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# FLAVIVIRUSES

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## INTRODUCTION

The Flaviviridae are the most medically important group of arthropod-borne viruses. Previously classified as members of the Togaviridae, in 1984 flaviviruses were put into the new virus family Flaviviridae (1). This reclassification was the result of recent research that clearly demonstrated the significant differences in genome structure, gene order, replication strategy and virus morphogenesis between the flaviviruses and other togaviruses. Because of inherent, as well as technical difficulties, the analysis of flaviviruses at the molecular level has been particularly slow. However, the recent successful cloning and sequencing of several flavivirus genomes (2, 3, 4) has dramatically increased our knowledge of the genetic organization of the flaviviruses. Current research efforts are focused on identifying the proteases which are responsible for processing the viral precursor polypeptide, characterizing the functions of the 7 viral non-structural proteins, delineating the nucleic acid signals which regulate viral RNA transcription, determining whether host cell proteins function as components of the viral replication complexes, and analyzing viral mutations which affect virulence. Although it is difficult to predict the clinical applications of the information to be gained from such studies, a novel approach for future anti-flavivirus drug therapy may result from a better understanding of the molecular details of the virus-host interaction.

To date, there are 64 recognized flaviviruses, of which yellow fever (YF) virus is the prototype (Table 1). The family name is derived from the Latin word flavus, which means "yellow". Flaviviruses are characterized by the presence of a serologically cross-reactive group-specific epitope present on the virion envelope glycoprotein. The flaviviruses are further subdivided on the basis of serological cross reactivity at subgroup-specific epitopes and by the type of arthropod vector (mosquitoes or ticks)

D.H. Gilden and H.L. Lipton (eds.), *Clinical and Molecular Aspects of Neurotropic Virus Infection.* 

Table 1. Antigenic and Vector Classification of Flaviviruses[a]

| Tick-borne | Antigenic group[b] | Mosquito-borne | Antigenic group[b] | Vector unknown | Antigenic group[b] |
|---|---|---|---|---|---|
| Kadam | I | Alfuy | III | Carey Island | I |
| Karshi | I | Bussuquara | III | Negishi | I |
| Kyasanur Forest disease | I | Japanese encephalitis (JE) | | Phnom Penh bat | I |
| Langat | I | Kunjin | III | Cowbone Ridge | II |
| Louping ill | I | Murray Valley encephalitis (MVE) | | Jutiapa | II |
| Omsk hemorrhagic fever | | Saint Louis encephalitis (SLE) | III | Moc | II |
| Powassan | I | Stratford | III | Sal Vieja | II |
| Royal Farm | I | Usutu | III | Erlita | II |
| Saumarez Reef | I | West Nile (WN) | III | Koutango | III |
| Tick-borne encephalitis(TBE) | I | Bagaza | IV | Israel turkey meningoencephalitis | VII |
| Russian spring-summer | | Kokobera | IV | Apoi | VII |
| Tyuleniy | U | Ntaya | IV | Bukalasae bat | VII |
| | | Tembusu | IV | Dakar bat | VII |
| | | Yokose | IV | Entebbe bat | VII |
| | | Banzi | V | Rio Bravo | VII |
| | | Bouboui | V | Saboya | VII |
| | | Edge hill | V | Aroa | U |
| | | Uganda S | V | Cacipacore | U |
| | | Dengue-1 | VI | Gadgets Gully | U |
| | | Dengue-2 | VI | Montana myositis leukoencephalitis | U |
| | | Dengue-3 | VI | Rocio | U |
| | | Dengue-4 | VI | Sokuluk | U |
| | | Ilheus | U | | |
| | | Jugra | U | | |
| | | Naranjal | U | | |
| | | Sepik | U | | |
| | | Sponweni | U | | |
| | | Yellow fever (YF) | U | | |
| | | Wesselsbron | U | | |
| | | Zika | U | | |

[a] After Brinton, 1986.
[b] Antigenic groups are designated I, II, III, IV, V, VI, VII, and U (unknown).

that transmits the virus during its natural cycle (Table 1). Many of the flaviviruses are human pathogens that regularly cause significant morbidity and mortality (5,6). Yellow fever, Kyasanur Forest disease, and Omsk hemorrhagic fever viruses frequently cause hemorrhagic febrile illness (Monath, 1986). While the majority of dengue virus infections cause a non-hemorrhagic febrile illness, the hemorrhagic complications associated with some dengue infections are thought to occur by a pathological immune enhancement mechanism which is the consequence of a secondary infection with a different dengue serotype virus (5,8). Other flaviviruses such as West Nile virus frequently cause human non-hemorrhagic febrile illness, while Banzi, Bouboui, Bussuquara, Ilheus, Sepik, Spondweni, Wesselsbron, Zika, Rio Bravo and Tyuleiny viruses are occasionally associated with human febrile illness. Seventeen of the flaviviruses, as indicated in Table 2, cause human encephalitis, with Japanese encephalitis and tick-borne encephalitis viruses responsible for most of the reported cases. Although only 18 of the flaviviruses are associated with domestic animal and/or human encephalitis during natural transmission cycles, all flaviviruses are neurotropic under experimental conditions of intracerebral inoculation of laboratory rodents. Also, the brain and ganglia of arthropod vectors are often major sites of flavivirus replication. The universal neurotropic potential of flaviviruses may reflect conservation of viral envelope protein epitopes which can interact with CNS cell surface receptors. However, invasion of the host's nervous system by flaviviruses seems unnecessary and even counterproductive for virus transmission to a new host under natural conditions. It is possible that neurological infection of vectors and reservoir host animals could cause behavioral changes that could alter virus transmission patterns (7,9).

Although the pathogenesis of hemorrhagic fevers associated with some flavivirus infections is interesting, only aspects of flavivirus encephalitis will be discussed in this chapter. For information on other aspects of flavivirus pathogenesis, the reader is referred to previous reviews (5,6,7).

Both host and viral factors influence the type of infection and disease produced by a particular flavivirus. Natural flavivirus isolates display heterogeneity in phenotype as assessed by neurovirulence, plaque size, and temperature sensitivity (10) and in

their RNase-$T_1$ resistant RNA oligonucleotide fingerprint patterns (11). Under conditions of natural transmission, infection occurs by a peripheral route usually via the bite of an arthropod. Initial virus replication takes place in the tissue adjacent to the bite site as well as in regional lymph nodes. Virus is transported by the lymphatic system to the thoracic duct and then enters the blood (12). This primary viremia leads to infection of additional tissues such as connective tissue, striated and smooth muscle, vascular endothelium, lymphoreticular cells, and endocrine and exocrine glands (6,13,14). Virus is cleared from the blood by macrophages and viremia terminates with the appearance of humoral antibodies (15).

Table 2. Flaviviruses associated with human encephalitis[a]

| Total cases reported to 1986 | Flavivirus |
|---|---|
| $> 10^5$ | Japanese encephalitis |
| $10^4$ to $10^5$ | tick-borne encephalitis |
| $10^2$ to $10^4$ | St. Louis encephalitis, Rocio, Murray Valley encephalitis, Kyasanur Forest disease |
| $< 10^2$ | West Nile, Powassan, Omsk hemorrhagic fever, Kunjin, louping ill, Ilheus, Apoi, Negishi, dengue, Rio Bravo, (? Modoc) |

[a] Modified from Monath (7).

The route by which flaviviruses invade the central nervous system following a peripheral infection has not been unequivocally established. One study has indicated that flaviviruses may gain access to the CNS by an olfactory pathway (16). Once virus has entered the central nervous system, neurons and glial cells become infected. In man and monkeys, the most susceptible neuronal centers are located in the thalamus, substantia nigra, and cerebellum (17,18), while in mice the hippocampus is the most suscep-

tible region. Although infected neurons and glial cells are killed directly as a result of virus replication, the elicited virus-specific inflammatory response can enhance the severity of the CNS lesions and accelerate death.

## FLAVIVIRUSES ASSOCIATED WITH HUMAN ENCEPHALITIS

As indicated in Table 2, Japanese encephalitis virus and tick-borne encephalitis virus account for the largest number of reported cases of human encephalitis. The clinical aspects of disease caused by these two flaviviruses will be discussed in detail. Only unique aspects of the pathology associated with the remaining 15 flaviviruses associated with human encephalitis will be included.

### Japanese encephalitis virus (JEV).

Japanese encephalitis is the most medically important of the arbovirus encephalitides, because of the high level of human morbidity and mortality associated with it. Epidemics of Japanese encephalitis occur periodically in the temperate regions of Asia and in the northern part of tropical Southeastern Asia. Pigs and birds are the principal hosts. The mosquito vector responsible for transmission between these vertebrates and from them to man is Culex tritaeniorhynchus (6,19). Transplacental infection can occur in humans and results in abortion. In documented cases, virus has been isolated from the fetuses (20).

Formalin-inactivated JEV vaccines have been developed. However, isolated strains of JEV have been found to represent at least three distinct antigenic types. The vaccines produced in Japan are all derived from the prototype Nakayama strain and their efficacy against antigenically different wild strains is probably low (6).

Clinical CNS disease caused by JEV infection in humans can manifest as an acute syndrome characterized by fever (oral temperature 37.8° C) and headache, aseptic meningitis, or encephalitis (6,21-23). The incubation period ranges from 5 to 15 days. Patients with headache and acute febrile illness may also experience nausea or vomiting, but have no symptoms of meningeal irritation or neurological dysfunction. Acute febrile illness accompanied by aseptic meningitis is characterized by a stiff neck with or without positive Kernig's or Brudzinski's signs, but no symptoms of neurological dysfunction.

Cerebral spinal fluid (CSF) pressure in patients with CNS involvement may be elevated. The peripheral white blood cell number is mildly elevated and 50 to 90% of the white cells are neutrophils. CSF contains 10 to 500 white blood cells per $mm^3$ which are predominantly lymphocytes. The protein concentration in CSF can be slightly elevated. Decreased electrical activity, slowing and dysrythmia are observed on electroencephalograms.

The onset of encephalitis is rapid and begins with a 2 to 4 day prodromal phase characterized by fever, chills, headache, anorexia, nausea and vomiting, dizziness, and drowsiness. Children may also develop abdominal pain and diarrhea. Photophobia, nuchal rigidity and one or more signs of an altered state of consciousness or of neurological dysfunction follow the initial symptoms. Altered consciousness can be manifested as hyperexcitability, confusion, disorientation, delirium, lethargy, stupor, or coma. Objective neurological signs include convulsion, cranial nerve palsy, tremor, muscular rigidity, mask-like facies, tremulous eye movements, involuntary body movements, abnormal reflexes, generalized and/or localized paresis and incoordination. Sensory functions are rarely affected. Occasionally, spinal cord involvement is observed. Paralysis of the legs is less common than paralysis of the arms. Convulsions are rare in adults, but common in children. Patients sometimes display severe hypothermia. Death can occur as rapidly as the fifth day after disease onset and is a more likely outcome in patients displaying respiratory dysfunction due to cardio-pulmonary complications, frequent or prolonged seizures, prolonged fever, positive Babinski's signs, or albuminuria (6,21).

Pathological changes have been found in both extraneural and neural tissues as a consequence of JEV infection. Hyperplasia of lymph node germinal centers, enlargement of spleen malpighian bodies, swelling of Kupffer cells, interstitial myocarditis, pulmonary interalveolitis, and focal hemorrhages in the kidneys have been observed. Neuronal degeneration, neuronophagia, glial nodule formation and perivascular inflammation are evident in the gray matter of the diencephalon, mesencephalon, and brain stem. Cerebellar Purkinje cells may be preferentially destroyed (6,22,24). Seventy to 80% of those who survive JE encephalitis, have neurologic sequelae, including parkinsonism, convulsive disorders, impaired intellect, motor disturbances, and emotional disorders. Sequelae are particularly severe in children (22).

Virus-specific IgM antibodies in serum or CSF can be measured by RIA or ELISA assays. IgM antibodies appear during the first week of infection and usually disappear by 3 months (25). Detection of IgM antibodies allows a rapid and accurate diagnosis even in patients who have had a prior heterologous flavivirus infection (26).

Tick-borne encephalitis (TBEV).

There are two distinct subtypes of TBEV, Russian-spring-summer encephalitis virus and Central European encephalitis virus. These two subtypes can be distinguished from each other antigenically and molecularly (27, 28). Within each subgroup, isolates obtained in different years and geographic locations show a surprising degree of antigenic and genetic homogeneity (28). In contrast, significant variation has been found among different isolates of the various mosquito-borne flaviviruses collected from close geographic regions at the same time (29,30). TBEV causes epidemics in Europe and the Soviet Union. The distribution of disease corresponds to that of the tick vectors, Ixodes ricinus for Central European encephalitis and Ixodes persulcatus for Russian-spring-summer encephalitis (6,31). In geographic areas in which Ixodes ticks are not present, Dermacentor and Haemaphysalis ticks may transmit TBEV (6). TBEV transmission has also been associated with consumption of unpasteurized milk from sheep or goats (32).

In general, human disease caused by TBEV is similar to that described for JEV. The incubation period is 7 to 14 days. Clinically, Russian spring-summer encephalitis differs somewhat from Central European encephalitis. Disease associated with Russian spring-summer encephalitis infections may mimic poliomyelitis. Disease onset is often gradual rather than acute. The prodromal phase characterized by fever, headache, anorexia, nausea, vomiting and photophobia is followed by stiff neck, visual disturbances, and neurological dysfunction. The extent of dysfunction varies and can include paresis, paralysis, sensory loss, and convulsions. The case-fatality rate is 20%, with death occurring within the first week of onset (6,33). Thirty to 60% of survivors experience sequelae usually consisting of flaccid paralysis of the shoulders and arms.

Disease caused by Central European encephalitis is usually milder. A diphasic course is observed in about 50% of the cases. An initial flu-like illness is followed by a 1 to 3 day recovery period. The onset of the

second phase is abrupt and varies from a benign meningitis to a severe encephalitis characterized by tremor, dizziness, sensory alterations, diplopia, and paresis. The case-fatality is 1 to 5%. Mild, objective neuropsychiatric sequelae occur in 20% of survivors (6,34).

The pathological changes associated with TBEV infections are similar to those previously described for JEV infections. Anterior horn cells are particularly susceptible to infection with Russian spring-summer encephalitis virus (6).

TBEV may induce persistent infections in experimental animals (35-37) as well as in humans. Chronic progressive human encephalitis has been attributed to TBEV based on serological evidence (38), and TBEV has been isolated from the CSF of an amyotrophic lateral sclerosis patient (39).

St. Louis encephalitis virus (SLEV).

Since 1933 there have been regular documented outbreaks of SLEV in the western United States, Texas, Florida and the Ohio-Mississippi valley. Epidemics occur at approximately 10-year intervals and have involved as many as 1800 cases. Infection can lead to a febrile headache syndrome, aseptic meningitis, or encephalitis. The severity of illness increases with age, and while the case-fatality rate is only 2% in young adults it is more than 22% in persons over 60 years of age. Disease onset occurs between 4 and 21 days after infection and is characterized with malaise, fever, headache, drowsiness, anorexia, nausea, myalgia, sore throat and cough. Urinary tract symptoms (frequency, urgency, dysuria) occur in about 25% of patients. Neurological signs may develop within 1 to 4 days after disease onset. The disease is similar to that described previously for JEV. About 10% of patients manifest convulsions. If death occurs, it is within the first two weeks of disease onset. In 30 to 50% of survivors, convalescence is prolonged (up to 3 years) and characterized by aesthenia, irritability, tremors, sleeplessness, depression, memory loss, and headaches. About 20% of patients display sequelae lasting longer than 3 years which are characterized by sensory and motor impairment, speech disturbances, tremors, and psychoneurotic complaints.

Rocio virus.

The clinical features of human encephalitis caused by Rocio virus infection are very similar to those described for JEV. Epidemics occur on the south coast of Sao Paulo State, Brazil (40,41). Aedes serratus and

Aedes scapularis mosquitoes are thought to be the major vectors for transmission of this virus (6).

The case-fatality rate among hospitalized patients is about 4%. Approximately 20% of survivors develop sequelae characterized by persistent cerebellar, motor, and neuropsychiatric signs (6).

Murray Valley encephalitis virus (MVEV).

Epidemics of Murray Valley encephalitis occur primarily in the Murray Valley region of Australia. However, cases have also been reported in other parts of Australia and New Guinea (42, 43). Culex annulirostris mosquitoes are the vectors of virus transmission.

Although humans can develop inapparent infections, mild febrile illness without neurological signs has not been associated with MVEV (42). Disease onset is characterized by fever, headache, myalgia, malaise, anorexia, and nausea. Nuchal rigidity and neurologic signs appear within 2 to 5 days. Patients with mild disease display an altered level of consciousness and variable neurologic abnormalities, but do not develop coma or respiratory depression. Severe cases are characterized by coma, paresis, respiratory impairment, and paralysis. Fatal cases are associated with spastic quadriplegia and progressive CNS damage. Neurologic sequelae, such as paraplegia, impaired gait and motor function, and intellectual impairment, occur in 40% of patients recovering from mild cases and all of those recovering from severe cases (44).

Kyasanur Forest disease virus.

Although Kyasanur Forest disease virus is usually associated with human hemorrhagic fever, in some patients a biphasic disease course is observed. The initial febrile disease is characterized by parenchymal degeneration of the liver and kidneys, hemorrhagic pneumonitis, reticuloendothelial proliferation in spleen and liver, and erythrophagocytosis. This phase lasts 6 to 11 days. After an afebrile period of 9 to 21 days, some patients again develop fever and show signs of meningioencephalitis. The clinical disease in these patients is similar to that described for TBEV. The case-fatality rate is 3 to 5%. No sequelae are associated with recovery (6,45).

So far, infections with Kyasanur Forest disease virus only occur in Mysore State, India, but the area in which the virus is endemic is expanding. The natural virus transmission cycle involves ixodid tick vec-

tors and wild rodents or insectivore reservoir hosts.

Other flaviviruses associated with human disease.

As indicated in Table 2 a number of additional flaviviruses occasionally cause human encephalitis. However, these viruses most frequently cause febrile disease with no neurological involvement. Dengue, West Nile, Banzi, Bussuquara, Ilheus, Kunjin, Rio Bravo, Sepik, Spondiveni, Wesselbron and Zika usually induce acute disease characterized by fever, arthralgia and rash. Yellow fever, Kyasanur Forest, Omsk hemorrhagic fever viruses and sometimes dengue viruses induce hemorrhagic fever. For further information on the pathology associated with these infections see the review by Monath (6).

## VIRION COMPOSITION

Flaviviruses are spherical, enveloped virions of about 40 to 50 nm in diameter. The outer surface of the virion is studded with 5 to 10 nm long projections (46) formed by the glycosylated envelope (E) protein. Differences in the extent of glycosylation of the flavivirus E protein have been reported and appear not to adversely affect virion antigenicity or attachment functions (28,47,48). The membrane (M) protein is also associated with the virion envelope. Intracellular virions contain a glycosylated precursor (preM) of this protein. During exit of the virions from infected cells the glycosylated portion of this protein which extends beyond the virion surface is cleaved off (2). Therefore, while mature intracellular virions contain two different surface glycoproteins, extracellular virions contain only one. PreM-containing extracellular virions are observed if infected cells are cultured in Tris-buffered medium (49). Neither the need for nor mechanism of cleavage of preM to M is currently understood. The central nucleocapsid is an icosahedral shell composed of capsid (C) protein subunits with the single-stranded genome RNA coiled inside.

The primary immune response to flaviviruses is directed toward surface epitopes of the virion E protein. Recently, panels of monoclonal antibodies have been generated against a number of different flaviviruses, such as TBEV (48,50,51), YFV (48), WNV (52), SLEV (53) and the four serotypes of dengue virus (54). These monoclonal antibodies have been utilized to analyze E protein epitopes that are involved in virus neutralization,

hemagglutination, immune enhancement, and antigenic cross-reactivity among different flaviviruses. At least 8 distinct E protein epitopes have so far been defined (48,53).

The atomic structures of several naked icosahedral RNA viruses, including human rhinovirus 14 (55), Mengo virus (56), and polio virus, type 1 (57) have been determined by x-ray crystallography. The folded capsid proteins of all of the studied viruses form an eight-stranded antiparallel β-barrel (56). The finding of significant depressions on the virion surfaces was unexpected (56). Whereas picornavirion shells are composed of multiples of 4 different capsid proteins, flavivirus icosahedral nucleocapsids are formed from a single capsid protein. Unfortunately adequate resolution has not yet been obtained after crystallization of any of the enveloped virion nucleocapsids for determination of their atomic structures. How much of a role the internal topography of the capsid shell plays in the packaging of the genome RNA or whether the topography of the outer nucleocapsid shell of enveloped viruses is involved in interactions with the virion envelope proteins has not yet been determined.

## FLAVIVIRUS REPLICATION CYCLE

Flaviviruses presumably attach to receptors on the cell surface and then enter the cell by adsorptive endocytosis. Virions are delivered to intracellular vacuoles and lysosomes and the viral nucleocapsid is released into the cytoplasm by a low pH-induced fusion of the viral and lysosomal vesicle membranes (58,59). Since the flavivirus genome is a functional messenger RNA (plus strand RNA), flavivirions need not carry replicative enzymes. The infecting flavivirus genome RNA is first translated to provide the viral proteins needed to synthesize viral RNA. Whereas the infecting genome RNA is the initial template from which minus-strand complementary RNA is synthesized, the minus-strand RNA is the template for synthesis of progeny plus-strand RNA. The newly synthesized plus-strand RNAs can function as templates for the synthesis of minus-strand RNA, as messenger RNA for the translation of viral proteins, and as molecules for encapsidation into progeny virions. It has been estimated that the ratio of plus- to minus-strand RNAs produced by infected cells is 10:1 (60). Nothing is known about how these various functions are regulated. It is interesting to note that among the plus-strand RNA animal viruses, only the

picornaviruses and the flaviviruses produce no subgenomic messenger RNAs and encode the polymerase(s) at the 3' end of the single long open reading frame. The flavivirus proteins are each made in equal quantities since they are cleaved from a single polyprotein precursor.

The macromolecular synthesis of the host cell is not dramatically affected during a flavivirus infection. Flavivirus protein and RNA synthesis as well as virion assembly occur in the perinuclear region of infected cells in association with endoplasmic reticular membranes (46; Figure 1). Hypertrophy of the rough and smooth endoplasmic reticulum and sometimes the Golgi membranes is a characteristic feature of flavivirus infections. Although it is generally thought that flavivirions obtain their envelopes by an intracellular budding process, flavivirions in the process of budding have not yet been observed with the electron microscope by either thin-section or freeze-fracture studies (46).

GENOME STRUCTURE AND PROTEIN CODING CAPACITY.

The flavivirus genome RNA is single-stranded and almost 11 kilobases in length (61). This genome is infectious, and a functional messenger RNA (mRNA) and, therefore, is characterized as "plus-stranded". No subgenomic mRNAs have been identified in flavivirus-infected cells and all encoded viral proteins are translated from the genome RNA. The RNA has a type 1 5'-terminal $M^7GppAmpN_1$ cap structure (62,63) and lacks internal adenine residues that are methylated (62). The cap appears to be added to nascent RNA strands by a viral methyltransferase (Brinton and Grun, unpublished observation). It is not currently known which of the viral proteins provides this function. The 3'-terminus of the flavivirus genome is not polyadenylated as are cellular mRNAs. Instead, this RNA terminates with $U_{OH}$ (2,64,65). The flavivirus genome has a single long open reading frame which codes for a single polypeptide. The individual functional proteins are processed from this polypeptide by proteolytic cleavage. Sequence data indicates that three types of proteolytic cleavages occur in processing the flavivirus polyprotein (Figure 2). The initiating methionine is removed, presumably by a cellular protease (70). The cellular protease, signalase, cleaves after serine, alanine, or other short side-chain amino acid residues and viral proteins, as yet unidentified, catalyze cleavages after two basic amino acid residues (70). The number and order of viral proteins

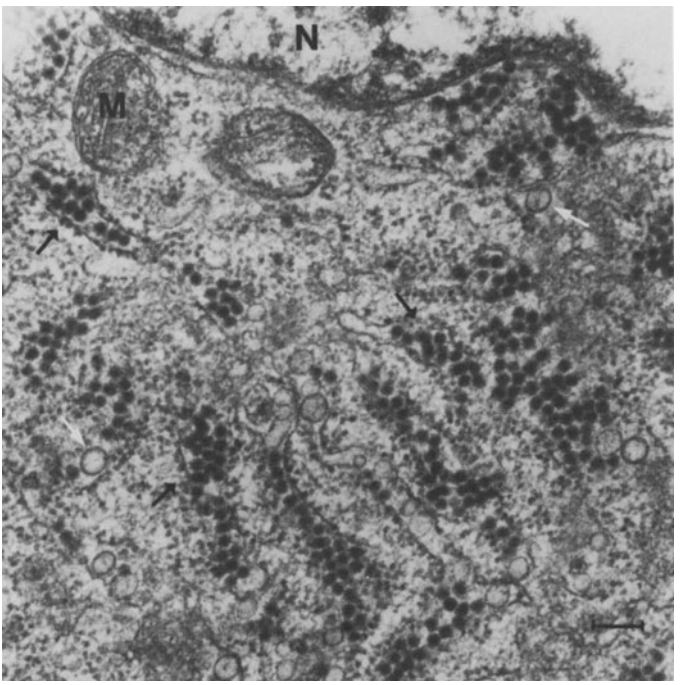


**Figure 1.** Electron micrograph of virus-specific structures seen in thin sections of BHK-21/WI2 cells 18 hr after infection with WNV at a multiplicity of infection of 5. Virions (indicated by black arrows) are located within cisternae of the endoplasmic reticulum in the perinuclear area of the cell. Round lucent, double-membrane vesicles (indicated by white arrows) are often observed in regions where virus maturation is occurring. N = nucleus, M = mitochondria, Magnification = x 43,000, Bar corresponds to 200 nm.

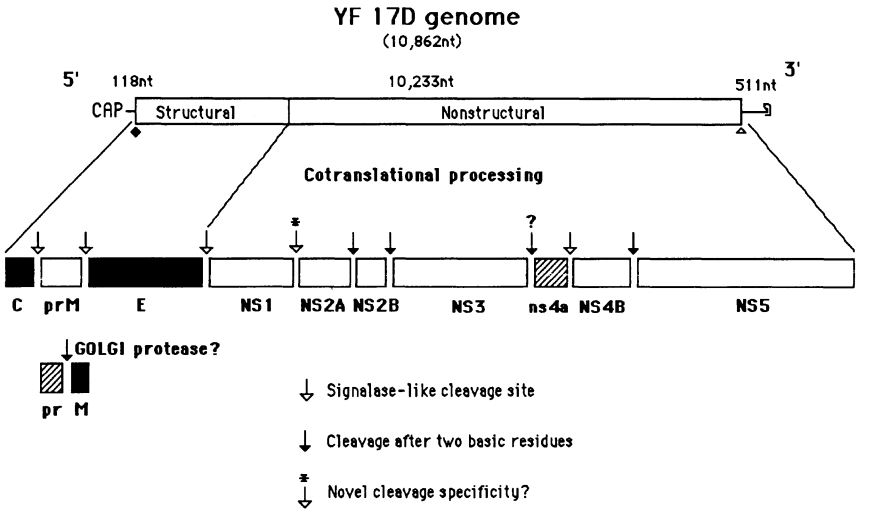


Figure 2. Genome organization and the proposed cleavage sites of the polyprotein encoded by YFV. Terminal untranslated regions of the genome RNA are indicated by a line, while the single open reading frame is represented as an open box. Structural proteins, identified nonstructural proteins, and predicted non-structural proteins are indicated below the genome RNA by solid, open, and hatched boxes, respectively. (nt) nucleotide, (Δ) AUG-translation initiation codon, (◆) UGA-translation termination codon. The single-letter amino acid code is used to indicate amino acids which flank polyprotein cleavage sites. From Rice et al (2; Copyright 1985 by AAAS).

encoded by the genome remained unresolved until Rice et al. (2) unequivocally mapped the flavivirus structural proteins and the 3 largest nonstructural proteins by comparing the genome nucleic acid sequence with the N-terminal amino acid sequences of these proteins (Figure 2). Coding sequences for 4 additional nonstructural proteins were observed by Rice et al. (2; Table 3). The location of the structural proteins at the 5' end of the coding region is similar to the protein arrangement on the genomes of the picornaviruses (66).

The terminal regions of RNA genomes do not code for proteins, but contain signals for the initiation and control of viral RNA transcription. Such signals would be expected to be present within the 3'-terminal regions of genome plus-strand RNA and its complementary minus-strand RNA. The 3'-non-coding region of flavivirus genomes is about 500 nucleotides long, whereas the 5'-non-coding region is about 100 nucleotides in length (2,67). Both sequences and nucleic acid secondary structures can serve as transcription signals (68). Flavivirus transcription signals would be expected to be unique to flaviviruses, and highly conserved among different flaviviruses.

The first 80 to 90 3'-nucleotides of the flavivirus genome RNA form a very stable secondary "stem and loop" structure (Fig. 3B; 2,4,65). The existence of this structure was demonstrated by RNase digestion patterns of 3'-end-labeled WNV genome RNA. Nucleotides within the stem structure were inefficiently digested, while nucleotides within the loops located at the top of the stem were cut efficiently by various RNases (65). Comparison of the 3'-terminal regions of 3 flavivirus genome RNAs indicated 7 regions of sequence conservation (indicated by brackets, Figure 3 A and B). However, only regions 1, 2, and 5 contained highly conserved sequences. These 7 regions were all located in loops, or, in the case of region 7, outside the terminal secondary structure. It has now been demonstrated that the 3'-terminal sequences of YFV (2), WNV, SLEV (65), MVEV (69) and dengue-4 (4) virus RNA can be folded into secondary structures of similar size and shape, even though there is no conservation of the sequences which constitute the stems of these structures. This suggests that the form of the secondary structure serves an important function for flaviviruses. The conservation of sequences located within loops implies that these sequences may be conserved by the specificity of their interactions with viral or

Table 3. Flavivirus Proteins

| Protein[a] | Mr[b] | Characteristics |
|---|---|---|
| **Structural proteins** | | |
| C | 13,000 - 16,000 | Nucleocapsid protein |
| preM | 19,000 - 23,000 | Glycosylated precursor to M protein |
| M | 8,000 - 8,500 | Nonglycosylated virion membrane (M) protein |
| E | 48,000 - 60,000 | Glycosylated envelope (E) protein |
| **Nonstructural proteins** | | |
| NS1 | | Glycosylated, soluble, complement-fixing antigen |
| ns2a[c] | | Hydrophobic protein |
| ns2b | | Hydrophobic protein |
| NS3 | | Polymerase protein (?) |
| ns4a | | Hydrophobic protein |
| ns4b | | Hydrophobic protein |
| NS5 | | Polymerase protein (?) |

a Protein nomenclature proposed by Rice et al. (2; Copyright 1985 by AAAS) for YFV.

b Range of molecular weights (in daltons) estimated for proteins encoded by several flaviviruses.

c The lower case indicates only tentative identification based on possible cleavage sites (2); three of the four hydrophobic, non-structural proteins (nS2B, nS2A, and nS4B) have been recently mapped for Kunjin virus (117).

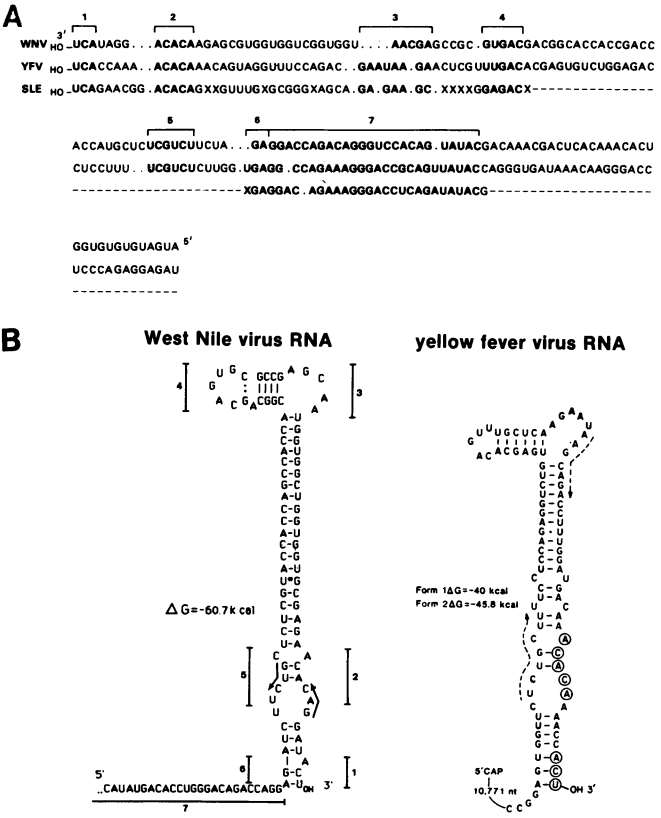


**Figure 3.** The flavivirus genome RNA 3'-terminal region. **A.** Comparison of the 3'-terminal sequences of the genomes of three flaviviruses: WNV, E101; SLEV, 75V-14532; and YFV, 17D. Gaps introduced to align homologous sequences are indicated by x's or dashes. Regions of conserved sequence are indicated by numbered brackets and correspond to those shown in the lower portion of this figure. **B.** Secondary structures formed by the 3'-terminal nucleotides of WNV and YFV RNAs. From Brinton et al. (65) and Rice et al. (2; Copyright 1985 by AAAS).

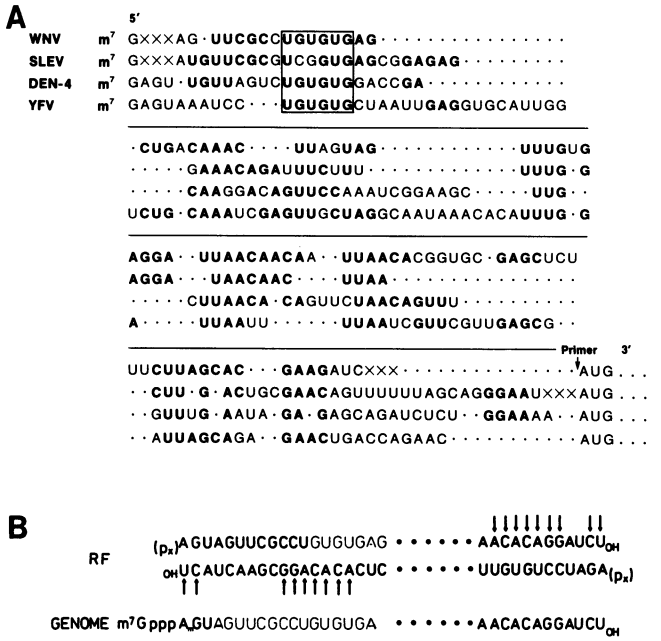


**Figure 4.** The flavivirus genome RNA 5'-terminal region. **A.** Comparison of the 5'-terminal sequences of the genome RNAs of four flaviviruses: WNV, E101; SLEV, 75V-14532; dengue virus, type 4; and YFV, 17D. The YFV sequence was determined by Rice et al (2) and the dengue virus, type 4 sequence was determined by Zhao et al (4). Gaps introduced to align homologous sequences are indicated by dots. Regions of conserved sequence are indicated by bold type. From Brinton and Dispoto (67) with permission. **B.** Comparison of the terminal regions of the WNV genome RNA with those of the complementary minus-strand RNA. The first two nucleotides and seven subsequent nucleotides indicated by arrows are conserved at the 3'-ends of both the plus-and minus-strand RNAs. From Wengler and Wengler (64) with permission.

cellular proteins involved in the replication of viral RNA. In addition, three repeats of a 49-nucleotide-conserved region have been identified within the remaining 400 nucleotides of the 3'-non-coding region (2) and it has been suggested that these may also represent replication signals. The non-coding region located at the 5' end of the genome does not contain these 49-nucleotide sequences. However, the 5'-non-coding region is 5 times shorter than the 3'-non-coding region (Figure 2).

Sequence conservation among flavivirus genome RNAs was also observed within the 5'-non-coding region (Figure 4A; 67). Nucleotides complementary to the first three 3'-terminal nucleotides as well as to the region 2 nucleotides shown in Figure 3 were present at the 5'-terminus (Figure 4B). These two regions represent the only sequences conserved at both the 3'-terminus of the plus-strand genome RNA as well as at the 3'-terminus of the complementary minus-strand. A stable stem and loop structure can be formed by the first 70 to 80 5'-nucleotides of WNV, SLEV, and YFV RNAs. Among the RNAs analyzed, two forms of the structure were observed (Figure 5). The structures consisted of a stem with a small top loop and a large, A-rich, side loop that could be located on either the left or the right side of the stem. A second small stem and loop structure was also conserved. This structure is formed by the 3'-portion of the 5'-terminal non-coding region and includes the genome translation initiation AUG codon (Figure 5, indicated by arrows). So far, only in the YFV RNA 5'-terminal sequence has a third small stem and loop structure been observed (Figure 5).

No flavivirus RNA has yet been found whose 3' and 5'-terminal sequences do not form the characteristic secondary structures. Much additional study is necessary before we will understand the functions of the various flavivirus terminal secondary-structure and sequence signals.

## FUNCTIONS OF VIRAL NON-STRUCTURAL PROTEINS

Functions have not yet been assigned to any of the 7 flaviviral non-structural proteins nor have the protein components of the viral replication complexes yet been identified. One or more of the flavivirus proteins are thought to have protease activity for processing the viral polypeptides (2), but which protein(s) has this activity has not been established. It seems unlikely that NS1 is involved in viral RNA replication because it is

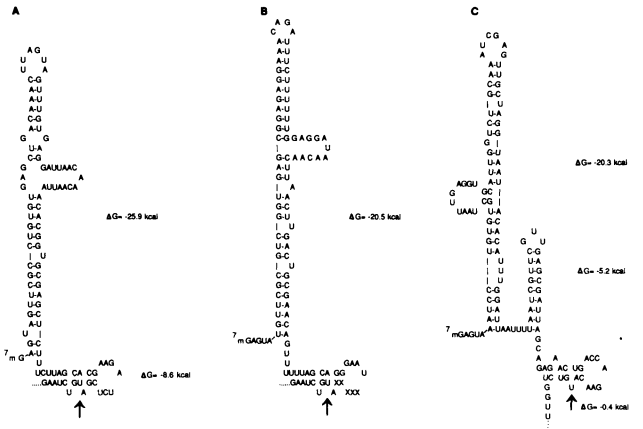


**Figure 5.** Secondary structures formed by the 5'-terminal nucleotides of the genome RNA of three flaviviruses (A) WNV, E101, (B) SLEV, 75V-14532, (C) YFV-17D. The AUG, which is the translation initiation codon for the single, long open reading frame, is indicated by arrows. The optimal ΔG is given separately for each stem and loop structure. From Brinton and Dispoto (67) with permission.

glycosylated, has complement-fixing activity and is localized on the surfaces of infected cells as well as of intracellular virions (47,48). Flaviviruses encode two large nonstructural proteins, NS3 and NS5, both of which could function separately as polymerases (70). Since the 3' and 5' terminal RNA secondary structures differ in their stability and shape constraints, it is possible that two different replication complexes are involved in the initiation and transcription of the flavivirus plus-and minus-strand RNA templates. Flavivirus RNA and protein synthesis occurs on endoplasmic reticular membranes in the perinuclear region of infected cells (71,72) and proliferation of these membranes is a characteristic feature of flavivirus infections (73). Nothing is yet known about how flaviviruses accomplish transcriptional initiation of their RNAs, nor how the preferential synthesis of genome (plus-strand) RNA is regulated. Crude cytoplasmic extracts from infected cells contain a viral-specific transcriptional activity which can be demonstrated in vitro (74,75). Optimal reaction conditions for in vitro transcription have been established (75). Attempts to further purify the viral replication complexes in these crude extracts has indicated that NS5 and NS1 are much more soluble than the other viral proteins. Removal of the majority of NS5 did not significantly reduce the in vitro transcriptional activity (76). This data suggests that only a small proportion of the total amount of NS5 produced functions in replication complexes rather than that NS5 is not a polymerase. The NS5 protein shares some amino acid homology with the polymerases of a number of other RNA viruses (Rice et al., 1986). Although these regions of sequence homology are quite short and are separated from one another by intervening sequences of varying length depending on the virus, they may represent conserved functional regions. One of these regions contains the Gly-Asp-Asp sequence thought to be characteristic of at least one class of RNA-dependent RNA polymerases. The NS3 protein which does not contain the homologous amino acid sequences may represent a different class of RNA-dependent-RNA polymerase.

It has been demonstrated for at least two other types of plus-strand RNA viruses, picornaviruses and RNA bacteriophages, that functional components of viral-replication complexes are of host as well as viral origin. The Qβ replicase is a stable complex consisting of three cell proteins and one viral protein (77,78). The viral-encoded enyzme provides the elonga-

tion function, while the cellular proteins recognize, bind and initiate the viral RNA templates. In in vitro assays of the activity of the purified picornavirus replicase protein, a host protein has been shown to be able to initiate transcription from a single-stranded template RNA in the absence of a nucleic acid primer (79,80).

The existence of a flavivirus-specific murine resistance gene (81,82) strongly suggests that cell proteins may also play a role in flavivirus RNA replication. Flavivirus resistance is inherited as a single Mendelian dominant allele which is not linked to the major histocompatibility locus. Cells from congenic resistant and susceptible mice are equally infectible, thus the resistance gene does not affect virus attachment or penetration. However, resistant animals and cultures prepared from their cells produce less virus. In resistant cells, viral RNA synthesis is less efficient and defective-interfering viral RNAs are preferentially amplified (81). These cell-specific differences could be explained by the existence of two isotypes, one in resistant cells and one in susceptible cells, of a cellular protein that is involved in flavivirus RNA synthesis. The two isotypes might be equally able to provide a host cell function but differ in their ability to provide a function required by flavivirus RNA replication.

## FACTORS INFLUENCING VIRAL NEUROVIRULENCE

Both host and viral factors are important in determining the outcome of a particular infection.

### Host factors.

The host immune response can certainly limit viral disemination, since it has been demonstrated that immunosuppression can convert a subclinical flavivirus infection to a lethal encephalitis. Flavivirus infections in humans are usually subclinical with 1 apparent infection occurring per 100 to 1000 inapparent infections. The age of the host is important in determining disease severity. In general, the very young and the very old are the most susceptible to clinical disease. As mentioned previously, a gene confering flavivirus-specific resistance has been identified in mice (82). It has been demonstrated that resistant mice as well as cells from resistant animals preferentially synthesize defective interfering virus, which in turn diminishes the production of virus thus giving the immune response a better chance to effectively limit virus dissemination and

pathogenesis (81,83). Flavivirus infections that induce permanent impairment or death in their hosts may well exert a selective pressure for the maintenance of host alleles that fortuitously confer a reduced susceptibility to virus-induced pathology (82). Virus selection of resistant host populations under natural conditions has been observed. Woodland and forest birds in Scotland have apparently developed resistance to louping ill virus (84,85) and African primates have developed resistance to YFV (86). It is quite possible that flavivirus resistance genes may also segregate within human populations.

Viral factors.

Heterogeneity among isolates of a particular flavivirus obtained from natural hosts and vectors has been demonstrated using phenotypic markers such as neurovirulence in experimental animals, plaque size, temperature sensitivity and $T_1$ resistant RNA fingerprints. Isolates are characterized as having high, intermediate, or low virulence based on differences between the intracerebral and subcutaneous minimum lethal dose fifty percent ($LD_{50}$). With JEV isolates, neurovirulence was associated with a high level, prolonged viremia after peripheral infection and with thermostability of the virus (87). Studies with SLEV (88) and YFV isolates (89) showed that the level of neurovirulence for a particular isolate was similar in mice and primates. Analysis of SLEV isolates also showed a high degree of concordance between the level and duration of viremia in birds (90) and mosquitoes (91) and neurovirulence for mice and monkeys. However, in one study a correlation between neurovirulence in mice and primates with several strains of WNV (92) could not be demonstrated.

No studies have yet been done to determine which flaviviral gene(s) confer the virulent or avirulent phenotype. Recent studies with two other RNA viruses, the alpha togavirus, Sindbis (96), and the rhabdovirus, rabies, (97) have demonstrated that a single mutation in a virion envelope glycoprotein can convert a virulent phenotype to an avirulent one.

RNA virus genomes can rapidly undergo mutation because of the lack of a proofreading mechanism within their replication complexes (93). Even though this allows for rapid virus evolution, dominant variants of a particular flavivirus have been found to persist in a geographic area for 10 to 20 or more years (94,95). It may be that the constraints placed on flaviviruses to survive during repeated cycles of replication in vertebrate

and arthropod hosts provide a means of maintaining a genetic stability in the viral genome (7).

## FLAVIVIRUS PERSISTENT INFECTIONS

Persistent cell culture infections have been successfully established with a number of different flaviviruses and a variety of mammalian, arthropod, and reptilian cell lines (reviewed by Brinton, reference 73). Interferon production does not appear to be directly involved in the maintenance of flavivirus persistant infections (98-100). However, the generation of defective interfering (DI) virus particles does appear to play an important role in the establishment of persistence (98,100). Virus produced by persistently infected cultures usually undergoes progressive phenotypic change from the parental phenotype. The generation of avirulent, temperature sensitive and less cytopathic virus variants by persistently infected cultures has been reported (100-103).

Persistence of flaviviruses in experimental animals, such as mice, monkeys and hamsters has also been observed. Very little is known about the mechanisms by which persistent infections are established and maintained in animal hosts. SLEV has been observed to persist in the brains of infected mice up to 5 months after infection (104,105). JEV can persist for prolonged periods in the lymph nodes of athymic nude mice in the absence of clinical signs (106). Transplacental transmission of JEV was demonstrated in female mice during consecutive pregnancies occuring as long as 6 months after infection by the intraperitoneal route (107). Kyasanur Forest disease virus normally causes fatal infections in mice, but occasionally paralyzed survivors are observed (36). Although virus can be isolated from these mice many months after infection, serum neutralizing antibody is often not detected. Persistence of louping ill virus in immunosuppressed guinea pigs for more than 50 days after infection has been reported (37). These results suggest that there may be a relationship between virus persistence and suppression of immune elimination of virus. In monkeys chronic encephalitis induced by TBEV lasting more than 2 years (108-110) and asymptomatic or postencephalitic persistent infections induced by WNV (111) have been reported. There are some indications that flaviviruses may also persist for long periods of time in humans (38,112,113). Such observations indicate that the use of live attenuated

vaccines for neurotropic flaviviruses may be problematic.

## NEUROINVASION BY FLAVIVIRUSES

The mechanism by which flaviviruses invade the central nervous system following peripheral inoculation has not been unequivocally established. Several studies support the idea that virus spreads from the blood to the central nervous system. The incidence of brain infection often increases with the level of viremia (114). Viral antigen has been found to appear simultaneously at multiple sites in the brain (115). How flaviviruses cross the blood-brain barrier is not known. Vascular endothelial cells can replicate virus and infection of such cells could allow virus to cross capillary walls located in the brain parenchyma. The olfactory tract can also act as a pathway for virus spread to the brain (116). Neuroinvasion by the olfactory route has been demonstrated in rodent models and a natural avian host species (6). In both adult hamsters and weanling mice infected peripherally with SLEV virus, the infection proceeded from extraneural tissues to sensory neurons in the olfactory epithelium (16). Bowman gland cells within the olfactory neuroepithelium are very susceptible to flavivirus infection. Virus was observed to spread from the neurons in the olfactory bulb throughout the CNS (16).

## CONCLUDING REMARKS

Our understanding of flaviviruses has increased dramatically in the last three years, since the first cloning and sequencing of a flavivirus genome. Additional flavivirus nucleic acid and protein sequences are rapidly being obtained and serve to further refine our knowledge of the flavivirus genome structure and organization. Recombinant DNA technology will eventually allow the mutational manipulation of complete infectious viral genomes for direct analysis of regions controlling viral virulence and of the signals controlling viral replication. Advances in flavivirus immunobiology, through the use of synthetic peptides, are also expected.

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