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I. **Introduction**

Within the flavivirus family, viruses that cause natural infections of the central nervous system (CNS) principally include members of the Japanese encephalitis virus (JEV) serogroup and the tick-borne encephalitis virus (TBEV) serocomplex. Neuroinvasion follows infection of host organisms in the periphery by bites of chronically infected mosquito and tick vectors. Syndromes that result from CNS infection in humans range from mild aseptic meningitis to acute encephalitis of variable morbidity and mortality and are often complicated by neurologic sequelae among survivors. The pathogenesis of these diseases involves complex interactions of viruses, which differ in neurovirulence potential, and a number of host factors, which govern susceptibility to infection and the capacity to mount effective antiviral immune responses both in the periphery and within the CNS. Animal models...
have been instrumental for providing insight into how virus-specific and host factors influence the course of disease. Rodent models have been used in classic experiments on pathogenesis and continue to be relied upon for studies of viral neurovirulence determinants and immune system requirements for a successful antiviral response, particularly because of readily available knockout strains. Nonhuman primates have also been useful for some studies of peripheral immune responses to encephalitic viruses and for quantitating the disease burden caused by replication of virus in the CNS (monkey neurovirulence testing) and have also been applied as models for immunization and challenge. This review summarizes progress in the field of flavivirus neuropathogenesis since previous reviews on this topic (Monath, 1986; Monath and Heinz, 1996). Mosquito-borne and tick-borne viruses are considered together, although it is important to note that there are differences in the pathogenesis of these two groups of viruses.

II. Host Factors

It is generally acknowledged that the ratio of apparent to inapparent infections with flaviviruses is quite low (on the order of 1:100 to 1:300), implying that a number of host factors are involved in protection against CNS disease. The most well known of these are age, genetic factors, and preexisting flavivirus immunity. The effect of age has been recognized in both clinical and experimental studies of flavivirus encephalitis (Eldadah et al., 1967a; Grossberg and Scherer, 1966; Luby et al., 1967; O’Leary et al., 1942; Powell and Kappus, 1978; Weiner et al., 1970), in some cases accompanied by effects of gender (Andersen and Hanson, 1974). In terms of human infections, clinical disease with JEV is primarily a pediatric entity, suggesting that certain features of the developing nervous system predispose to pathogenesis of encephalitis. Experimental evidence in support of this is based on the known propensity of arboviruses to infect and spread more rapidly through nervous tissue of young rodents; however, resistance to fatal infection occurs abruptly soon after the weanling stage. This resistance occurs in conjunction with neural ontogeny and is associated with restricted replication of virus in neurons as a function of their degree of differentiation, with low levels of virus being observed in mature neurons in some models (Hase et al., 1993; Ogata et al., 1991). However, this phenomenon is very dependent on the intrinsic level of viral neurovirulence, being most apparent with strains of lower
virulence (Eldadah et al., 1967a; Ogata et al., 1991; Oliver et al., 1997),
and is also affected by the dose and route of inoculation (Eldadah et al.,
1967a; Fitzgeorge and Bradish, 1980). Changes in the expression of
cellular receptors and/or intracellular factors required for efficient rep-
lication have been invoked as explanations for the age-related resis-
tance. In support of these hypotheses, it is known that the replication
block can be overcome by the neuroadaptation of flaviviruses, which
results in genetic changes in the envelope region, as well as in non-
structural regions of the genome (Chambers and Nickells, 2001;
McMinn, 1997; Schlesinger et al., 1996).

Studies with the mouse model of Sindbis virus encephalitis suggest
that the differential expression of neural genes in young versus older
mice is the determinant of age-related resistance. Candidate genes in-
volved in this process include apoptotic regulators, interferon-respon-
sive genes, and other classes that are regulated developmentally
(Labrada et al., 2002; see Section IX). These factors are presumed to
operate by activating innate antiviral effector systems in neurons
and promoting survival of these cells after viral infection.

Despite these experimental data, age-dependent resistance to en-
cephalitic viruses in areas endemic for human disease is influenced
by the effects of immunization and recurrent inapparent infections
with homologous and heterologous viruses, such that the cumulative
immune responses in adults may confer protection or ameliorate the
severity of disease (Kurane, 2002; Solomon and Vaughn, 2002). How-
ever, susceptibility of young children (below the age of 9 months) to
yellow fever virus (YFV) 17D vaccine-associated encephalitis is well
documented (Freestone, 1994), and resistance to this adverse event
in older individuals is clearly not based on immunity from recurrent
subclinical infections. This supports the phenomenon of age-related
susceptibility to encephalitis. A predisposition of elderly individuals
to develop encephalitis from West Nile Virus (WNV) and St. Louis
encephalitis (SLE) virus has also been observed. In this case, lack of
previous cross-reactive immunity may be an important factor, but co-
morbid illnesses may contribute to the risk of complications, and the
decline in immune function with advanced age is also likely to play a
role (Grubeck-Loebenstein and Wick, 2002). In support of this, im-
munocompromised individuals encountering flavivirus infections
appear to be less able to mount effective immune responses, as in
reports of HIV-infected subjects with complicated CNS disease (Neogi
et al., 1998; Okhuysen et al., 1993; Szilak and Minamoto, 2000; Wasay
et al., 2000) and in transplant recipients sustaining WNV infection
(Iwamoto et al., 2003). YFV 17D vaccine appears to be tolerated in
HIV infection, provided immunosupression is not severe, although this issue deserves further investigation (Kengsakul et al., 2002; Receveur et al., 2000).

Coexistent infection with other infectious agents has been suggested as a modifying factor for flavivirus encephalitis. The association between cysticercosis and JEV infection was investigated in a controlled study that did not provide evidence for predisposition to viral encephalitis (Azad et al., 2003). Association of TBEV encephalitis with borreliosis infection has been described (Korenberg et al., 2001), but probably reflects coexistence of these pathogens in the tick vector. Autopsy studies have suggested that herpes simplex infection may predispose to JEV encephalitis by altering the integrity of the blood–brain barrier (Hayashi and Arita, 1977). Experimentally, infection of mice with Toxocara canis or Trichinella spiralis predisposes to JEV encephalitis as a result of T-cell immunosuppression (Cypess et al., 1973; Gupta and Pavri, 1987; Lubiniecki et al., 1974; Pavri et al., 1975), suggesting the possibility of increased disease severity in endemic areas where parasitic pathogens and encephalitic viruses coexist.

The susceptibility of mice to flavivirus encephalitis is controlled genetically and is associated with host factors that map to chromosome 5 at the oligoadenylate synthetase (OAS) gene cluster (Mashimo et al., 2002; Perelygin et al., 2002; Sangster et al., 1994). Although OAS is involved in the activation of RNase L, the mechanism of resistance associated with the gene is not known and could conceivably relate to other potential functions of OAS proteins in cellular responses to viral injury (Samuel, 2002). The importance of the OAS gene in human susceptibility to these viruses requires investigation, as differences exist between murine and human gene clusters. Evidence shows that 2', 5'-OAS is activated in response to peripheral flavivirus infection in humans (Bonnevie-Nielsen et al., 1989, 1995), but studies to correlate this with the effectiveness of the antiviral responses are not yet available.

The effect of different class I HLA-A and B alleles on the immune response to flavivirus infection has been investigated for dengue viruses, where associations have been found with increased and decreased susceptibility to dengue hemorrhagic fever (Loke et al., 2001; Stephens et al., 2002), suggesting a relationship between the extent of T-cell activation and severe disease. It is unclear whether similar results will be found for encephalitic viruses, as these associations occurred in the context of secondary infections and presumably affect the strength of the memory rather than the primary T-cell response.
Interactions between flavivirus untranslated regions (UTRs) and intracellular proteins indicate another potential level of host-mediated control over virus infection (Brinton, 2000). Multiple proteins have been reported to bind to the 3' UTR of the positive strand of various flaviviruses, including EF-1α and Mov34, which bind to the positive strand 3' UTR of WNV and JEV, respectively (Blackwell and Brinton, 1995, 1997; Ta and Vrati, 2000), and as many as eight cellular proteins in the case of dengue (De Nova-Ocampo et al., 2002). Several proteins have also been reported to bind to the 3' UTR of the negative strand of dengue virus (Yocupicio-Monroy et al., 2003) and four proteins in the case of West Nile virus (Li et al., 2002). These proteins have been suggested to participate in flavivirus replication by influencing viral transcription and/or translation in conjunction with host intracellular membranes. There is some evidence that interactions of the viral RNA with such proteins are in fact important for virus replication (Li et al., 2002). It is not known whether compartmentalization of their interactions in different cell types is a determinant of tissue tropism in infected hosts.

III. ARTHROPOD FACTORS AFFECTING PATHOGENESIS

It has become evident from studies on arthropod vectors that certain components of their saliva influence pathogenesis in vertebrate hosts. For mosquitoes, flaviviruses are deposited principally in the extravascular tissue during probing, as virus that is injected intravascularly is reingested rapidly during the blood meal (Burke and Monath, 2001; Turell and Spielman, 1992; Turell et al., 1995). Instead of a rapid dissemination of virus via the bloodstream, flaviviruses may then undergo some replication locally in subcutaneous tissues accompanied by spread to regional lymph nodes through lymphatic channels. Dendritic cells in the skin are likely to serve as a vehicle for the transport of virus to lymphoid tissues.

Components of the insect saliva modulate the earliest steps in flavivirus infection by altering the local host immune response. Feeding by Aedes aegypti or Culex pipiens mosquitoes or administration of sialokinin-I, a mosquito salivary protein, downregulates interferon-γ (IFN-γ) production and upregulates the T_H2 cytokines interleukin (IL)-4 and IL-10 (Zeidner et al., 1999). Salivary gland extracts from Dermacentor and Ixodes ticks decrease natural killer activity (Kubes et al., 1994, 2002), suppress the antiviral actions of interferons in cell culture (Hajnicka et al., 2000), and enhance the transmission of TBEV in rodents (Labuda et al., 1993). Overall, these findings suggest that insect
factors facilitate flavivirus transmission by interfering with aspects of both innate and adaptive responses. Whether these effects are required to establish systemic infection and neuroinvasion is not known.

IV. EXTRANEURAL INFECTION

Flavivirus neuropathogenesis involves both neuroinvasiveness (capacity to enter the CNS) and neurovirulence (replication within the CNS) (Monath, 1986), both of which can be manipulated experimentally. In rodent models, neurovirulence is an inherent property of most of these viruses, and the quantity of virus needed to cause infection in the CNS is usually quite small. In classic studies of arbovirus pathogenesis (Albrecht, 1968; Huang and Wong, 1963), distinctions were made among neuropathogenic phenotypes on the basis of replication efficiency and pathogenic potential in the peripheral tissues versus the CNS, with various phenotypes being distinguished (high peripheral susceptibility, low neurotropism; low neuroinvasiveness, high neurotropism; and high neuroinvasiveness, high neurotropism). These phenotypes were related to different clinical outcomes, which ranged from inapparent infection to acute encephalitis of varying severity, and are influenced in the rodent model by host age and species (Kozuch et al., 1981). These concepts have held up over time and have been supplemented by additional data concerning viral determinants of virulence and host innate and adaptive immune mechanisms. A main principle that applies is the relationship between peripheral virus burden and the propensity to cause neuroinvasion. Viruses with a low capacity to replicate in the periphery generally can be classified as low in neuroinvasive potential, regardless of their intrinsic level of neurovirulence. A relationship between systemic virus burden and viremia is also apparent (Albrecht, 1968), with the potential of the virus to generate viremia being a correlate of neuroinvasion as it applies to most naturally acquired encephalitic infections. Aerosol-acquired infections are probably an exception, but some of these may also cause systemic infection with viremia after gaining access to the lower respiratory tract. Mucosal infection of the alimentary tract has also been implicated both experimentally and in naturally acquired cases of TBEV encephalitis (Gresikova et al., 1975; Odeola and Oduye, 1977). Furthermore, data from a number of studies indicate that such factors as the time of onset, magnitude, and duration of the viremia, as well as the integrity of the host innate immune system, also influence the risk of entry into the CNS, prior to the onset of the virus-specific
immune response (see Section VIII). Type I interferons and macrophages in particular have been identified as important factors in limiting infection and clearing systemic virus. Thus this process is largely a balance between the replicative efficiency of the virus and the effectiveness of early host defenses in clearing viremia.

V. CELLULAR RECEPTORS FOR FLAVIVIRUSES

The cellular receptors that mediate attachment and entry of flaviviruses have been only partially characterized, and to date there has been no definitive identification of the molecules required for these processes in either peripheral or CNS tissues. Although several groups have used biochemical approaches to identify candidate protein receptors (Kopecky et al., 1999; Martinez-Barragan and del Angel, 2001; Ramos-Castaneda et al., 1997) on mammalian cells, their physiologic relevance remains unclear, as there is heterogeneity in the proteins that bind flaviviruses in different cell types. Most recently, CD209 or DC-SIGN (the dendritic cell-specific intercellular adhesion molecule 3–grabbing nonintegrin) has been proposed as a cell surface ligand for dengue virus (Navarro-Sanchez et al., 2003; Tassaneetritip et al., 2003). Additional studies are required to evaluate the in vivo significance of DC-SIGN as an attachment or entry ligand and whether this is a common determinant of tropism for other flaviviruses. One possibility to reconcile these observations is that multiple independent cellular receptor molecules are utilized either during the spread of flaviviruses within the host or during entry into the CNS. A number of early studies have described binding of flaviviruses to mouse brain substances (reviewed in Albrecht, 1968), suggesting that this tissue is enriched for a unique receptor activity, which may enhance tropism for this organ, particularly in developing brain (Kimura-Kuroda et al., 1992). In this regard, it has been shown that mouse and monkey brain membrane-receptor preparations preferentially bind neurovirulent strains of flaviviruses, but not attenuated variants (Ni and Barrett, 1998; Ni et al., 2000). Receptor variability in vivo may be a general mechanism for promoting wide tissue tropisms of arthropod-borne viruses, which require cycling in both arthropod and vertebrate hosts. Some data suggest that different cell surface proteins may be utilized for the entry of insect versus vertebrate cells (Martinez-Barragan and del Angel, 2001; Munoz et al., 1998). Tissue tropism in mosquitoes has also been observed to correlate with expression of a specific receptor molecule (Yazi-Mendoza et al., 2002).
Heparan sulfate has been proposed as a flavivirus receptor based on studies showing the dependence of dengue virus infectivity on binding of the E protein to heparan sulfate on target cells (Chen et al., 1997; Hilgard and Stockert, 2000). Subsequent reports have demonstrated that infectivity of TBEV, yellow fever virus (YFV), JEV, and Murray Valley encephalitis (MVE) viruses is affected by cell surface interactions with glycosaminoglycans that are proposed to mediate initial low-affinity binding to the cell surface (Germi et al., 2002; Lee and Lobigs, 2000; Mandl et al., 2001; Su et al., 2001), but the role of heparin as an authentic receptor for virulent flaviviruses remains uncertain. For instance, the serial passage of JEV and MVE viruses in cell culture results in selection for viruses that exhibit increased binding to heparin but decreased virulence in vivo (Lee and Lobigs, 2002). Similar observations have been made with alphaviruses (Bernard et al., 2000; Klimstra et al., 1998), where heparin binding was associated with cell culture adaptation of primary virus isolates and attenuation of viral virulence. Thus enhanced binding to glycosaminoglycans is a marker for attenuation of JEV and MVE viruses in the mouse model and correlates with rapid clearance of the glycosaminoglycan-binding variants from the circulation compared to more pathogenic strains (Lee and Lobigs, 2002). The mechanism responsible for this process has not been defined. The relationship of this observation to classic studies on neuroinvasion is also unclear, as virulent strains were originally characterized by their ability to undergo rapid uptake from the circulation, presumably as a result of highly efficient binding and entry to target cells (Albrecht, 1968). Wild-type and glycosaminoglycan-binding variants may differ, however, with respect to their entry into cells that are permissive for replication or are involved in virus clearance (Lee and Lobigs, 2002).

Antibody-dependent enhancement (ADE) of infection has been reported for some encephalitic flaviviruses. ADE is believed to occur through FcγRI (CD64) and FcγRII (CD32), although a second type of ADE that requires complement has also been described (Cardosa et al., 1983, 1986). In tissue culture, ADE occurs with several flaviviruses in cells of myeloid lineage (Brandriss and Schlesinger, 1984; Brandt et al., 1982; Cardosa et al., 1983, 1986; Diamond et al., 2000b; Halstead and O’Rourke, 1977; Halstead et al., 1980, 1984; Schlesinger and Brandriss, 1983). Most of these data are relevant to the pathogenesis of dengue infection, and the significance for encephalitic viruses is less certain. However, there is some experimental evidence that ADE may be involved (Hawkes, 1964), and this notion is consistent with the concepts described for dengue viruses based on the wide antibody
cross-reactivity among flaviviruses with respect to the E protein. Neutralizing homologous or cross-reactive antiflavivirus antibodies can enhance neurovirulence and mortality associated with YFV and JEV infection (Gould and Buckley, 1989; Gould et al., 1987; Lobigs et al., 2003b). However, in some cases, the pathogenesis was associated with complement-mediated cytolysis and not with enhancement of infection in vivo (Gould et al., 1987). The strongest data in support of ADE in dengue infections are epidemiological in nature. In this regard, there is not abundant evidence to support a phenomenon of enhancement of JEV or other encephalitic viruses by preexisting cross-reactive antibodies in natural infections. There is evidence of cross-protection in experimental models, particularly among JEV serogroup members, and for amelioration of JEV encephalitis by prior immunity to related viruses, including dengue (Kurane, 2002; Solomon and Vaughn, 2002). Experimentally, the protective effect is presumably antibody mediated and is most apparent following infection with live virus or transfer of serum from animals infected with virus (Broom et al., 2000; Tesh et al., 2002). Antibody responses elicited by inactivated virus do not exhibit much cross-protection in animals and may lead to enhanced infections (Broom et al., 2000; Lobigs et al., 2003b). Consistent with these observations, recipients of inactivated JEV vaccine or live-attenuated dengue vaccine did not generate neutralizing activity in sera against WNV (Kanesa-Thasan et al., 2002). Severe forms of TBEV encephalitis have been observed after passive immunization with hyperimmune globulin (Waldvogel et al., 1996), but it is not clear if this represents an enhancement phenomenon as opposed to either failure of antibody to penetrate the CNS or suppression of peripheral or CNS immune responses by high-titer immunoglobulin. The entire issue is somewhat limited by the fact that the encephalitic viruses may not necessarily target cells with abundance of Fc receptors, such as monocyte–macrophages, which are generally considered more important for the pathogenesis of dengue viruses.

VI. CELLULAR TROPISM OF ENCEPHALITIC FLAVIVIRUSES

In cell culture, flaviviruses readily infect a variety of cell types, including epithelial, endothelial, and fibroblasts (Avirutnan et al., 1998; Bielefeldt-Ohmann, 1998; Diamond et al., 2000b; Kurane et al., 1992), but the relationship of these findings to in vivo replication is uncertain. After peripheral inoculation, flaviviruses probably do not replicate extensively in the skin, but are spread from local lymph
nodes by immature dendritic or Langerhans cells, which are permissive for infection (Byrne et al., 2001; Johnston et al., 1996, 2000; Libraty et al., 2001; McMinn et al., 1996; Wu et al., 2000). Within 1 day of infection, epidermal Langerhans cells that express viral antigens migrate from the skin to the draining lymph node (Byrne et al., 2001; Wu et al., 2000) while expressing maturation markers such as B7-1, B7-2, class II MHC molecules, CD11b, and CD83 (Ho et al., 2001). These cells produce tumor necrosis factor (TNF)-α and IFN-α (Ho et al., 2001; Libraty et al., 2001) and become more resistant to flavivirus infection (Wu et al., 2000). Thus infected dendritic cells probably serve to promote antigen presentation in the lymph node and also participate in the spread of infection to lymphoid compartments. The consequences of DC infection, whether apoptosis (as for alphaviruses) or persistent infection, as in the case of Kunjin virus replicons (Varnavski and Khromykh, 1999), and their effects on subsequent shaping of the immune response remain important areas for further investigation. For instance, data from other viral models indicate that there is a quantitative requirement for activated dendritic cells in order to induce T-cell responses (Ludewig et al., 1998). Survival versus death of these cells as a result of virus infection may have an important impact on this requirement. After replication in lymphoid tissue, encephalitic viruses are believed to exit via efferent lymphatics (Malkova and Frankova, 1959) and gain access to the circulation, whereby systemic infection is established.

Although a tropism of encephalitic viruses for lymphoid tissues has been observed, the identities of the cell types in other compartments that support replication to the levels needed to generate a viremia sufficient to cause neuroinvasion have not been determined definitively. Replication in various peripheral tissues occurs, but vascular endothelial cells have not necessarily been implicated as important sites of replication (Albrecht, 1968 and references therein). However, it should be noted that dengue, JEV, and probably other flaviviruses can enter and, in some cases, establish infection in endothelial cells (Dropulic and Masters, 1990; Liou and Hsu, 1998) and modulate their activation state and cytokine production (Anderson et al., 1997; Avirutnan et al., 1998; Bosch et al., 2002; Huang et al., 2000). In vitro studies with endothelial cells are complicated by the fact that variable responses can be observed depending on the cell types and assay systems; however, given the potential role of these cells in immune activation, further studies on the effects of flavivirus infection on cytokine and chemokine production by these cells are needed.
In the CNS, neurons are the primary targets for encephalitic flaviviruses (Eldadah et al., 1967b; Hase et al., 1987; Iwasaki et al., 1986; Kimura-Kuroda et al., 1992; Wang et al., 1997, 1998; Weiner et al., 1970; Xiao et al., 2001). Although viral replication and antigen production have also been observed in cultured oligodendrocytes (Jordan et al., 2000) and astrocytes (Chen et al., 2000; Liu et al., 1988; Suri and Banerjee, 1995), the significance of these reports is difficult to judge, as there is scant evidence for infection of glial cells in vivo. Neurotropism and neurovirulence are governed to a large extent by determinants in the viral E protein, as indicated by an abundance of genetic data indicating that mutations in this protein modulate these phenotypes (McMinn, 1997; Ni and Barrett, 1998; Ni et al., 2000), presumably through their effects on receptor targeting and postreceptor events involved in virus entry. In the absence of an experimental system to manipulate the virus receptor on neurons, one cannot conclude whether high neurotropism of these viruses is dependent solely on the binding of the E protein. Comparison of sequence data from virulent and attenuated strains of encephalitic viruses also suggests that the nonstructural region and 3′ UTRs contain determinants that influence pathogenesis. It is important in these types of studies to differentiate effects of genetic mutations on overall replication fitness of the virus versus specific effects in terms of interactions of viral proteins and RNA structures with host factors that affect pathogenesis uniquely. Thus the molecular basis for neurotropism is not understood adequately, and further studies using genetic clones of well-characterized viruses in animal models should help address this issue. Furthermore, the use of primate models to investigate the issue is needed greatly, as the observations in rodent models may not have direct correlates to human infections.

VII. Immune Responses to Flaviviruses and Their Role in Pathogenesis

Susceptibility to flavivirus encephalitis implies a failure at some stage of the immune response that theoretically may be defined in either qualitative or quantitative terms. There is substantial clinical and experimental evidence for a correlation between protection against encephalitic disease and the presence of virus-specific antibodies, but the molecular and cellular basis for the development of this response has not been defined thoroughly. In studies that have shown protection by antibodies, the roles of other immune system components in
the process have not often been assessed. Furthermore, there is increasing evidence that flaviviruses have evolved mechanisms to manipulate the effector functions of both innate and adaptive immune responses. The magnitude and importance of these responses probably vary from one experimental model to another and account for differences observed in studies that have examined the immune system in the context of a either a primary or a memory response.

### A. Innate Immunity

#### 1. Interferons

*In vitro* and *in vivo* studies have demonstrated that interferon-dependent responses are relevant to protection against flavivirus infections. Dendritic cells in the skin may be the first cells to produce type I interferons (IFN-α or -β) in response to flavivirus infection and initiate this antiviral response (Libraty *et al.*, 2001). These interferons inhibit flavivirus infection by preventing translation and replication of infectious viral RNA at least partially through an RNase L-, Mx1-, and Protein Kinase R (PKR)-independent mechanism (Anderson and Rahal, 2002; Diamond and Harris, 2001; Diamond *et al.*, 2000a). These studies have been supported by experiments in immunodeficient and therapeutic mouse models of disease. Pretreatment of mice with IFN-α or its inducers prevents or ameliorates flavivirus infections (Brooks and Phillpotts, 1999; Charlier *et al.*, 2002; Harrington *et al.*, 1977; Leyssen *et al.*, 2003b; Lucas *et al.*, 2003), and mice that are deficient in type I IFNs or their receptor have increased susceptibility to flaviviruses (Johnson and Roehrig, 1999; Lobigs *et al.*, 2003a). The role of type II IFN (IFN-γ) in protection versus immunopathogenesis of flavivirus encephalitis is less clear, as this cytokine has a multitude of effects on the host response to these viruses. In part, this includes induction of proinflammatory and antiviral molecules, including nitric oxide (Lin *et al.*, 1997), and enhancement of the phagocytic activity of monocytes/macrophages through increased Fc receptor expression (Rothman and Ennis, 1999). Data from various models support the importance of IFN-γ production in the context of a T<sub>H</sub>1 virus-specific immune response for the control of infection with encephalitic viruses (Johnson and Roehrig, 1999; Liu and Chambers, 2001; Lobigs *et al.*, 2003a). Distinctions should be made, however, concerning the effects of IFN-γ in the periphery and in the context of the CNS immune response (see Section X).
Flaviviruses appear to be capable of attenuating some of the IFN-dependent antiviral effector mechanisms. Treatment of cells or animals with IFN-α as few as 4 h after infection with dengue or SLE viruses resulted in almost a complete loss of antiviral activity (Brooks and Phillpotts, 1999; Diamond et al., 2000a). Similarly, IFN-α treatment of patients with documented JEV encephalitis had no significant effect on outcome (Solomon et al., 2003), despite anecdotal reports of its benefit (Harinasuta et al., 1985). The mechanisms by which the antiviral effect is avoided remain uncharacterized, but data suggest that it may act at a very early step in infection. Future studies should also help determine if the role of type I IFNs in these infections lies only in innate intracellular effector defenses or whether there are effects on the quality and magnitude of the subsequent cell-mediated immune response through their immunoregulatory effects.

2. Macrophages

Activation of macrophages and modulation of their effector functions are integral parts of flavivirus pathogenesis (Rothman and Ennis, 1999; Spain-Santana et al., 2001). In addition to their role in nonspecific defense, macrophages are targets of infection by some flaviviruses and have the potential to contribute to pathogenesis through antibody-dependent enhancement of infection mediated by Fc and complement receptors (Cardosa et al., 1986; Gollins and Porterfield, 1984; Hawkes, 1964; Peiris et al., 1981). The preponderance of data supports the protective role of macrophages in control of infection by means of cytokine production and antigen presentation to B and T cells (Kulkarni et al., 1991a; Marianneau et al., 1999). Classic studies have shown that abrogation of phagocytic activity of macrophages results in higher viremia, neuroinvasion, and more severe encephalitis (Ben-Nathan et al., 1996a; Khozinsky et al., 1985; Monath, 1971; Zisman et al., 1971). Some of the protective effect provided appears to be mediated by the stimulation of inducible nitric oxide synthetase (NOS-2) to produce nitric oxide (NO) and other reactive oxygen intermediates such as peroxynitrites (Saxena et al., 2000). Pretreatment of macrophages with agents that induce NO synthesis have been shown to inhibit JEV infection in vitro (Lin et al., 1997). Moreover, treatment of mice with a NOS-2 inhibitor increased mortality after JEV infection (Lin et al., 1997). However, other studies suggest that the inflammatory actions of NO and other reactive oxygen intermediates may, in some cases, contribute to flavivirus pathogenesis. The in vivo administration of a competitive inhibitor of NOS-2 improved survival in mice infected with TBEV (Kreil and Eibl, 1995, 1996). Activation of macrophages in response to flavivirus
infection promotes not only production of NO, but also release of TNF-\(\alpha\), IL-1\(\beta\), IL-8, and other mediators of acute inflammation that may contribute to tissue damage where macrophages accumulate (Atrasheuskaya et al., 2003; Bosch et al., 2002; Raghupathy et al., 1998; Rothman and Ennis, 1999). These various studies indicate that the behavior of macrophages is fundamental to the pathogenesis of flavivirus disease, but the contribution to virus clearance versus deleterious effects driven by IFN-\(\gamma\) and other proinflammatory stimuli depends on regulation of their activity, and the properties of this innate defense may therefore vary from one context to another.

3. Natural Killer Cells

Natural killer (NK) lymphocytes lyse infected cells by releasing cytotoxic granules that contain perforin and granzymes or by binding to apoptosis-inducing receptors on target cells (Orange et al., 2002). NK cell activation is finely regulated through a balance of activating (Ly49D, Ly49H, and NKG2D) and inhibitory receptors [killer cell immunoglobulin-like receptors (KIR), immunoglobulin-like inhibitory receptors (ILT), and CD94-NKG2A] (Smith, et al., 2001). A decrease in expression of class I MHC molecules on a cell may prompt NK cell activation by attenuating the inhibitory signals. Thus NK cell target recognition occurs after ligation of activating receptors and repression of inhibitory receptors on the cell surface. NK responses have been analyzed in various experimental models of flavivirus infection, but as noted (Hill et al., 1993), the characterization of the responding cells has been limited. Studies of NK cell activity during WNV infection have revealed blunted cytolytic activity against virus-infected cells, associated with upregulation of MHC antigen and ICAM-1 expression on the target cells by interferon-independent mechanisms (Müllbacher et al., 1989). However, NK cell-dependent lysis of dengue virus-infected target cells by both natural killer and antibody-dependent cell-mediated cytotoxicity has been observed (Kurane et al., 1984). Infection of mice with Langat, WNV, and TBEV transiently activated and then suppressed NK cell activity (Vargin and Semenov, 1986). Despite these conflicting observations, the bulk of evidence currently suggests that flaviviruses have evolved a mechanism to evade NK cell responses through an augmentation of cell surface class I MHC expression (King and Kesson, 1988; King et al., 1989; Liu et al., 1988, 1989b), driven by a TAP-dependent process (Momburg et al., 2001; Müllbacher and Lobigs, 1995; Lobigs et al., 1999) and NF-\(\kappa\)B-dependent transcriptional activation of MHC class I genes (Kesson and King, 2001). Thus, flaviviruses may overcome susceptibility to NK cell-mediated lysis at the
expense of increased class I MHC expression and later recognition by virus-specific cytotoxic lymphocytes (CTLs). Consistent with this hypothesis, splenocytes from WNV-immunized mice had poor NK cell lytic activity (Momburg et al., 2001), and mice that are genetically deficient in NK cells demonstrate no increased morbidity or mortality compared to wild-type controls in response to WNV infection (M. Engle, W. Yokoyama, and M. Diamond, unpublished results). Some residual NK cell function may still be important during flavivirus pathogenesis, as suggested by studies with perforin and Fas knockout mice, which are partially protected from encephalitic disease, through events that operate at the level of neuroinvasion. This may involve the cytotoxic activities of NK cells and/or CTLs (Lincon Luna et al., 2002). In addition, the lack of a substantial effect of IFN-α in the therapy of flavivirus encephalitis is also consistent with inhibition of the NK response, as type I interferons are normally potent activators of these cells.

4. Natural Antibody

Natural antibodies are primarily of the IgM class, although activity of IgG has also been described. They are secreted constitutively by CD5+ B-1 cells without specific stimulation, have widely variable binding avidities, and represent an initial nonspecific defense against pathogens (Baumgarth et al., 2000; Casali and Notkins, 1989; Ochsenbein et al., 1999a) through direct neutralization of some bacteria and viruses in the circulation (Gobet et al., 1988; Ochsenbein et al., 1999a), enhancement of phagocytosis (Navin et al., 1989), and complement activation (Baumgarth et al., 2000). Although the role of natural antibody in flavivirus infection remains unexplored, mice that genetically lack secreted IgM (sIgM−/−), but in which cell surface IgM and IgG responses are intact have increased mortality in certain viral infections, involving a defect in the antiviral IgG responses (Baumgarth et al., 2000; Boes et al., 1998). Such mice are also very susceptible to infection with WNV (Fig. 1; M. Engle and M. Diamond, unpublished data). This observation, along with other data, suggests an important role for natural antibody and complement during the early antiviral defense against flaviviruses, although a virus-specific IgM response is likely to be more important (see Section VII,A,5).

5. Complement

The complement system is an important innate defense for limiting infection by fungal, bacterial, and viral pathogens. Complement inhibits viruses by several mechanisms (Volanakis, 2002), including lysis
enveloped viral particles and virus-infected cells by the C5–C9 membrane attack complex; recruitment and activation of monocytes and granulocytes by C3a and C5a; clearance of virus from circulation after opsonization by proteolytic fragments of C3, C3b, and C3bi, followed by uptake into cells that express complement receptor; and C3-facilitated uptake of antigen and presentation by macrophages and dendritic cells (Ochsenbein and Zinkernagel, 2000) during priming of T and B lymphocytes (Da Costa et al., 1999; Kopf et al., 2002; Ochsenbein et al., 1999b). Preliminary studies indicate that complement plays an essential role in limiting WNV infection. Mice that are genetically deficient in C3 uniformly succumb to infection even at low inoculating doses (Fig. 2; E. Mehlhop, M. Engle, and M. Diamond, unpublished data). Additional studies must be performed to determine which individual mechanisms are most critical for this complement-mediated control. A deficiency of C3 could exacerbate WNV infection because of depressed C5–C9 lytic or C3 opsonic activity that results in a failure to clear virus from the circulation. Alternatively, C3 may play important roles in linking the innate and adaptive immune responses (Barrington et al., 2001; Carroll, 1998; Ochsenbein and Zinkernagel, 2000) against WNV. C3 is required for normal IgG production and T-cell priming against influenza and herpes viruses (Da Costa et al., 1999; Fischer et al., 1996; Kopf et al., 2002; Ochsenbein et al., 1999), and a deficiency in C3 decreases opsonization and viral antigen presentation, leading to deficits in the adaptive B-and T-cell responses (Ochsenbein and Zinkernagel, 2000). Although the lytic and proinflammatory activity of complement

Fig 1. Soluble IgM-deficient mice are highly sensitive to peripheral infection with WNV. Mice were infected by subcutaneous inoculation of the footpad with 100 plaque-forming units of WNV NY 99 and monitored for mortality from CNS disease.
may contribute to the defense against WNV, preliminary data indicate that a deficiency in either C3 compromises the antiviral B-cell immune response, as mice that lack C3 have markedly depressed titers of WNV-specific IgG (E. Mehlhop, M. Engle, and M. Diamond, unpublished data).

### B. Adaptive Immunity

The roles of both humoral and cellular immunity during the pathogenesis of flavivirus disease have been studied in various models involving active and passive immunization in normal and immunodeficient animals, and understanding of the immunologic basis for protection against acute encephalitis is beginning to advance. B cells, CD4^+^ cells, and CD8^+^ T cells have all been implicated in contributing to protection, although as stated earlier, their relative importance seems to vary depending on the context of the experimental model under investigation.

#### 1. B Cells and Antibody

Antibody responses to the E and NS1 proteins involve many epitopes, and both neutralizing and nonneutralizing antibodies (including those against NS1) can prevent fatal encephalitis, as demonstrated in many passive transfer and active immunization experiments in experimental animal models (Brandriss et al., 1986; Diamond et al., 2003; Gould et al., 1987; Henchal et al., 1988; Kimura-Kuroda and
A protective effect can occur even after establishment of infection in the CNS. Mechanisms of antibody-mediated protection during flavivirus infection include direct neutralization of receptor binding (Crill and Roehrig, 2001), blocking of virus uncoating (Gollins and Porterfield, 1984), and Fc receptor-dependent virus clearance via the reticuloendothelial system. Most neutralizing antibodies recognize the structural E protein, and antibody epitopes appear to be broadly distributed over its surface (Heinz, 1986; Roehrig et al., 1989); however, these do not all represent potent sites for neutralization. A subset of neutralization epitopes is found on the prM protein (Colombage et al., 1998; Falconar, 1999; Pincus et al., 1992). The presence of nonneutralizing, yet protective antibodies against NS1 is also well documented (Cane and Gould, 1988; Després et al., 1991; Falgout et al., 1990; Henchal et al., 1988; Putnak and Schlesinger, 1990; Schlesinger et al., 1986, 1987). Antibodies to NS1 are proposed to mediate the lysis of virus-infected cells, which express this protein on their surface, by complement-mediated lysis and/or antibody-dependent cellular cytotoxicity (Hill et al., 1993; Kurane et al., 1984; Schlesinger et al., 1990). These humoral responses are believed to be important components of the protective immune response to flavivirus infection, but antibodies to NS1 are not often measured in experimental models of infection or immunization and challenge.

Although the neutralizing antibody responses are considered correlates of protection (Markoff, 2000), this process is probably also a function of additional innate and adaptive immune effector systems whose roles in the control of infection are less easily demonstrated. In this regard, challenge in the context of passively administered virus-specific antibodies is not necessarily associated with sterilizing immunity, indicating that antiviral defenses other than antibodies are involved in protection (Kreil et al., 1998a, 1998b). Definitive experiments to determine the extent of immune activation in this setting are likely to expand our understanding of the correlates of protection and the immunological basis for a successful antiviral response.

Mice that lack B cells are very vulnerable to flavivirus infections and encephalitis (Diamond et al., 2003; Liu and Chambers, 2001), purportedly as a consequence of lacking antibodies. However, these models must be explored further to determine whether T-cell responses are otherwise fully activated and effective, as it is possible that B cells could influence CD4+ and CD8+ T-cell responses through antigen presentation or other immunoregulatory events, as has been observed in other neurotropic viral infections (Bergmann et al., 2001).
Factors that drive B-cell activation and maturation during flavivirus infections are not well understood. Multiple T helper epitopes have been identified in the E protein of JEV and MVE viruses, some of which are dominant, broadly reactive among JE serogroup viruses, and prime for the neutralizing antibody (Kutubuddin et al., 1991; Mathews et al., 1991, 1992; Roehrig et al., 1992). Immunization with plasmid DNA encoding the E region is more effective than viral E antigen itself, presumably due to the induction of T-cell responses to the E protein (Chen et al., 1999). Immunization of HIV-infected individuals with inactivated flavivirus vaccines has suggested that CD4+ T cells may not be critical for the induction of protective antibodies (Panasiuk et al., 2003). However, others have observed that antibody responses to inactivated or live viral vaccines are weak in these subjects (Rojanasuphot et al., 1998; Sibailly et al., 1997). Immunization of CD4 knockout or class II knockout mice with YFV markedly diminished or abrogated the neutralizing antibody response, respectively (Chambers and Liang, unpublished data), suggesting that there is dependence on functional CD4+ T cells to generate long-lasting B-cell memory against flaviviruses. Differences in immunization schedules and numbers of residual CD4+ lineage T cells in these various reports may explain the discrepancies. Studies on the role of dendritic cells in early B-cell activation and the nature of the toll-like receptor signals induced in response to viral antigens on these cells may provide new insights into the determinants that establish and drive the memory response to flavivirus antigens.

An IgM response to flaviviruses is a feature of most clinical and experimental infections (Martin et al., 2002) and has been reported to be a correlate of protection during clinical JEV encephalitis in some studies (Burke et al., 1985a, 1985b; Ravi et al., 1993). Flavivirus infections typically elicit IgM responses that can often persist for prolonged periods (Edelman et al., 1976; Monath, 1971; Roehrig et al., 2003). However, experimentally, the initial IgM response to encephalitic viruses may possess variable neutralization activity and protective capacity (Diamond et al., 2003; Hofmann et al., 1978; Ishii et al., 1968); in some studies, the complement-fixing activity was limited compared to that provided by the ensuing IgG response (Ishii et al., 1968; Lee and Scherer, 1961). This contrasts with what has been observed with YF 17D vaccination (Monath, 1971) and may reflect differences in early B-cell activation among these infections or differences between humans and mice with respect to the process. However, the role of a vigorous IgM response may be in providing temporary neutralizing activity, while more importantly activating complement-dependent pathways involved in
programming virus-specific B- and T-cell responses. Studies with the WNV model (see later) suggest a critical early function of both IgM and complement in the control of extraneural infection. The neutralizing activity of sera rises in conjunction with the appearance of IgG, and this response includes a variety of biological activities, including hemagglutination inhibition, complement fixation, and virus neutralization. The memory or “antigenic sin” response elicited by cross-reactive antigens involves all of these responses, but is weakest for the neutralization response (Innis, 1997), suggesting that there is some hierarchical pattern of B-cell epitopes or some selectivity in recognizing the most cross-reactive antigens. This phenomenon is probably evolutionarily adaptive for recurrent infections with heterologous flaviviruses, but is capitalized upon by dengue viruses during the pathogenesis of dengue hemorrhagic fever and shock syndrome. In any case, the anamnestic antibody response has been demonstrated to be critical to a defense against encephalitic viruses in the context of immunization and challenge models, which are surrogates for the efficacy of vaccines (Konishi et al., 1999; Lee and Sherer, 1961; Pan et al., 2001). It nevertheless remains unclear at the present time the extent to which antibody responses alone contribute to the control of acute infections.

2. T Cells

T-cell responses to flavivirus proteins have been best studied for members of the DEN and JEV serogroups. Both CD4+ and CD8+ T lymphocyte responses involve broad flavivirus cross-reactivity, although this varies significantly among different MHC haplotypes (Hill et al., 1992; Kulkarni et al., 1992; Kurane et al., 1991; Uren et al., 1987; reviewed in Hill et al., 1993). Multiple epitopes for T-cell responses have been identified on both structural and nonstructural proteins; however, genetic factors restrict the number of targets and fine specificities differ considerably. Determinants for class I responses are more frequent within the viral nonstructural region, particularly the NS3 protein, which contains dominant epitopes in both humans and mice. In contrast, viral structural protein antigens elicit class II responses more consistently. The role of virus-specific CD4+ T cells in flavivirus encephalitis is not well understood, although experimental models indicate a requirement for such cells in protection against acute disease (see Section X). Some of the cytotoxic T-cell response to dengue and JE viruses is contained in the CD4+ compartment and is probably mediated by Fas/FasL interactions (Aihara et al., 2000; Gagnon et al., 1999), which has implications for possible immunopathogenic responses both in the periphery and in the CNS.
Flavivirus-specific CD8\(^+\) T cells have multiple effector functions, including cytotoxic activity and production of IFN-\(\gamma\) (Douglas et al., 1994; Kesson et al., 1987; Kulkarni et al., 1991b; Kurane et al., 1989, 1991, 1995; Liu et al., 1989a; Murali-Krishna et al., 1996; Takada et al., 2000), suggesting that polarization of the immune response toward a T\(_{H1}\) phenotype is involved in control of these viruses. Although the importance of type I interferons in protection against these viruses would suggest its involvement in promoting this T-cell response, as has been described for other viruses (Cousens et al., 1999), the role of IL-12 in driving this process has received only limited study (Chen et al., 2001; Phillpotts et al., 2003), and its importance may vary from one context to another (Dalod et al., 2002). Cellular immunity clearly contributes to the control of virus infection in experimental animal models, but this varies depending on the context examined and with respect to the virulence of the challenge virus. T-cell-deficient mice fail to generate protective immunity after a sublethal challenge with YFV strains (Bradish et al., 1980). Moreover, animals that are treated with drugs that impair T-cell function develop a rapidly progressive flavivirus encephalitis (Camenga et al., 1974; Cole and Nathanson, 1968; Nathanson and Cole, 1970). The adoptive transfer of immune spleen cells can protect against encephalitis, but the lymphocyte subpopulations that mediate this protection have not been very well characterized in classic studies (Bradish et al., 1980; Camenga et al., 1974; Jacoby et al., 1980).

More recent studies have, to some degree, clarified the role of CD8\(^+\) T cells in these infections. Quantitation of the CD8\(^+\) T-cell response to YFV in experimental mice (van der Most et al., 2002) reveals activation by immunodominant epitopes and is supported by the observation that CD8\(^+\) knockout mice exhibit a defect in the clearance of infectious YFV from the CNS (Liu and Chambers, 2001; T. J. Chambers, unpublished data) and also have increased mortality after WNV infection (B. Shrestha and M. Diamond, manuscript in preparation). Human recipients of YFV 17D vaccine exhibit an increase in CD8\(^+\) T cells as well, and epitopes have been mapped to multiple proteins (Co et al., 2002). While data from most of these models would indicate that CTL responses are primarily protective in vivo, their potential for immunopathogenic effects requires further investigation. Some studies with MVE virus (Licon Luna et al., 2002) and dengue virus (Rothman and Ennis, 1999) suggest that the cytotoxicity of CTL may contribute to the disease pathogenesis. Studies with knockout mice continue to provide novel information on the role of T cells in flavivirus pathogenesis and immunity. However, an important limitation of these experiments
is that the effect of gene knockout on immunologic development and function is not known. Better attempts to assess the entire range of properties of the immune response in these types of experimental models are needed, as gene knockouts could have multiple effects beyond simply loss of the targeted function.

Human studies on immune responses to JEV or JEV structural protein antigens encoded in recombinant vaccinia virus revealed proliferation and induction of cytolytic activity in the CD8$^+$ T-cell compartment (Konishi et al., 1995, 1998a), similar to what has been observed in mice (Konishi et al., 1997, 1998b). The significance of these responses is uncertain, as another study found no correlation between T-cell proliferation and either the antibody response or the clinical outcome (Desai et al., 1995a). Inactivated JEV vaccine induced CD4$^+$, class II-restricted T cells with cytolytic activity (Aihara et al., 2000), suggesting that it is not capable of inducing high levels of CD8$^+$ CTLs; in mice, this vaccine elicits a T$_{H2}$ immune response (Ramakrishna et al., 2003). As noted earlier, in the context of clinical infections with flaviviruses, the integrity of cellular immune function appears to be important (Iwamoto et al., 2003; Neogi et al., 1998; Okhuysen et al., 1993; Szilak and Minamoto, 2000). However, better understanding of the effector properties of T cells and their role in protection in humans and in experimental animals is needed.

VIII. NEUROINVASION

Neuroinvasiveness is a critical step in the pathogenesis of flavivirus encephalitis and is affected by both viral and host factors. In terms of viral factors, characterization of various virulent and attenuated strains of JEV, TBEV, YFV, and WNV has revealed that viral determinants of neuroinvasiveness map principally the E protein (reviewed in McMinn, 1997). The mechanisms associated with these genetic determinants have not been completely determined, but are believed to relate to increased viral infectivity toward important target cells through enhanced binding and penetration. Entry into the CNS has been proposed to involve a number of potential processes, none of which has been definitively demonstrated in vivo. The proposed pathways include (1) transport across the cerebrovascular endothelium, or infection of these and other cells constituting the blood–brain barrier (Dropulic and Masters, 1990; Liou and Hsu, 1998); (2) access to the CNS after loss of blood–brain barrier integrity (Kobiler et al., 1989; Lustig et al., 1992); and (3) entry through the olfactory epithelium.
(McMinn et al., 1996; Monath et al., 1983). There are regions of the CNS where lack of a blood–brain barrier may constitute sites of vulnerability to infection in the presence of viremia (i.e., the choroid plexus and the circumventricular organs). Some of these may be supported by nonspecific defenses that are sufficient to withstand a low level of viremia, as the choroid is a site of IFN-α expression and OAS induction (Asada-Kubota et al., 1997; Khan et al., 1989). Entry of virus into the CNS by passage across the small cerebrovascular vessels is consistent with the accumulation of perivascular infiltrates of inflammatory cells, which is a hallmark of flavivirus encephalitis (Johnson et al., 1985). However, access through the olfactory bulb is believed to occur either after infection by the aerosol route or intranasally (Hambleton et al., 1983; Myint et al., 1999; Nir et al., 1965; Raengsakulrach et al., 1999) or in the context of hematogenous dissemination of virus (McMinn, 1996; Monath, 1986; Monath et al., 1983). The olfactory bulb is especially vulnerable to infection because of the exposure of its nerve terminals within the olfactory mucosa, and this route is exploited by other neurotropic viruses (Fazakerley, 2002).

Disruption of the blood–brain barrier facilitates the entry of noninvasive flaviviruses into the CNS (Lustig et al., 1992), suggesting that neuroinvasion may be influenced by host factors that alter the permeability of this barrier as a result of systemic infection (Chaturvedi et al., 1991; Kaiser and Holzmann, 2000; Mathur and Chaturvedi, 1992), including IFN-γ, TNF-α, and possibly effector functions of CTLs and NK cells (Lincon Luna et al., 2002). Neuroinvasion is also influenced by physical stress and other agents, including inhalational anesthetics (Ben-Nathan et al., 1989, 1992, 1996b, 2000). Immunosuppression by corticosterone and other endogenous immunomodulators is an important factor, as involution of lymphoid tissue has been observed and probably facilitates the generation of viremia by attenuated viral strains. Perturbation of the blood–brain barrier may also be involved in this process (Ben-Nathan et al., 2000). Early viremia and sustained viremia are correlated with neuroinvasion in animal models, consistent with the belief that replication to high titers in peripheral tissues is an important property of invasive strains, at least in immunologically normal hosts (Albrecht, 1968; Huang and Wong, 1963; Monath, 1986). However, the timing of peak viremia is probably critical, as levels sufficient to cause neuroinvasion are dissipated concurrent with the appearance of adaptive immune responses in the periphery (Diamond et al., 2003; Halevy et al., 1994). This is also evident from studies of immunodeficient mice lacking T- and B-cell responses, which exhibit neuroinvasion in the presence of viremia (Chambers and
Nickells, 2001; Charlier et al., 2002; Diamond et al., 2003; Johnson and Roehrig, 1999; Halevy et al., 1994; Lin et al., 1998). At present it appears that the mechanisms responsible for entry into the CNS may vary, depending on a specific virus and the level of host immunocompetence. Continued research with the use of knockout strains harboring single and multiple defects in these redundant immune system defenses will improve the understanding of how this process occurs.

The mechanisms of flavivirus spread within the CNS are not established. Studies with alphaviruses in the mouse model suggest that this occurs in a circuit-specific manner among olfactory neurons that are undergoing developmental synaptogenesis (Oliver and Fazakerley, 1998). Spread of MVE virus in immature mice exhibited a pattern consistent with these observations, but occurred throughout the CNS (McMinn et al., 1996). An unusual accumulation of WNV particles in myelin lamellae was observed in spinal cord cultures (Shahar et al., 1990), suggesting the possibility of virus transport by mechanisms not only involving the axoplasm.

IX. Neuropathology

Flaviviruses, particularly members of the JEV and TBEV serogroups, cause viral encephalitis in many vertebrate species, a dead-end transmission pathway believed to reflect an evolutionarily conserved capacity of these viruses to grow in the CNS of arthropods and vertebrates (Monath, 1986). In contrast to the noncytopathic nature of infection in arthropods, a spectrum of acute and chronic CNS pathologic changes occur in vertebrates and have been documented extensively (Chu et al., 1999; Dominguez and Baruch, 1963; Hase et al., 1993; Levenbook et al., 1987; Manuelidis, 1956; Nathanson et al., 1966; Pogodina et al., 1983; Reyes et al., 1981; Vince and Grevic, 1969; Zlotnik et al., 1971, 1976). Virus can be demonstrated within neurons throughout the brain and spinal cord, but infection of other cell types has been less well characterized. In humans, neuroinvasive flaviviruses cause an acute, often fatal encephalomyelitis (Monath, 1986) associated with characteristic inflammatory changes and often targeted to specific regions (Johnson et al., 1985; Miyake, 1964; Suzuki and Phillips, 1966; Zimmerman, 1946). The pathological changes have been characterized in many experimental animal models, as well as in fatal human cases. These viruses can evoke inflammatory infiltrates extending from the meningeal layers into the brain substance, with features typical of other viral encephalitides, including leptomeningitis,
perivascular lymphocytic accumulation, parenchymal infiltrates, and microglial nodules associated with neuronophagia in regions of viral infection (Figs. 3, 4, and 5). The neuropathology can, in some cases, include the destruction of vascular structures with focal hemorrhage, suggesting a vasculitis. Loss of regional blood flow, as well as disruption of the blood–brain barrier, has been described in clinical cases of TBEV (Gunther et al., 1998; Kaiser, 2002; Kaiser and Holzmann, 2000). The nature and degree of the inflammatory disease depend on many factors, including the virulence of the virus, the route of infection, and the age and immunocompetence of the host. Varying levels of CNS inflammation without frank evidence of neuronal damage have been described in some models, including human autopsy cases, suggesting that viral infection can induce lethal pathophysiology prior to or in the absence of recruitment of the peripheral immune response (reviewed in Monath, 1986). Although cytopathic effects have been observed primarily in virus-infected neurons, other noninfected cells can also exhibit pathologic changes, presumably through bystander injury (see later). A number of studies have documented the distribution of the neuropathology and the clinical manifestations that characterize flavivirus infection of the CNS. For instance, infection in cortical

Fig 3. Yellow fever virus meningoencephalitis in the rhesus monkey showing leptomeningeal accumulation of acute inflammatory cells. Courtesy of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).
FIG 4. Yellow fever encephalitis in the rhesus monkey showing focus of perivascular infiltrate with mononuclear cells in the cerebral cortex. Courtesy of USAMRIID.

FIG 5. Yellow fever encephalitis in the rhesus monkey showing microglial nodule with neuronophagia of a cortical neuron stained for viral antigen. Courtesy of USAMRIID.
regions typically gives rise to depressed consciousness and seizures, but the involvement of subcortical regions, including the midbrain and brainstem, as well as thalamic and basal ganglial involvement, can give rise to a variety of movement disorders (Asher, 1975; Kalita and Misra, 2000; Misra and Kalita, 1997a; Murgod et al., 2001; Ogata et al., 2000; Pradhan et al., 1999). WNV encephalitis in the United States and JEV encephalitis have both been characterized by a poliomyelitis-like syndrome, suggesting infection of lower motor neurons in association with flaccid paralysis (Glass et al., 2002; Leis et al., 2002; Misra and Kalita, 1997b; Solomon et al., 1998). Lower motor neuron disease is also typical of TBEV infection. Factors governing the differences in neuronal susceptibility to flaviviruses are not known, but may be similar to those that operate in the case of other neurotropic arboviruses, such as Sindbis, where the neuronal response to viral injury may be variable (Griffin and Hardwick, 1999). Neuronal death associated with flavivirus infection has classically been ascribed to degenerative necrosis. Pathologic changes that accompany this process include vacuolization and proliferation of intracellular membranes, which produces a characteristic ultrastructural appearance (Fig. 6; Murphy et al., 1968). Whether there is a greater propensity to cause necrosis versus apoptosis requires further study.

![Image](image.png)

**Fig. 6.** Electron micrograph of a mouse CNS neuron infected with SLE virus showing characteristic cytoplasmic pathology but integrity of the nuclear (N) envelope. Arrow indicates virions within inner and outer nuclear membranes. From Murphy et al. (1968), with permission.
Several flaviviruses have been shown to induce apoptosis, both in vitro (Isaeva et al., 1998; Liao et al., 1998; Parquet et al., 2001; Prikhod’ko et al., 2002) and in vivo, in the rodent CNS (Andrews et al., 1999; Després et al., 1998; Duarte dos Santos et al., 2000; Isaeva et al., 1998; Xiao et al., 2001; Fig. 7). In this regard, reports describing primarily degenerative pathology at the light or electronmicroscopic

Fig 7. WNV infection in the Syrian golden hamster. (A) Viral antigen-positive neurons in the cerebral cortex. (B) TUNEL-positive apoptotic neurons in the cortex. Courtesy of Dr. Shu-Yan Xiao. From Xiao et al., 2001.
level, typically involving cytoplasmic changes such as chromatolysis, swelling, and dissolution of Nissl substance, also mentioned nuclear pathology, including pyknosis, disruption of the nuclear envelope, and alterations in chromatin (Dominguez and Baruch, 1963; Manuelidis, 1956; Mathews et al., 2000). These findings are of interest given reports that neuronotropic viruses such as Sindbis virus may not induce typical apoptotic morphology in neurons (Griffin and Hardwick, 1999; Havert et al., 2000; Kerr et al., 2002; Sammin et al., 1999) and that cell death may occur through both apoptotic and necrotic mechanisms (Havert et al., 2000; Nargi-Aizenman and Griffin, 2001; Sammin et al., 1999), depending on the integrity of the apoptotic pathways of a given population of neurons, their profile of apoptotic modulators, and the presence of excitotoxic stimuli. The most detailed in vivo characterization of neuronal apoptosis by flaviviruses has been reported for dengue. Neuroadapted dengue virus induces apoptosis in infected neurons as well as in noninfected cells (Després et al., 1998), suggesting that indirect mechanisms of cellular injury occur in areas of heavy virus burden. It is important to note that these findings were demonstrated in very young mice, whose neurons are highly susceptible to apoptotic stimuli, and may not reflect the response of more mature cells in older mice. However, studies with WNV in the adult hamster model provide evidence that highly neurovirulent strains are potent inducers of apoptosis (Xiao et al., 2001; Fig. 7). A relationship between viral virulence and the extent of apoptosis has not been clearly established for flaviviruses; however, the general capacity of neuroadapted strains to produce high virus burdens in association with cytopathology suggests that the situation is likely to resemble that of alphaviruses and other neuronotropic viruses (Lewis et al., 1996; Oberhaus et al., 1997; Theerasurakarn and Ubul, 1998). Although the molecular details of this process are not fully known, the expression of some flavivirus proteins appears to directly induce apoptotic cell death of neurons, including the WNV capsid protein (Yang et al., 2002), the Langat virus NS3 protein, which causes apoptosis through the activation of caspases 3, 8, and 9 (Prikhod’ko et al., 2002), and the E proteins of neuroadapted dengue-2 virus (Duarte dos Santos et al., 2000) and JEV, which appear to stimulate an ER unfolded protein response (Su et al., 2002) and a component of oxidative stress (Raung et al., 2001). However, there are many other potential mechanisms for provoking apoptosis by neuronotropic RNA viruses, including signaling through interferon-α-dependent pathways, phospholipase A2 activation, activation of NF-κB and p53-regulated genes (Fazakerley, 2001), and activation of apoptosis during
viral entry (Jan et al., 2000). Some of these processes may also be involved in the pathogenesis of encephalitic flaviviruses.

The role of apoptotic regulators bcl-2, bax, bcl-X, and related gene products in modulating neuronal death has been only partially characterized for flaviviruses. Forced expression of bcl-2-related genes promotes the survival of neuronal cell lines infected with JEV and dengue virus and facilitates viral persistence, primarily by restricting virus-induced cytopathic effects and not viral replication (Liao et al., 1997, 1998). These outcomes vary in neuronal versus nonneuronal cell lines, and differential effects of bcl-2 and bcl-X appear to operate. Such findings suggest that the effects of apoptosis modulators on flaviviruses are similar to those observed with alphaviruses, where it is known that the expression of these proteins can vary in their effects from one cell type to another. Conclusions about the role of these proteins in different in vivo models therefore require careful evaluation (Griffin and Hardwick, 1999; Levine, 2002). Furthermore, because neuronotropic viruses can induce both necrosis and apoptosis, neuronal death may require assessment by several criteria. Neuronal injury as a result of bystander effects may also be a factor during flavivirus neuropathogenesis given that microglial activation and elaboration of inflammatory mediators, including IL-1β and TNF-α, occur in the CNS during these infections (Andrews et al., 1999; Liu and Chambers, 2001; Ravi et al., 1997) and may accompany the production of nitric oxide and peroxynitrite, which can cause neurotoxicity. Other potential mechanisms include excitatory cell death due to the activation of NMDA receptors, which has been implicated in the pathogenesis of Sindbis virus and HIV (Nargi-Aizenman and Griffin, 2001). Thus, although it is likely that the neurovirulence phenotype of flaviviruses is linked to the extent of neuronal cell death caused during the encephalitis, there appear to be multiple independent mechanisms by which neuronotropic viruses cause cell death. This process can be affected by the region of the brain affected, the degree of neuronal maturity, the factors that regulate cell death signaling receptors and their pathways, and levels of apoptosis modulators and other innate responses of virus-infected neurons (Fazakerly, 2001; Griffin and Hardwick, 1999; Levine, 2002; Liang et al., 1998). In some cases, changes in the expression of neurotrophins may also be involved in the CNS response to viral injury (Zocher et al., 2000); however, the relevance of this phenomenon to other types of viral encephalitis has yet to be widely investigated. The process is also subject to additional influence by the properties of the immune response recruited into the CNS, including CD4+ and CD8+ T cells, which may be involved in
cytotoxicity toward virus-infected and, in the case of CD4$^+$ cells, perhaps even noninfected cells (Després et al., 1998; Gagnon et al., 1999), through Fas-dependent mechanisms under certain circumstances (Medana et al., 2000). The effects of nonspecific inflammation, such as release of toxic substances from neutrophils (Andrews et al., 1999), may also contribute to cellular injury.

X. THE CENTRAL NERVOUS SYSTEM IMMUNE RESPONSE

Flavivirus infections induce a CNS inflammatory response of variable intensity. Data from most experimental models suggest that this inflammation is a requirement for protection from lethal infection with neurovirulent strains. The characteristics of the inflammation in experimental models have been shown to be affected by numerous factors, which include the endogenous CNS response, as well as the adequacy of the peripheral immune response and its timely recruitment into the CNS. In some cases of encephalitis, relatively scant inflammatory disease has been noted. This has also been observed experimentally as, for example, with TBEV under conditions where viral neuroinvasion and CNS involvement progress rapidly (Vince and Grevic, 1969). Immunosuppression also dramatically reduces the intensity of the CNS inflammation (Hirsch and Murphy, 1967; Leyssen et al., 2003a). However, acute inflammation may become severe in response to a heavy antigen load and has been implicated in immunopathologic reactions in the CNS (reviewed in Monath, 1986).

A. Innate Responses

Viral infections of the CNS commonly result in the induction of innate responses, which include activation and proliferation of microglia and activation of astrocytes and cerebrovascular endothelium, with ensuing production of chemokines and proinflammatory cytokines (Benveniste, 1997). A consequence of this activation is the conditioning of cells in the CNS parenchyma and the blood–brain barrier to accommodate and modulate the influx of activated lymphocytes from the periphery by the upregulation of adhesion molecules and class I and II antigens. Activation of innate responses within the CNS during flavivirus encephalitis has been suggested by several human and experimental animal model studies in which the expression of chemokine and cytokine genes or their proteins has been analyzed. The production of IL-8 and macrophage inhibitory factor (MIF) has
been detected in cerebrospinal fluid (CSF) or brain tissue during early stages of encephalitis with JEV and MVE viruses (Andrews et al., 1999; Singh et al., 2000; Suzuki et al., 2000), with levels of IL-8 correlating directly with the number of neutrophils in the CSF. TNF-α and IL-1β are also elicited in response to flavivirus infection of the CNS (Liu and Chambers, 2001; Ravi et al., 1997; Suzuki et al., 2000), presumably representing intrinsic responses of microglia and astrocytes to the acute injury (Benveniste, 1997). Thus the early stage of the encephalitis involves the endogenous expression of mediators that results in the recruitment of nonspecific acute inflammatory cells with the potential for the production of neurotoxic substances, such as reactive oxygen intermediates, and probably facilitates further stimuli that intensify the inflammation. For instance, IL-1β and TNF-α can also mediate the release of IL-8 from astrocytes (Aloisi et al., 1992). The induction of the nonspecific acute inflammation may be a deleterious process because levels of IL-8 are predictive of fatal disease (Suzuki et al., 2000) and treatment of infected mice with inhibitors of NOS-2 in the acute stage of encephalitis lessened mortality in association with reduced inflammation (Andrews et al., 1999). Because viral infections of the CNS induce the expression of chemokines, which have been implicated in inflammatory cell recruitment, (Liu and Lane, 2001; Liu et al., 2000, 2001), the intense level of inflammation observed in flavivirus encephalitis may be driven by the induction of one or more chemokines and their receptors. The expression of monocyte and T-cell chemokines has not been reported in these infections; however, studies with other models suggest that MCP-1, IP-10, RANTES, and other chemokines are involved in the trafficking of leukocytes into areas of virus infection in the CNS. In conjunction with the effects of TNF-α and IFN-γ, both of which can lead to loss of integrity of the blood–brain barrier, this collection of stimuli may be sufficient to drive the commitment phase of the inflammatory response, during which the unrestricted entry of T lymphocytes then proceeds (Hickey, 1999).

The intrinsic defensive response of the CNS to viral injury also includes the induction of other classes of genes likely to influence the antiviral activity of this compartment through direct effects and by shaping the virus-specific immune response to viral injury (Johnston et al., 2001; Labrada et al., 2002). These include IFN-α and IFN-regulated genes such as ISG12 (Labrada et al., 2002). IFN-α itself does not seem to be strongly upregulated in acute encephalitis. However, IFN-α has been reported in the CSF and brain tissue of human cases of encephalitis with JEV serogroup viruses and, in such cases, appeared to represent a marker of severe infection with a fatal outcome (Burke and
The role of type I interferons in the CNS response to injury is complex, and differences in the activities of IRFs and IGSFs toward target response elements in neurons and glia can affect the range of genes involved in the response, including MHC antigens, the OAS1b protein, and antiapoptotic factors (Baron-Delage et al., 2000; Hirsch et al., 1986; Lucas et al., 2003; Massa et al., 1999; Njenga et al., 1997). Studies of viral infection in IFN-α and IFN-α-regulated gene knockout mice should help clarify the role of this defense system in directly controlling the viral infection of neurons versus effects on promoting the activity of the immune response recruited from the periphery. The importance of IL-12 in the innate CNS response to flavivirus encephalitis has not been investigated extensively; however, the outcome of CNS disease appears to vary among different viruses when this cytokine is used as an experimental therapy (Phillpotts et al., 2003). Because of the importance of IL-12 in antiviral defense against other CNS viral infections, through its ability to stimulate NOS (Reiss et al., 2002), more studies are needed to determine if it is implicated in protection against flaviviruses.

B. Virus-Specific Responses

Compared to information available on other neurotropic viruses, there has been relatively little work done to characterize the properties of virus-specific T cells recruited into the CNS in response to flavivirus infection (Scheider-Schaulies et al., 1997). This response is subject to a number of influences, including previous immunologic experience with related viruses, the level of immunocompetence, immunogenetic host factors, and the virulence of the infecting virus. Available information comes from a limited number of human and animal model experiments, which partially characterized lymphocytes or soluble markers of activated T cells and cytokines in the CNS (Burke et al., 1985b; Carson et al., 2003; Gunther et al., 1996; Iwasaki et al., 1993; Johnson et al., 1985, 1986; Kuno et al., 1993; Sampson et al., 2000), and other studies that have evaluated the requirements for these cells in the control of infection of this compartment in mouse models (Liu and Chambers, 2001; Liu et al., 1989a; Murali-Krishna et al., 1996; van der Most et al., 2000, 2003). T cells bearing both CD4+ and CD8+ surface markers have been visualized in perivascular infiltrates, in CSF, and in brain parenchyma during human flavivirus encephalitis (Johnson et al., 1985, 1986; Sampson et al., 2000). These cells include a larger proportion of CD4+ to CD8+ T cells and moderate numbers of B cells and macrophages. However, the composition of cells differed regionally
in the CNS, with macrophages and T cells more abundant in brain parenchyma and B cells more common within perivascular infiltrates. Factors governing the distribution of these cells and their effector functions are not well understood. Data from other experimental models suggest that CD4+ T cells are important in directing the recruitment of lymphocytes (Hickey, 1999) and in maintaining the effector function of CD8+ T cells within the brain parenchyma (Stohlman et al., 1998). CD8+ T cells have clearly been isolated from the brains of flavivirus-infected mice and, in some cases, demonstrated to have cytolytic activity (Liu et al., 1989a); however, the contributions of such cells to both virus clearance and cellular injury within this compartment have not been defined. This remains a fundamental question, as the role of CD4+ and CD8+ T cells in different viral infections of the CNS and different experimental paradigms can vary considerably (Schneider-Schaulies et al., 1997). For instance, virus clearance from mouse brains acutely infected with JEV has been reported by virus-specific CD8+ T cells adoptively transferred into the CNS; however, CD4+ T cells were also required (Murali-Krishna et al., 1996). The relationship of this adoptive response to the natural immune response recruited from the periphery remains unclear, as the route of transfer may not reflect the normal pathway of lymphocyte trafficking. A requirement for CNS-associated CD4+ and CD8+ T cells was also observed in an immunization/challenge model of dengue virus (van der Most et al., 2000). However, in the context of a memory response to YFV in the mouse model, CD4+ T cells and B cells were required for the control of viral infection, whereas CD8+ T cells were not required, although CD8-deficient mice exhibited a defect in virus clearance (Liu and Chambers, 2001; T. J. Chambers, unpublished data). Part of the effector activity of CD8+ T cells may be mediated by the production of IFN-γ, which has a range of effects on the immunological properties of the CNS, including the upregulation of class I and II antigens on glial cells and injured neurons, activation of microglial, priming of astrocytes for cytokine production, and increasing permeability of the blood–brain barrier, as well as antiviral activity in the brain parenchyma (Benveniste, 1997; Kundig et al., 1993; Popko et al., 1997). IFN-γ knockout mice are defective in the clearance of YFV from the CNS and exhibit decreased inflammatory cell recruitment to this compartment (Liu and Chambers, 2001), indicating an important role of this cytokine in flavivirus encephalitis. IFN-γ may have a primary role in these processes because elimination of virus-infected neurons by CTLs is very tightly constrained by the absence of constitutive expression of MHC class I in these cells and the lack of susceptibility to perforin-mediated lysis (Medana et al., 2000; Neumann et al.,
Although killing can occur through Fas–FasL interactions (Medana et al., 2000), induction of FasL expression by neurons can also confer protection against CTL attack under certain conditions (Medana et al., 2001) and even induce apoptosis in infiltrating lymphocytes (Flugel et al., 2000). Virus-specific CD8+ T cells have been shown to persist in the brains of mice recovering from dengue encephalitis for prolonged periods and differentiate into effector–memory cells that lose CTL activity while still producing IFN-γ (van der Most et al., 2003). Thus it appears that cellular immune-mediated mechanisms of neuronal cell death are subject to tight regulation in the presence of acute viral injury.

The role of antibody responses in the control of flavivirus encephalitis has been investigated in many classic studies, and it has been demonstrated repeatedly that immune serum can arrest viral infection in the CNS (Roehrig et al., 2001). Mechanisms responsible for this antibody-mediated control of infection remain unclear; however, studies with YFV encephalitis indicate that the Fc region and the IgG subclass are critical for protection in the mouse model (Schlesinger et al., 1993, 1995). These data suggest that direct effector functions associated with IgG, including interactions with cells bearing Fc receptors, such as microglia and recruited macrophages, are involved in this process. It is also conceivable that antibody–dependent cellular cytotoxicity directed against cell surface NS1 or even complement-mediated lysis also contributes to the protective effect. The antibody-mediated control of neuronal infection is a well-established mechanism for alphaviruses and coronaviruses, in which case the role of antibody in preventing reactivation of viral infection has been demonstrated (Griffin et al., 1997; Levine et al., 1991, 1992; Lin et al., 1999). In the case of Sindbis virus, there is inhibition of viral release and eventual sequestering of viral RNA within neurons without clearance. This process occurs preferentially at cortical sites of infection and obligates the local retention of virus-specific B cells (Tyor et al., 1992) in contrast to infection in the spinal cord where the cytokine-mediated (IFN-γ) elimination of viral RNA apparently predominates (Binder and Griffin, 2001). Antibody-mediated mechanisms may also operate in flavivirus encephalitis, as reactivation or recrudescence of CNS disease has been documented in some experimental animal systems and in clinical cases (Section XII). Factors responsible for the prolonged survival of B cells in the CNS are not known, particularly whether these cells represent a resident population of memory cells or require replenishment from peripheral sites (Tschen et al., 2002).

The beneficial role of antibody responses within the CNS is also supported by clinical studies of JEV and TBEV encephalitis (Burke et al., 1995).
High levels of IgM correlate with an improved outcome in some cases, presumably reflecting the recruitment of virus-specific B cells into the CNS, as was reported in JEV cases (Burke et al., 1985a; Gunther et al., 1997), and the presence of IgG1 in particular is associated with the control of infection (Thakare et al., 1991), although IgM can be elevated in severe cases with a fatal outcome (Desai et al., 1994a). The functional activities provided by IgM in the CNS that result in clinical benefit remain unclear, as experimentally, IgM may have limited neutralization activity against encephalitic viruses at least early in infection (Diamond et al., 2003; Hoffman et al., 1978; Ishii et al., 1968). The contribution of complement-fixing activity could be important, as discussed earlier, in view of the fact that complement proteins may be upregulated in the CNS as a result of viral infection (Johnston et al., 2001).

The inflammatory response to flavivirus infection of the CNS may, in some cases, cause deleterious effects on neuronal function and survival. Immune complexes and autoantibodies to neurofilaments and myelin basic protein in the CSF and serum have been reported in severe JEV and TBEV infections (Desai et al., 1994a, 1994b; Fokina et al., 1991; Thakare et al., 1988) and may reflect an immunopathogenic process rather than nonspecific reactions to viral injury, particularly because of their association with poor outcome. Mechanisms leading to resolution of the acute inflammation in CNS viral infections remain undefined (Bradl and Flugel, 2002). At least three potential factors could be involved, including the expression of IL-4, IL-10, and TGF-β, which have been observed in the CNS of Sindbis virus-infected mice (Wesselingh et al., 1994) and are known to have immunomodulatory activities that reduce CNS inflammation; induction of apoptosis of infiltrating T cells; or possibly entry of NKT cells, which have been associated with the suppression of inflammatory responses. Further studies are needed to determine whether these or other factors are required to downregulate potentially harmful immune responses in cases where viral disease is eventually controlled.

**XI. NEUROPATHOGENESIS: WEST NILE VIRUS AS A MODEL**

Similar to JEV, infections with WNV can be characterized as protean, involving an extraordinary host range, and an abundance of pathologic and virologic data has been obtained from experiments with
WNV in birds (Kramer and Bernard, 2001; Steele et al., 2000), horses (Bunning et al., 2002), and humans (Asnis et al., 2000; Hubalek and Halouzka, 1999). WNV has emerged as dimorphic in its clinical disease, with the apparent shift from typical WN fever to disease of greater severity, including more frequent cases of acute encephalitis in conjunction with the emergence of New World lineage I strains (Asnis et al., 2000; Cernescu et al., 1997). Studies with virulent strains such as New York 1999 WNV have demonstrated the neuropathogenic potential inherent in this virus. It is therefore important to note that lineage differences (type I versus type II) and perhaps genetic variation within lineages may influence the development of CNS disease.

After peripheral inoculation in the mouse model, WNV is believed to infect Langerhans dendritic cells (Johnston et al., 2000), which migrate to draining lymph nodes, and within 12 to 24 h of infection, viral replication is observed in secondary lymphoid tissue (Diamond et al., 2003; McMinn et al., 1996). Infectious virus is detected in serum within 24 to 48 h of infection (Diamond et al., 2003; Kramer and Bernard, 2001). The course of the early infection is slightly different in wild-type (WT) versus B-cell-deficient mice, as the peak of viremia occurs later in the latter case (day 2 versus days 4 to 6; Fig. 8). Shortly afterward, in WT mice, infectious virus is detected in visceral organs such as the spleen, kidney, and heart but not the liver (Diamond et al., 2003; Kramer and Bernard, 2001; Weiner et al., 1970), which may reflect restricted tropism or a high level of reticuloendothelial clearance in this organ. The levels of infectious virus in visceral tissues and serum peaked by day 4 after infection and thereafter diminished (Diamond et al., 2003; Kramer and Bernard, 2001; Xiao et al., 2001), concurrent with a rise in the titer of neutralizing antibodies (Fig. 9). In B-cell-deficient mice, replication in peripheral lymphoid tissue and visceral organs follows kinetics similar to those of WT mice; however, virus is not cleared from these sites. These data indicate the profound susceptibility of mice to WNV in the absence of antibody-producing B cells.

Virus can first be detected in the CNS by 4 days after peripheral subcutaneous infection in both WT and B-cell-deficient mice. Infectious virus is detected simultaneously in multiple sites in the brain, as well as in the inferior and superior spinal cord, suggesting a hematogenous route of dissemination and/or rapid spread within the CNS (Diamond et al., 2003). However, the route of peripheral inoculation influences the rate of spread to the brain and spinal cord. Infection via an intraperitoneal or intravenous route results in the spread of infectious virus to the brain within 2 days of infection (Kramer and Bernard, 2001), with these animals succumbing to infection several days earlier than
those infected by a subcutaneous route. These differences probably reflect the fact that the interaction of virus with peripheral tissues results in the engagement of nonspecific and virus-specific defenses that have an impact on the course of disease. Models of infection that bypass the physiologic route of inoculation must be considered with this limitation in mind, as the balance of viral replication and dissemination versus immune activation can be skewed greatly by intraperitoneal or intravenous injection. Regardless of the route of administration, the course of disease after the onset of CNS infection is rapid and leads to fatal encephalitis beginning by days 9 to 10 postinfection, associated with high burdens of brain-associated virus.

Neutralizing activity associated with the IgM class is detectable by day 4 postinfection in the mouse model (Fig. 9), and this antibody response can confer partial protection against virus challenge in naïve mice (Diamond et al., 2003). Neutralizing IgG is detectable by day 8 and reaches levels of activity that are 10-fold higher than that of IgM by day 12 postinfection. The importance of secreted IgM in protection against disease is indicated from these data, as well as data presented

Fig 8. Wild-type or B-cell-deficient (uMT) mice were inoculated with WNV in the footpad, and the virus content in serum, peripheral tissues, and brain was measured serially using plaque assay or quantitative polymerase chain reaction. From Diamond et al. (2003), with permission.
earlier (Fig. 1). The relative importance of the mechanisms involved, including neutralizing activity per se or complement-assisted priming of the B-cell response to facilitate IgG production, is currently under investigation.

Fig 9. Neutralizing IgM and IgG antibody responses in acute WNV encephalitis in the mouse model. From Diamond *et al.* (2003), with permission.
Persistent infection in vitro and in vivo has been described in a number of experimental and clinical settings involving flaviviruses. The phenomenon of persistent infection in cell cultures of vertebrate and arthropod origin is well documented (Brinton, 1982; Chen et al., 1996; Jarman et al., 1968; Igarashi, 1979; Katz and Goldblum, 1968; Lancaster et al., 1998; Loginova et al., 1980; Poidinger et al., 1991; Randolph and Hardy, 1988a, 1988b; Schmaljohn and Blair, 1977, 1979; Shah and Gadkari, 1987; Vlaycheva and Chambers, 2002; Zhang et al., 1993), and the findings are analogous to many other models of viral infections in vitro where this process can be observed. Flavivirus persistence in vitro typically arises following a cytocidal infection, with survival of a residual population of cells that harbor low levels of replicating virus for long periods of time. The majority of cells in such cultures usually express viral antigen, but only a minority actually are productive of infectious virus. The cultures are generally resistant to superinfection with homologous but not heterologous virus, although in some cases, superinfection can drive virus production in quiescent antigen-positive cells (Schmaljohn and Blair, 1979). The persistence is not dependent on the expression of IFN-α, rather there is evidence for the involvement of host cell antiapoptotic pathways (see later). The infection is not necessarily deleterious for host cells, but in some cases, reduced growth efficiency occurs. Viruses detected in persistently infected cultures frequently undergo phenotypic alterations, including a reduction in plaque size, temperature sensitivity (Randolph and Hardy, 1988b; Shah and Gadkari, 1987), host-range restriction (Randolph and Hardy, 1988b), and loss of neurovirulence for mice (Igarashi, 1979). The genetic basis for the phenotypic change of viral variants has, in some cases, been demonstrated (Vlaycheva and Chambers, 2002). However, these infections are also associated with the emergence of defective viral particles or defective RNAs (Brinton, 1982; Debnath et al., 1991; Lancaster et al., 1998) that form the basis for superinfection resistance, and perhaps in part for the attenuation of mouse neurovirulence in flavivirus–resistant strains of mice. Some persistently infected cultures exhibit alterations in the composition of viral proteins or produce truncated forms of NS1 (Chen et al., 1996), which presumably are believed to reflect deletions in the viral genome associated with the generation of defective interfering viruses. The genome of defective MVE virus in Vero cells was shown to lack a large segment encompassing the prM-E region and a portion of the NS1 region, which leads to the production of truncated NS1 (Blitvich et al.,
1999; Lancaster et al., 1998). This finding is entirely consistent with the fact that replicons such as those engineered for Kunjin virus are designed to delete the corresponding region of the genome and are able to establish persistent replication in cell culture without any typical viral cytopathic effects (Varnavski and Khromykh, 1999). At least part of the explanation for the loss of cytotoxic activity therefore may result from the deletion of structural proteins, which may provoke an ER overload response and/or trigger apoptosis (Duarte dos Santos et al., 2000; Prikhod’ko et al., 2001; Su et al., 2002). The production of infectious virus from persistently infected cultures can be reduced by treatment with a neutralizing antibody (Randolph and Hardy, 1988a), but it is not clear whether this shifts the cells to preferentially harbor defective viral genomes or whether these are actually eliminated.

The expression of apoptotic modulators is an important factor influencing the establishment and maintenance of persistent infection. In a model of JEV infection, the persistence in some cell lines was facilitated by the expression of bcl-2 (Liao et al., 1997), indicating that the mechanism is analogous to that of alphaviruses, where lytic infection can be converted to persistence in the presence of antiapoptotic proteins (Levine et al., 1993). However, the mechanisms that confer resistance to JEV-induced apoptosis must differ in some way from those of Sindbis and other viruses, as bcl-2 could fully protect N18 neuroblastoma cells against Sindbis, but not JEV. It is likely that the difference involves divergent pathways for activating the apoptotic process (Jan et al., 2000; Su et al., 2002). It is also possible that some cell lines represent variants that are defective in their apoptotic pathways, allowing virus to adapt in the absence of apoptotic events. At present, there is no evidence that flaviviruses encode proteins with antiapoptotic properties that might influence the process of the persistence.

The relevance of data on persistently infected cells in vitro to the issue of persistent infection in animal models and in apparent human cases is not straightforward. Although evidence for such clinical entities has been reported, the situation is complicated by the fact that there may be overlap between infections that have protracted convalescence and those that have frank neurological sequelae because flavivirus encephalitis is associated with neurologic complications that confer long-term disability among those who survive acute infection (Baruah et al., 2002; Finley and Riggs, 1980; Greve et al., 2002; Haglund and Gunther, 2003; Huy et al., 1994; Kumar et al., 1993; Richter et al., 1961; Vaneeva, 1969). In some cases, there is radiographic and pathologic evidence of permanent neurologic injury, including reduced cerebral blood flow, areas of abnormal signal density, and cellular dropout and gliosis.
(Gunther et al., 1998; Ishii et al., 1977; Kumar et al., 1997; Shoji et al., 1990). Second, the possibility that a recrudescent latent infection really represents reinfection in a host with suboptimal immunity is a difficult question to evaluate. Also, the criteria for defining persistence are somewhat arbitrary. The presence of prolonged expression of IgM has been invoked as a basis for concluding persistence, but only infrequently has the presence of infectious virus been documented in situations where the duration of CNS disease exceeds that which is expected in acute uncomplicated flavivirus encephalitis (2 to 3 weeks).

Mechanisms of flavivirus persistence in vivo may theoretically also involve the formation of DI particles, which reduce infectious viral load, but like their in vitro counterparts, may not be sufficiently cytopathic to cause neuronal cell death. Such defective viruses may be capable of stimulating immune responses and thus be detected serologically. Experimentally, there is certainly evidence that DI particles can inhibit the production of infectious virus in the CNS (Atkinson et al., 1986; Barrett and Dimmock, 1984; Smith, 1981). The failure to detect infectious virus in both clinical and experimental situations where prolonged evidence of viral activity in the CNS was suspected is consistent with this hypothesis (Pogodina et al., 1983; Ravi et al., 1993). In a study of viral RNA in the brains of mice with the Flv resistance allele, a reduction in genome-length RNA rather than appearance of DI RNAs was observed (Urosevic et al., 1997), even though flavivirus–resistance in mice is associated with production of D1 virus (Smith, 1981). Thus, data implicating the role of DI particles in in vitro persistence may not correspond directly to an in vivo process.

Encephalitic flaviviruses that have been implicated in the occurrence of persistent infection of the CNS include members of the JEV serogroup and TBEV serogroup (Iliyenko et al., 1974; Ogawa et al., 1973; Pogodina et al., 1983; Slavin et al., 1943; Zlotnik et al., 1971), with the latter having a particular propensity to cause chronic infections. In humans, there have been reports of various types of persistent infection. These include (1) reactivation of latent disease, as in children with JEV encephalitis who experienced recurrent infection months after the primary infection and, in some cases, gave positive virus isolations (Sharma et al., 1991); (2) chronic progressive disease with cognitive and motor disturbances that resembled subacute sclerosing panencephalitis, years after a primary infection with Russian spring–summer encephalitis (RSSE) virus (Ogawa et al., 1973); (3) prolonged infection in primary cases of acute encephalitis, with elevation of CSF IgM for as long as 10 months and, in some cases, with virus isolated from spinal fluid (Ravi et al., 1993); and (4) evidence of prolonged
circulation of virus-infected cells without clear-cut CNS disease (Southam et al., 1958). Protracted cases of TBEV encephalitis, with either cognitive dysfunction or spinal nerve paralysis, occur frequently (Haglund and Gunther, 2003), although the relationship of these outcomes to persistent viral infection is not known. These different types of clinical infections have been mimicked by various experimental models, and it has been documented that virus can be recovered after a protracted phase of infection. For instance, strains of WNV with differing neurovirulence properties were capable of causing prolonged encephalitis of variable severity, ranging up to 5 months in duration in monkeys (Pogodina et al., 1981, 1983). Eventually the viruses were cleared or were rendered replication defective and detectable only by the presence of viral antigen. Some viruses recovered from brains of these animals had undergone attenuation of mouse neurovirulence, indicating a selection for genetic variants and/or defective interfering particles. Mechanisms preventing the efficient clearance of virus were not determined, but did not involve defects in the production of neutralizing antibodies. Immunosuppression, either by cytotoxic drugs or even that associated with pregnancy, has been demonstrated to cause prolonged flavivirus disease (Mathur et al., 1986; Zlotnik et al., 1971), and reactivation of infection can be elicited during or after the primary infection by immune depletion (Mathur et al., 1986). In some cases, as in JEV infection of mice, viral latency was established in T lymphocytes, which could be subsequently activated by immunosuppression to produce infectious virus (Mathur et al., 1989). These latter findings are consistent with reports of recurrent JEV infection observed in children, but the stimulus for reactivation in such cases is not known (Sharma et al., 1991).

In the CNS, experimental persistent infections have been related to immune response factors, as well as the ability of neurons to survive viral infection by mechanisms involving apoptotic modulators. Immunological tolerance is also a potential factor that has been associated with other viruses such as LCMV that establish persistent CNS infection, but this does not seem to play any obvious role in flavivirus persistence. Failure to clear brain-associated virus may result from a limited effectiveness of the innate CNS defenses and the peripheral immune responses that are activated in response to flavivirus infections. In addition, elimination of viral RNA may require CD8+ T-cell functions [as described for alphaviruses (Binder and Griffin, 2001)], which are severely restricted due to a lack of class I expression on neurons. The generally resistant state of differentiated neurons toward apoptotic stimuli is also likely to be an important factor (Griffin and Hardwick, 1999).
Together, these processes may facilitate the persistence of viral RNA and perhaps foster the evolution of DI particles and noncytopathic-attenuated viral variants. The host immune response, together with properties of the neuronal cellular environment, influences the likelihood of virus persistence and may both contribute to differences in phenotypes of persisting viruses. For instance, WNV variants that emerged from persistent infection in monkeys tended to acquire attenuation phenotypes (Pogodina et al., 1983), whereas it has been observed in persistent infection of mice with Sindbis virus that neurovirulent mutants arise (Levine and Griffin, 1992, 1993). Selection pressures imposed by neutralizing antibodies that are produced intraparenchymally by virus-specific B cells may facilitate the emergence of viral variants in some cases, but tissue-specific adaptations are also involved. At present, it is reasonable to believe that the phenomenon of flavivirus persistence in the CNS is a function of many variables, which include the heterogeneity of the neuronal response to injury, encompassing innate interferon-regulated antiviral defenses (Johnston et al., 2001) and the competence for and propensity towards apoptosis. Interaction of virus–infected cells with virus-specific T cells possessing cytokine-mediated effector functions that can eliminate viral RNA and B cells that can provide antibodies capable of downregulating viral replication influences this process in a region-specific manner. The balance among these factors results in a spectrum of outcomes that may range from either clearance to merely suppression of viral infection, with variable consequences for long-term neurologic function.

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