). By using high multiplicities of infection, peak virus titers are usually obtained 3 to 6 days postinfection depending on the host-virus combination. At the peak of virus production, the yield of virus per cell at 37°C rarely exceeds 200 to 500 PFU/cell (238). While large numbers of "virionlike" particles accumulate intracellularly, 80% or more of the infectivity is found in the extracellular medium (38, 169, 238). Intracellular virus may consist of immature, less infectious virions containing prM instead of M protein in the viral envelope (238, 247, 276).

Mammalian cell lines that have been used extensively with the dengue viruses were previously reviewed by Schlesinger (238) and include the following: LLC-MK2 (monkey kidney), VERO (monkey kidney), BHK-21 (baby hamster kidney), various human cell lines, FRhL (fetal rhesus lung) (162), and more recently, PDK (primary dog kidney) cells (21, 101). In each of these cases, a period of adaptation after isolation is often required, and maximum titers rarely exceed $10^5$ to $10^6$ PFU/ml. The LLC-MK2 cell line has proven value for plaque titration assays or plaque reduction neutralization assays (220, 260). A microtitration method using BHK-21 cells has been described (180). FRhL cells are a popular substrate for the production of candidate live, attenuated viruses (72, 162). Recently, the PDK cell line has been used extensively to produce live, candidate-attenuated, vaccine strains (21, 72, 101).

Continuous mosquito cell lines have been shown to be highly susceptible to dengue virus infection. C6/36 (*A. albopictus*), AP-61 (*A. pseudoscutellaris*), and TRA-284 (*Toxorhynchites amboinensis*) cells are among the most widely used in the field for the isolation of dengue viruses (126, 148, 273). Maximum titers as high as $10^8$ to $10^9$ PFU/ml have been obtained.

Persistently infected cell cultures, which produce infectious dengue virus for long periods of time can be subcultured indefinitely, have been established in vertebrate (14, 125, 160, 241, 248) and invertebrate (149, 252) cells. Dengue viruses, which were subcultured for a year in persistently infected, nonvector mosquito *T. amboinensis* cultures, showed changes in antigenic reactivity, increased temperature sensitivity, and decreased neurovirulence in mice as compared with the original viruses used to initiate the infections (32, 149). Kurane et al. have demonstrated the

ability of dengue-2 virus to establish persistent infections of human monocytes (153). Clinical correlates to persistence or cases of latent dengue virus infections have not been reported.

### Virus Variants and Mutants

The existence of dengue virus variants has been recognized by many investigators, using serological assays. Because of the mutability of the RNA genome, variation within one serotype is common. Dengue viruses were first differentiated in the laboratory by using "neutralization indices," and some dengue viruses differed from prototype reference viruses in such a manner that they were interpreted as distinct serotypes. Hammon and Sather (110, 111) characterized two dengue virus isolates, TH-36 and TH-SMAN, which they suggested were dengue types 5 and 6, respectively. Later, using specific reference serum in sensitive plaque reduction neutralization assays, Russell and Nisalak (219) conclusively demonstrated that these Thai dengue viruses could not be distinguished from prototype dengue-1 and dengue-2 viruses. However, individual dengue virus isolates which show significant variations in the extent of plaque reduction neutralization by a single prototype reference immune serum have been found frequently (217, 221). The existence of dengue virus subtypes was especially demonstrated when reference antibodies to certain Tahitian and Caribbean dengue-3 virus isolates were shown to cross-neutralize the Southeast Asian prototype poorly (208, 218). Similarly, using a plaque reduction neutralization assay (219), Henchal et al. (123) showed that a dengue-4 virus isolated during an extensive epidemic in the Caribbean in 1981–1982 differed significantly from the prototype virus.

Interest in intratypic variation was initially fueled by observations that some epidemics occur with explosive transmission and unusually severe symptoms (12, 158, 161, 178). While virulence factors have not been identified for the dengue viruses, dengue patients can be grouped according to disease severity, using carefully defined clinical criteria (see below and reference 292). Morens and Halstead (179) reported that dengue-2 viruses isolated from patients with different levels of disease severity had identifiable antigenic differences, detected by using monoclonal antibodies prepared with dengue-4 virus antigens. However, definitive virulence markers have not been identified.

Advances in molecular biology have aided the identification of dengue virus variants. Oligonucleotide RNA fingerprinting has been shown to be an effective tool for the identification of viruses that have similar genetic characteristics (200, 270, 271, 274, 275). Virus isolates that share RNA fingerprint patterns have been organized into groups called "topotypes." Numerous genetic topotypes for all four dengue serotypes have been identified. Dengue-2 viruses have been studied most, and 14 topotypes from different geographical regions have been described (271). Comprehensive comparisons of the sequence homology of different dengue viruses have now been completed (24, 25, 49, 69, 205, 270, 271), and evolutionary rates have been estimated (205). Additional characterization with monoclonal antibodies has aided the identification of variation occurring at specific epitopes. Antigen signature analysis assays, in which the binding of various monoclonal antibodies directed against distinct epitopes on the envelope glycoprotein was evaluated, demonstrated few differences between dengue-2 viruses from the same geographical region, but revealed significant antigenic differences between isolates from different regions (176). Walker et al. (275) were the first to demonstrate that significant antigenic variation may occur within a single epidemic year in a defined geographical area. Some of these changes occurred in the nonstructural protein, NS1. However, the significance of these changes with regard to the virulence of the viruses has not yet been determined.

Blok et al. (25) performed comprehensive comparisons of eight dengue-2 virus E-gene sequences from viruses isolated from patients with different levels of disease severity. However, none of the sequence changes, identified at either the nucleotide base sequence or the amino acid sequence level, could be correlated with perceived differences in disease severity. Additional studies are required to correlate these changes with other functional aspects of the envelope protein. Recently, it has been shown by limited sequence analysis that dengue viruses that participate in sylvatic transmission cycles can be differentiated from those viruses associated with epidemic outbreaks (205).

The selection of virus variants from natural populations or by mutagenesis has been used to develop vaccine candidate strains. Temperature sensitivity and a small-plaque morphology have been phenotypic characteristics often associated with attenuated viruses. Eckels et al. (73) showed that the dengue-2 vaccine candidate, PR 159, attached less efficiently to sensitive cells at higher temperatures, suggesting that it had some alterations in the envelope glycoprotein. Late assembly or the maturation phase of the virus replication cycle was also inhibited. However, these phenotypic characteristics are not reliable markers of virus attenuation and often were not stably transferred to virus progeny (10, 172).

## CLINICAL FEATURES, PATHOGENESIS, PATHOLOGY, AND DIAGNOSIS

### Clinical Features

Probably the first accurate description of true dengue fever was made by Benjamin Rush, who described a 1780 epidemic of "bilious remitting fever" or "breakbone fever" in Philadelphia (213). The spectrum of symptoms that we define as dengue was determined after hundreds of observations made since the 1920s of natural and experimental infections (124, 223, 250, 251). After an incubation period of 3 to 15 days (usually 5 to 8 days), classical dengue begins with an abrupt onset of fever (103 to 106°F [39.4 to 41.1°C]) accompanied by frontal or retroorbital headache. Prodromal signs of headache, myalgias, chilliness, backache, and malaise have been reported, but occur infrequently. Flushing of the face and a generalized, transient, macular rash which blanches under pressure may be seen during the first 24 to 48 h of fever. During days 2 to 6 of fever pronounced anorexia, nausea and vomiting, generalized lymphadenopathy, and cutaneous hyperalgesia may develop. In typical cases, fever persists for 4 to 6 days and usually terminates with a crisis. Viremia generally coincides with fever. Defervescence is usually lytic with intense sweating. On the last day of fever or within 24 h, a secondary morbilliform or macropapular rash lasting 1 to 5 days sometimes appears. Although itching, especially of the palms and soles, is common, desquamation rarely occurs. Upon appearance of the secondary rash, a second rise in temperature may occur, resulting in a saddleback fever profile. Toward the end of the febrile period or immediately after defervescence, as the generalized rash fades, localized clusters of pinpoint hemorrhagic lesions (petechiae) may appear over the dorsum of the feet, on the legs, hands, or fingers, or occasionally on the mucous

TABLE 2. World Health Organization criteria for classification of DHF patients

| Grade of disease | Signs and symptoms |
|---|---|
| I | Fever accompanied by nonspecific constitutional symptoms with a positive tourniquet test as the only hemorrhagic manifestation |
| II | Same as grade I, except with spontaneous hemorrhagic manifestations |
| III | Circulatory failure manifested by rapid, weak pulse with narrowing of the pulse pressure (<20 mmHg) or hypotension |
| IV | Profound shock with undetectable blood pressure and pulse |

membranes of the oral cavity (227, 238). Laboratory results reveal a depressed peripheral leukocyte count with an absolute granulocytopenia and a platelet count falling to <100,000/mm$^3$. Young children may present with respiratory symptoms including cough, sore throat, and rhinitis (102). Gastrointestinal bleeding, menorrhagia, and bleeding from other organs have been described in outbreaks involving adults (145, 204, 242).

The more serious illnesses of DHF and DSS are now common elements of dengue outbreaks in certain regions. These illnesses begin with symptoms indistinguishable from those of simple dengue fever, followed 2 to 5 days later by rapid deterioration, physical collapse, and sometimes death (57, 99, 185). This second phase of the disease is coincident with a period of defervescence. Petechiae, easy bruising, bleeding at venipuncture sites, and large spontaneous ecchymoses are frequently observed. Hepatomegaly is occasionally described. The World Health Organization, using data collected primarily in Thailand, has defined strict criteria for DHF and DSS and recognizes four grades according to the severity of disease (Table 2) (292). Hemorrhage, regardless of site and severity, when not accompanied by thrombocytopenia and hypovolemia, does not satisfy the criteria for DHF. The presence of thrombocytopenia and concurrent hemoconcentration differentiates grade I and II DHF from classical DF with hemorrhagic manifestations. Shock is the single criterion for discriminating between grades I and II and grades III and IV.

Specific treatment for dengue virus-infected patients does not exist. Supportive care includes bed rest, antipyretics, and analgesics. Fluid and electrolyte replacement should be carefully managed in the DHF patient. Aspirin and other salicylates should be avoided in view of diminished platelet numbers and, possibly, function. Convalescence from severe attacks of dengue is characterized by pronounced bradycardia and marked asthenia. Psychomotor depression may be evident in some patients, consistent with the name "breakheart fever" proposed by Rush in 1790 (213).

Since 1963, investigators have recognized that DHF patients from different geographical regions or epidemics do not always present with the same clinical findings (108). Hepatomegaly common in Bangkok is not a consistent feature of the disease elsewhere. Gastrointestinal bleeding prior to the onset of shock and not coincident with hemoconcentration is common in Indonesia, but not in other areas. Some investigations in Indonesia have suggested that only 60 to 70% of the DHF cases meet World Health Organization criteria (84, 261). Dengue cases with encephalitic disease have been reported in Indonesia, Malaysia, Burma, Thailand, Dominican Republic, and Puerto Rico (reviewed in reference 84). These variations may result from differences in racial or cultural environments, adequacy of clinical evaluations, or the immunological status of the affected population or other unrecognized factors.

Risk factors for DHF or DSS are controversial and not well understood. After extensive epidemiological studies conducted in Thailand, Halstead proposed the "secondary infection" or "immune infection enhancement" hypothesis (97). Results from numerous studies over 20 years have suggested that individuals who have had a previous dengue virus infection and have circulating non-neutralizing, cross-reactive antibodies are at significant risk for developing DHF or DSS (97, 98, 104, 140, 232, 291). However, cases of DHF or DSS have been documented in patients experiencing dengue virus infections for the first time (86, 181, 244), and infants under 1 year of age are at increased risk because of the presence of maternally acquired, infection-enhancing antibodies (100, 139, 291). Epidemiological evidence suggests that only anti-dengue antibodies play a role in infection enhancement (107, 197). While the presence of flavivirus cross-reactive antibodies appeared to enhance infection by an attenuated dengue-2 virus vaccine (71, 243), only antibodies against dengue viruses contribute to the risk for severe disease. Recent evidence has also indicated that the sequence of infecting serotypes, especially a dengue-1 infection followed by a dengue-2 infection, may influence the resulting disease severity (232). That the 1981 outbreak of epidemic DHF in Cuba was caused by a dengue-2 virus supports this general hypothesis. Cuba had previously experienced a large dengue-1 epidemic in 1977 (91). Although dengue-2 has been reported to be commonly associated with DHF and DSS in Thailand, all dengue virus serotypes have the potential for causing severe hemorrhagic disease (232). Obviously, factors contributing to an increased risk for more severe disease have serious implications for the development of dengue virus vaccines.

In addition to the humoral immune response, T-cell responses may play a significant role in the immunopathogenesis of DHF and DSS. Ennis and his associates have demonstrated that CD4$^+$ T cells from dengue virus-immune humans proliferate and produce gamma interferon in response to soluble dengue virus antigens (152). Serotype-cross-reactive, CD4$^+$ CD8$^-$ cytotoxic T-lymphocyte clones, which are class II restricted and secrete gamma interferon, have also been detected (34, 154). Gamma interferon has been shown to increase infection of human monocytes by immune infection enhancement (141). Recently, these investigators demonstrated the proliferation of peripheral blood mononuclear cells from a dengue-4 immune donor in response to a live dengue virus and the generation of serotype-cross-reactive, CD8$^+$, class I-restricted, dengue virus-specific cytotoxic lymphocytes (34). These results suggest that these cytotoxic lymphocytes may mediate viral clearance and contribute to shock by lysing dengue virus-infected cells in secondary infections (34).

Further identification of factors contributing to overall risk await comprehensive studies of the virulence of different dengue virus strains, identification of significant antigenic epitopes on the surface of virions, and definition of the role of immune cells in the regulation of the immunopathology of dengue virus disease.

### Pathology

Pathological examinations of skin lesion from classical dengue fever patients have shown swelling of endothelial

cells of small vessels, perivascular edema, and infiltration of mononuclear cells (175, 223, 238). However, neither virus nor viral antigen has been detected in biopsy materials. The data suggest that the maculopapular rash of dengue fever may be caused by involvement of immune globulins or by some mechanism other than direct viral infection of the skin (66).

Extensive pathological surveys have been made with tissues from fatal cases of DHF (8, 17–20). Gross pathological findings in cases of DHF or DSS include hemorrhages in the skin, subcutaneous tissues, gastrointestinal tract, and heart (18). Significant histopathological changes are found principally in three major organ systems: the liver, the reticuloendothelial system, and the vascular system (18). Hemorrhage, dilatation and congestion of vessels, and edema of arterial walls were common findings. Hemorrhagic manifestations in other organs and fluid accumulation in body cavities may be evident. Proliferation of young lymphocytes, plasma cells, and sinusoidal lining cells in the spleen and lymph nodes and accelerated phagocytic activity of lymphocytes have been reported. Degeneration of liver and Kupffer cells and the formation of Councilman bodies, similar to those of yellow fever, may be evident (20, 124). Hypoplasia of the bone marrow, acute atrophy and wasting of the thymus, and atrophy and depletion of cells in the periarterial lymphatic sheaths of the spleen and the paracortical areas of the lymph nodes are consistent findings. Since many of the tissues affected are thymus-dependent areas of the spleen and lymph nodes, and the thymus itself, it has been suggested that immunodepression may be an integral part of the pathophysiology of DHF (8). Consistent with these findings is the discovery that dengue antigens were localized in monocytelike cells associated with glomerular basement membranes and in mononuclear cells closely infiltrated around blood vessel walls in dermal papillae (26, 27). Electron microscopy has shown that endothelial cells in skin biopsies from DHF patients have increased numbers of vacuoles and pinocytotic vesicles; these are important in the transport of plasma fluids from the capillary to the pericapillary space (229).

Identification of the primary target cells of dengue virus replication in the human patient has proven to be extremely difficult. Dengue antigens have been visualized by fluorescent microscopy on the surface of human B lymphocytes during the acute stages of DHF or DSS. However, this observation could come about by the attachment of circulating immune complexes to Fc receptors. Human B lymphoblastoid cells and mitogen-treated lymphocytes can support dengue virus replication in vitro, but whether the same is true in vivo has not been demonstrated. Recently, it has been shown that dengue viruses can infect human hematopoietic cells and alter their proliferative capacity (184). Evidence that mononuclear cells, macrophages, or monocytes are targets for dengue virus infection is derived from studies showing that infiltrating mononuclear cells in affected tissues contain viral antigens (26, 27, 229), that dengue viruses can be regularly isolated from peripheral blood leukocyte fractions (245), and that monocyte cultures can regularly be infected with dengue viruses in the presence or absence of cross-reactive antibodies (65, 103).

### Laboratory Diagnosis

Laboratory diagnosis of dengue virus infections currently depends on isolation of infectious virus or identification of virus-specific antibodies. Dengue viruses have been isolated from patient specimens by using suckling mice (136, 173, 222, 223, 227, 228, 238), cultured cells (85, 267), or live mosquitoes (87, 147). By far the most sensitive isolation method is the intrathoracic inoculation of *T. splendens* or *A. aegypti* mosquitoes (146, 147). With this method, mosquitoes are inoculated with virus-containing material and incubated for 14 days at 30°C. Dengue antigens are detected in infected mosquitoes with reference antibodies in complement fixation or immunofluorescence assays (146, 147). The development of continuous cell lines of mosquito origin has simplified dengue virus isolations (85, 126, 151, 267). The cloned line of Singh's *A. albopictus*, C6/36, was developed especially for its ability to grow dengue and other arboviruses to high titer (126). Tesh compared titrations of low-passage dengue viruses, using C6/36 cells AP-61 (a *Toxorhynchites* cell line), and mosquitoes (267). He reported that, although mosquito cell lines are less sensitive than live mosquitoes, they make it possible to identify dengue viruses in 6 days. Virus has been isolated from 20 to 65% of serologically confirmed dengue patients by these methods. An improved mosquito cell line, TRA-284-SF, has been shown to be a more sensitive medium for virus isolations than similar cell lines (151). The virus isolation rate (the number of cultures positive per samples tested) in the TRA-284-SF (36%) cells was significantly higher ($P < 0.05$) than in C6/36 (28%) or AP-61 (33%) cells (151). By using antibody-mediated, infection enhancement of dengue viruses in a mouse macrophage cell line, virus isolations were obtained from more than 80% of clinically and serologically confirmed dengue patients (39). An improved mosquito inoculation system, using mosquito larvae, which identified dengue viruses in 4 to 6 days, has been described (156, 193).

Serotype identification of virus isolates has been performed with polyclonal reference antibody preparations in carefully standardized complement fixation assays (147) or in plaque reduction neutralization (PRNT) assays (219). Although laborious and time-consuming, the PRNT assay has long been considered the standard for virus typing. Virus identification was simplified after serotype-specific monoclonal antibodies were prepared by Henchal et al. (121). These specific reagents, available from the American Type Culture Collection, Rockville, Md., and distributed by the Centers for Disease Control, Fort Collins, Colo., rapidly identified virus serotypes even when significant changes at neutralization determinants made serotyping by PRNT difficult (123). Originally used in indirect immunofluorescent assays, they have now been used more widely in enzyme-linked immunosorbent assay formats (150).

Serological diagnosis of the dengue viruses is complicated by the existence of cross-reactive antigenic determinants shared by all four dengue virus serotypes and members of the flavivirus family. Even after a single exposure to a related flavivirus, convalescent patient sera usually contain detectable cross-reactive antibodies. The most widely used diagnostic test has been the hemagglutination inhibition assay (52). This assay depends on the inhibition of virus antigen-dependent hemagglutination by antiviral antibodies. A fourfold or greater increase in antibody titer is diagnostic for a recent flavivirus infection, but not for any specific agent. Because of extensive experience with the assay, standards that differentiate between primary (first exposure) or secondary dengue infections have been established. Primary dengue patients usually have convalescent hemagglutination inhibition antibody titers of $\leq$1:1,280 (84).

The PRNT assay is a sensitive and specific serological tool for detection of anti-dengue antibodies (220). While other

types of anti-dengue antibodies wane considerably with time, neutralizing antibodies have been detected in patients more than 60 years after their last identified exposure to dengue viruses (104). Since antibodies produced after primary dengue virus infections are commonly monospecific, PRNT assays have been used successfully to diagnose dengue virus infections. It has been suggested that the neutralization titer against the serotype responsible for the primary exposure is anamnestically greater than the neutralization titer against the virus responsible for the second illness (105, 182, 232, 240). These data suggest that the PRNT assay is a useful tool for epidemiological studies.

Recently, attempts have been made to adapt the enzyme-linked immunosorbent assay to detect dengue virus-specific IgM antibodies (35, 51, 128, 231). Extensive field testing of the assay suggests that the assay is 10 to 78% sensitive when admission sera are tested and >97% sensitive when convalescent sera are tested. The test has been used successfully by several investigators to distinguish between serum titers to Japanese encephalitis virus and dengue viruses in those geographical areas where these viruses cocirculate. However, the detection of cross-reactive IgM after dengue infection has been reported (35, 128).

The immunological parameters of flavivirus disease syndromes were previously reviewed by Halstead (98). Hemagglutination inhibition antibody is usually detected in primary dengue fever cases by day 5 to 6 after the onset of fever. In secondary cases, rises in HI titer may be detected two-to-three days after onset (98). By day 5 after onset, both primary and secondary patients have detectable IgM antibody, which may persist for as late as 90 days, a characteristic shared with other flaviviruses (84, 174). Antiviral IgG usually appears by day 14 in primary dengue cases and by day 2 in secondary cases. IgG antibody has been detected as late as 60 years after exposure to the virus (104). In light of the remarkably durable IgM response of some flaviviruses (84, 174), Gubler cautions that the presence of dengue-reactive IgM does not necessarily indicate a recent dengue virus infection (84).

Alternative methods of dengue virus detection have been reported. Among these are nucleic acid hybridization (122, 134, 189) and multi-epitope site competitive binding with monoclonal antibodies (36, 177). The polymerase chain reaction (230), which has the potential for sensitive detection of virus nucleic acid, is being evaluated for the rapid diagnosis of dengue virus infections. Because of the current complexity and cost of some of these assays, it is unlikely that their use will become widespread.

## PREVENTION AND CONTROL

Dengue virus transmission can be successfully prevented by the control of vector populations. It was specifically by this means that dengue and other arthropod-borne viruses were controlled in the Americas in the 1920s. Recent reintroductions of the vector mosquitoes and outbreaks of dengue and other flavivirus infections can be directly linked to decisions made more than two decades ago to merge mosquito control programs and de-emphasize *A. aegypti* eradication (83). Political and economic destabilization of the region have placed many of the remaining programs in limbo. As a result, countries in the Americas now have to bear the threat of increased transmission of arthropod-borne viruses. In many regions, vector control is deemed impossible given the intensity of the "jungle cycle" and the numbers of mosquito habitats available. Therefore, interest in the development of effective dengue virus vaccines has been almost continuous since the late 1940s.

Attempts to induce protection against experimentally transmitted dengue by inoculating human volunteers with unmodified virus by abnormal infection routes (i.e., nasal instillation, dermal scarification, and instillation of conjunctival sacs) were made by Sabin (223). These efforts were unsuccessful. In 1944–1945, Sabin and Schlesinger (228) prepared and tested the first live, attenuated (by serial mouse passage), dengue-1 virus vaccine. Vaccinated individuals experienced either no symptoms or a low-grade fever, with or without headache and malaise, lasting 24 h or less. All vaccinees developed maculopapular rashes and petechiae. All were fully immune to challenge with unmodified, homotypic dengue virus. Similar tests were performed independently on a limited scale in Japan during the 1940s (124). After strains of dengue-2 virus were successfully propagated to high titer in suckling mice, tests in humans with both the New Guinea C (226) and New Guinea B (240) strains indicated that the modified strains behaved like dengue-1 (227), Also, Schlesinger tested a dengue-1 virus vaccine propagated in embryonated eggs, using the successful 17D yellow fever chicken embryo-derived vaccine as a model (236). After 38 passages in chicken embryos, the virus retained the attenuated character of its mouse-adapted progenitor in that two nephrotic children, inoculated with it as a possible therapeutic measure, suffered no or mild (low-grade fever for 2 days and rash) effects but produced neutralizing antibody (236, 238). A live, attenuated dengue-1 vaccine derived from the 18th mouse passage (228) and then subjected to 13 to 14 additional passages by Wisseman et al. was tested in over 1,100 individuals during a dengue-3 epidemic in Puerto Rico (288, 290). This vaccine was shown to be well tolerated, did not cause systemic reactions (a mild rash appeared in some individuals), induced type-specific neutralizing antibodies, and reduced the incidence of dengue to about one-half that in a placebo group (16, 288, 290). Additional work with these and similar vaccines has been abandoned to avoid possible complications associated with mouse-brain-derived vaccines.

Attempts to protect humans or primates by hyperimmunization with related flaviviruses have generally proven unsuccessful (196, 289). Although vaccinated subjects produced high-titered cross-neutralizing antibodies after serial or simultaneous infections with related flaviviruses, none were protected against heterologous challenge viruses. Some transient protection, up to 6 months after a dengue virus infection, has been demonstrated; however, it has been convincingly established that effective, long-lasting protection is produced only in response to serotype-specific immunological stimuli (223). Serial infection with three different dengue virus serotypes has been reported (182). There is no evidence that homotypic reinfection occurs, and epidemiological evidence suggests that clinical illness in humans rarely follows challenge with a third or fourth virus serotype (107). Future dengue virus vaccines will undoubtedly contain protective epitopes from multiple serotypes.

Two dengue virus type 2 vaccines produced in cell culture have been successfully tested for safety and immunogenicity (11, 21). The dengue-2 PR-159/S-1 vaccine, which was derived by the selection of a stable virus clone after sequential passage in primary green monkey kidney cells, has been given to more than 145 volunteers and is a safe and moderately attenuated product (11). The vaccine strain used in this study had phenotypic markers of temperature sensitivity, produced small plaques, and caused a reduced viremia in

monkeys (70, 112). Efficacy trials have not been performed with this product. Dengue-1, dengue-3, and dengue-4 vaccine candidates with similar properties have also been prepared and evaluated in volunteers, but with unsatisfactory results (72, 127, 172). In the absence of a satisfactory animal model, which mimics human clinical signs, markers of attenuation for predicting the possible success of a dengue vaccine have not yet been established (10). Recently, dengue viruses attenuated by serial passage through PDK cells have been demonstrated to be promising vaccine candidates (6, 21, 72). Vaccine strains produced in this manner are now available for the dengue-1, dengue-2, and dengue-4 viruses. These vaccines strains are being extensively tested for safety and efficacy.

Recently, there has been considerable interest in the development of subunit vaccine preparations. Schlesinger et al. (235) have shown that mice can be protected from lethal challenge by immunization with purified dengue-2 NS1. Extensive work has now been completed which demonstrates that both dengue-4 structural and nonstructural protein antigens, expressed with the vaccinia virus or baculovirus virus vectors, can protect mice against lethal challenge with dengue virus (29, 30, 296, 298). However, trials with these preparations in the primate infection model have had only limited success. Attempts are being made to identify epitopes important for protection. Such epitopes have now been identified on the prM, E, NS1, and NS3 proteins by using monoclonal antibodies in passive protection assays (119, 132, 133, 266). Synthetic peptides composed of linear amino acid sequences have been used to identify antigenically reactive regions of the E protein (1, 129; Roehrig, et al., W.H.O. Programme for Vaccine Development, Dengue Steering Committee Meeting, 1989). Extensive work is still needed to define elements important for protective immune responses.

## CONCLUSIONS

During the past 10 years, we have seen numerous advances in our understanding of the dengue virus genome structure, the identity of viral proteins, and the composition of viral antigens. However, the incidence of serious dengue illness has increased, especially in the Western hemisphere. After more than 30 years of study, the immunopathogenesis of dengue and its complications remain unclear. The next decade may bring the development of improved safe and effective dengue virus vaccines, a goal since World War II, but prevention and containment of dengue outbreaks will require widespread vector control and aggressive epidemiological surveillance.

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