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Progress on the Development of Therapeutics against West Nile Virus

Michael S Diamond
Departments of Medicine, Molecular Microbiology, Pathology & Immunology, Washington University School of Medicine, St. Louis, MO

Abstract

A decade has passed since the appearance of West Nile virus (WNV) in humans in the Western Hemisphere in New York City. During this interval, WNV spread inexorably throughout North and South America and caused millions of infections ranging from a sub-clinical illness, to a self-limiting febrile syndrome or lethal neuroinvasive disease. Its entry into the United States triggered intensive research into the basic biology of WNV and the elements that comprise a protective host immune response. Although no therapy is currently approved for use in humans, several strategies are being pursued to develop effective prophylaxis and treatments. This review describes the current state of knowledge on epidemiology, clinical presentation, pathogenesis, and immunobiology of WNV infection, and highlights progress toward an effective therapy.

Keywords

Therapy; West Nile virus; Antiviral; Antibody; Pathogenesis

I. Biology of WNV Infection

A. Ecology, Epidemiology, and Clinical Manifestations

West Nile virus (WNV) was first isolated in 1937 in the West Nile district of Uganda from a woman with an undiagnosed febrile illness (Smithburn et al., 1940). It is an RNA virus that cycles in nature between *Culex* mosquitoes and birds but also infects and causes disease in humans, horses, and other vertebrate species. Although its enzootic cycle was believed to be almost exclusively between mosquitoes and birds, with vertebrate species serving as “dead-end” hosts because of low-level and transient viremia, one study demonstrated non-viremic transmission of WNV between co-feeding mosquitoes (Higgs et al., 2005). This suggests that vertebrates may also act as reservoirs for mosquito infection, resulting in further virus transmission.

Historically, WNV caused sporadic outbreaks of a mild febrile illness in regions of Africa, the Middle East, Asia, and Australia. However, in the 1990’s, the epidemiology of infection

Address correspondence to: Michael S. Diamond, MD, Ph.D, Departments of Medicine, Molecular Microbiology, Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8051, St. Louis, MO 63110, Tel: 314-362-2842. Fax: 314-362-9230, diamond@borcim.wustl.edu.

changed. New outbreaks in Eastern Europe were associated with higher rates of severe neurological disease (Hubalek and Halouzka, 1999). In 1999, WNV entered North America, and caused seven human fatalities in the New York area as well a large number of avian and equine deaths. Over the last ten years, WNV has spread to all 48 of the lower United States as well as to parts of Canada, Mexico, the Caribbean, and South America. Because of the increased range, the number of human cases has continued to rise: in the United States between 1999 and 2008, 28,961 cases that reached clinical attention were confirmed and associated with 1,131 deaths (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm).

Most (~85%) of human infections in the United States occur in the late summer with a peak number of cases in August and September. This reflects the seasonal activity of *Culex* mosquito vectors and a requirement for virus amplification in the late spring and early summer in avian hosts. In warmer parts of the country, virtually year-round transmission has been observed. Although more than 100 avian species are susceptible to WNV infection, some are particularly vulnerable with a large number of deaths in crows, blue jays, and hawks. The magnitude of dying birds in a community in the early summer often predicts the severity of human or equine disease weeks later (Komar, 2003). Ecology studies suggest that *Culex pipiens*, the dominant enzootic (bird-to-bird) and bridge (bird-to-human) vector of WNV in urbanized areas in the northeast and north-central United States, shifts its feeding preferences from birds to humans during the late summer and early fall, coincident with the dispersal of its preferred host, the American robin (*Turdus migratorius*) (Kilpatrick et al., 2006).

Seroprevalence studies suggest that most (~80%) cases are sub-clinical, without significant symptoms. Among clinical cases, many develop a self-limiting illness that is termed WNV fever. This syndrome begins after a 2 to 14 day incubation period and is characterized by fever accompanied with myalgias, arthralgias, headache, fatigue, gastrointestinal complaints, maculopapular rash or lymphadenopathy. This non-neuroinvasive form of WNV infection can be severe as 38% of patients with WNV fever were hospitalized with a mean length stay of 5.4 days (Huhn et al., 2005). A subset of the symptomatic cases progress to the neuroinvasive forms of WNV infection, including acute flaccid paralysis, meningitis, encephalitis, and ocular manifestations (Bakri and Kaiser, 2004; Sejvar et al., 2003); in many instances, a combination of these syndromes is present. Overall, about 1 in 150 WNV infections, result in the most severe and potentially lethal form of the disease. During an epidemic, on a human population scale, the seroconversion rate is ~3% (Mostashari et al., 2001; Tsai et al., 1998) and the attack rate for severe disease during an epidemic is ~7 per 100,000 (Huhn et al., 2005). The risk of severe WNV infection is greatest in the elderly (Chowers et al., 2001; Nash et al., 2001; Tsai et al., 1998). At least two studies have estimated a 20-fold increased risk of neuroinvasive disease and death in those over 50 years of age (Huhn et al., 2005; Nash et al., 2001).

Two human genes, *CCR5* and *OAS1* have been identified as susceptibility loci for WNV infection. In mice, a genetic deficiency of the chemokine receptor CCR5 was associated with depressed leukocyte trafficking, increased viral burden, and enhanced mortality (Glass et al., 2005). Analogous genetic deficiencies (e.g., CCR5Δ32, a deletion in the *CCR5* gene) are associated WNV-induced disease in humans (Glass et al., 2006). Although individuals that are homozygous for the CCR5Δ32 allele represent ~1% of the general United States population, 4–8% of individuals with laboratory-confirmed symptomatic WNV infection were homozygous for the mutant allele. Thus, CCR5 functions as an essential host factor to resist neuroinvasive WNV infection, which may have implications for the use of CCR5 antagonists (e.g., Maraviroc) in HIV therapy. In certain mouse strains, susceptibility to flaviviruses, including WNV, maps to a truncated isoform of the 2’5’ oligoadenylate sythetase (*OAS1b*) gene, a member of an IFN-regulated gene family involved in degradation of viral RNA. A recent study suggests that a hypomorphic allele of the human ortholog *OAS1* is associated with both symptomatic and asymptomatic WNV infection (Lim et al., 2009). Thus, in humans,

variation in *OAS1* is a genetic risk factor for initial WNV infection although not for disease severity.

Although most human WNV infections occur after the bite of an infected *Culex* mosquito, other routes including transfusion, organ transplantation, placental crossing, and through breast milk have resulted in transmission. In 2002, 23 cases of WNV infection were identified after transfusion of blood products (Pealer et al., 2003). These cases led to the development and implementation of nucleic acid amplification tests, which have been used to test pools or individual blood product samples (Busch et al., 2005b; Kleinman et al., 2009; Petersen and Epstein, 2005; Tobler et al., 2005) and largely prevent transmission by transfusion (Busch et al., 2005a). Nucleic acid screening of blood donors have not completely eliminated transfusion-transmitted WNV infections as "breakthrough" infections have occurred, and were attributed to units that had levels of viremia below the sensitivity of the screening assay (Busch et al., 2005b). In addition to transfusion associated WNV infection, several cases by organ transplantation have been reported (DeSalvo et al., 2004; Kleinschmidt-DeMasters et al., 2004; Kumar et al., 2004a; Kumar et al., 2004b). In 2007, the FDA approved a screening test for WNV in donated organs (Lang, 2007). Because of the relatively low incidence of WNV infection in organ transplantation and risk of false-positives that can occur with wide scale testing, screening is not mandated (Kiberd and Forward, 2004).

B. Diagnosis

Although clinical criteria for assessment of patients with suspected WNV infection have been defined (Granwehr et al., 2004; Sejvar et al., 2003), diagnosis depends on the detection of antibodies or viral nucleic in the blood or cerebrospinal fluid (Zhang et al., 2009b). Only a subset of clinical laboratories has the facilities to isolate virus directly from infected clinical samples. Because viremia is relatively transient and often precedes the severe neurological manifestations of the WNV infection, nucleic acid testing although quite specific has a relatively low sensitivity. The detection of WNV IgM in the serum or CSF is still the most utilized method for diagnostic confirmation (Kapoor et al., 2004; Martin et al., 2004). The tests are sensitive (90%) when carried out by day 8 of illness. Nonetheless, testing within the first 72 hours of clinical presentation may yield false negative results because of the inherent kinetics of the anti-WNV IgM response (Busch et al., 2008; Diamond et al., 2003b). Because the ELISA test also detects antibodies against related flaviviruses (e.g., St Louis and Japanese encephalitis virus), false positives are possible, and thus it is important to obtain a history of recent vaccination (e.g., yellow fever virus) or foreign travel. Definitive serological diagnosis of WNV infection requires a comparison of antigen or neutralization activity among related flavivirus family members. Investigational diagnostic assays that utilize purified WNV structural and non-structural proteins (Wong et al., 2003; Wong et al., 2004) may allow distinction between natural infection, vaccination, and immunity. One cautionary note is that WNV IgM can persist in serum up to 500 days after onset of infection (Prince et al., 2008; Prince et al., 2007; Roehrig et al., 2003); this could confound interpretation of serology results in patients presenting subsequently with clinical syndromes that resemble WNV infection.

C. Virology and Pathogenesis

The genus *Flavivirus* is composed of greater than 70 members, 40 of which are associated with human disease: dengue, yellow fever, Japanese encephalitis, tick-borne encephalitis, and West Nile encephalitis viruses are the most important globally, causing extensive morbidity and mortality (Burke and Monath, 2001). Flaviviruses are enveloped RNA viruses with a single-stranded, positive-polarity 11-kilobase genome. They are translated in the cytoplasm as a polyprotein, and then cleaved into structural and non-structural proteins by virus- and host-encoded proteases (Brinton, 2002; Lindenbach and Rice, 2001). The structural proteins include a capsid protein (C), an envelope protein (E) that functions in receptor binding, membrane

fusion, and viral assembly, and a transmembrane protein (prM) that assists in proper folding and function of the E protein. The role of the nonstructural (NS) proteins is not fully delineated but these proteins form the viral protease (NS2B, NS3), NTPase (NS3), RNA helicase (NS3), RNA-dependent RNA polymerase (NS5), and methyltransferase (NS5) and antagonize host immune responses.

WNV infection occurs following cellular attachment and receptor-mediated endocytosis. Although both DC-SIGN-R and the $\alpha_v\beta_3$ integrin have been suggested as WNV attachment ligands (Chu and Ng, 2004b; Davis et al., 2006), the cellular receptors for WNV on physiologically relevant cell types such as neurons or macrophages remain uncharacterized. Indeed, more recent studies have reported that WNV entry occurs independently of the $\alpha_v\beta_3$ integrin (Medigeshi et al., 2008). Cellular entry of WNV requires the formation of clathrin-coated pits (Chu and Ng, 2004a; Krishnan et al., 2007) and cholesterol rich lipid rafts (Medigeshi et al., 2008). Following a pH-dependent conformational change in the E protein (Modis et al., 2004; Zhang et al., 2004), the viral and endosomal membranes fuse, releasing the viral nucleocapsid into the cytoplasm (Allison et al., 1995; Gollins and Porterfield, 1986). Upon nucleocapsid release, viral RNA associates with endoplasmic reticulum (ER) membranes and is translated. Translation is a prerequisite for generating a negative-strand RNA intermediate that serves as a template for nascent positive-strand genomic RNA synthesis (Mackenzie and Westaway, 2001). WNV RNA synthesis is semi-conservative and asymmetric, as positive-strand RNA genome production is about ten times more efficient than negative-strand synthesis (Brinton, 2002). Positive strand RNA is either packaged within progeny virions or used to translate additional viral proteins. WNV assembles and buds into the ER to form enveloped immature particles containing the prM protein. During egress, immature virions undergo a maturation step in which a furin-like protease cleaves prM (Elshuber et al., 2003; Guirakhoo et al., 1992; Stadler et al., 1997), resulting in a reorganization of E proteins into a distinct homodimeric array (Mukhopadhyay et al., 2003).

Progress has been made on the structural organization WNV, and this has provided insight into the molecular transitions that occur during the virus life cycle (Mukhopadhyay et al., 2005). Three-dimensional reconstruction images from cryoelectron microscopy demonstrate that the WNV has a well-organized outer protein shell, a 40 Å lipid membrane bilayer, and a less-defined inner nucleocapsid core (Mukhopadhyay et al., 2003). The icosahedral scaffold consists of 180 E and M proteins arranged in a repeating herringbone pattern (Kuhn et al., 2002; Zhang et al., 2003a). Structural analysis of the soluble ectodomain of WNV E proteins reveals three domains (Kanai et al., 2006; Nybakken et al., 2006), consistent with earlier studies on related flaviviruses (Modis et al., 2003; Rey, 2003; Rey et al., 1995). Domain I is an 8-stranded β-barrel that participates in the conformational changes associated with the acidification of the endosome (Modis et al., 2004). Domain II, which contains 12 β-strands, has important roles in dimerization, trimerization, and virus-mediated fusion (Modis et al., 2003; Modis et al., 2004; Rey et al., 1995). Domain III adopts an immunoglobulin-like fold, contains the most distal projecting loops on the mature virion (Mukhopadhyay et al., 2003; Zhang et al., 2003a; Zhang et al., 2003b), and has been hypothesized to contain the binding site for cell attachment (Beasley and Barrett, 2002; Bhardwaj et al., 2001; Rey et al., 1995; Roehrig et al., 2001). The 180 E monomers lay relatively flat along the virion surface as sets of three anti-parallel homodimers. This mature flavivirus virion has quasi-icosahedral symmetry, such that three E monomers are found in the asymmetric unit resulting in distinct chemical environments that are available for antibody or receptor binding.

Most strongly neutralizing type-specific antibodies against WNV that have been generated in mice recognize epitopes on Domain III of the E protein (Beasley and Barrett, 2002; Choi et al., 2007; Li et al., 2005; Oliphant et al., 2005; Sanchez et al., 2005; Volk et al., 2004). The crystal structure of a Fab fragment of a neutralizing antibody (E16) in complex with Domain

III of WNV E protein provided structural insight into the type-specificity of antibody neutralization (Nybakken et al., 2005). The E16 Fab fragment engaged four discontinuous segments of Domain III including the N-terminal region (residues E302-E309) and three strand-connecting loops: BC (E330–E333), DE (E365–E368) and FG (E389–E391). Comparison of available WNV sequences revealed nearly complete conservation of the structurally defined E16 epitope. Sequence analysis of other flaviviruses revealed diversity in the four segments of the E16 epitope. As individual flavivirus-specific neutralizing antibodies have been mapped to analogous binding regions on Domain III (Gromowski and Barrett, 2007; Hiramatsu et al., 1996; Sukupolvi-Petty et al., 2007; Volk et al., 2004; Wu et al., 2003), this structural epitope, although specific for individual flaviviruses, may have an important role in neutralization of all flaviviruses. More recent studies with human and chimpanzee derived monoclonal antibodies against WNV and related flaviviruses suggest that additional strongly neutralizing antibody epitopes are located within DI, at the DI-DII hinge, and along the DII-dimer interface (Goncalvez et al., 2008; Lai et al., 2007; Vogt et al., 2009).

Infection experiments in animals have contributed to our understanding of the pathogenesis of WNV encephalitis (reviewed in (Diamond et al., 2009; Samuel and Diamond, 2006)). Based on studies with related flaviviruses, initial replication after mosquito inoculation is believed to occur in the skin in dendritic cells (Ho et al., 2001; Libraty et al., 2001; Marovich et al., 2001; Wu et al., 2000); these infected cells migrate to draining lymph nodes (Johnston et al., 2000) where infection and the risk of dissemination are countered by the development of an early immune response (Bourne et al., 2007; Purtha et al., 2008). After reaching secondary lymphoid tissues, a new round of infection occurs, leading to entry into the circulation via the efferent lymphatic system and thoracic duct. Viremia ensues and after spread to visceral organs (e.g., kidney, and spleen), WNV crosses the blood-brain barrier and enters the central nervous system (Diamond et al., 2003a; Wang et al., 2004; Xiao et al., 2001) through an incompletely understood mechanism. Although WNV likely enters the central nervous system via a hematogenous route (Diamond et al., 2003a; Johnson and Mims, 1968), perhaps as a result of TNF-α or matrix metalloproteinase-9 induced changes in capillary permeability (Wang et al., 2008a; Wang et al., 2004), other mechanisms of entry include retrograde axonal transport through peripheral nerves (Samuel et al., 2007b), transport across the brain microvascular endothelium (Verma et al., 2009), active replication in endothelial cells (Verma et al., 2009), or a “Trojan horse” mechanism in which WNV is carried into the brain by infected inflammatory cells (Burke and Monath, 2001; Dai et al., 2008; Wang et al., 2008b).

In animal models, WNV is first identified in the central nervous system about three to four days after infection. Infectious West Nile virus is detected in multiple sites in the brain and spinal cord. Patchy infection of neurons is observed in the cerebral cortex, hippocampus, basal ganglia, cerebellum, brain stem, and anterior horn of the spinal cord (Diamond et al., 2003a; Eldadah and Nathanson, 1967; Eldadah et al., 1967; Xiao et al., 2001). Neuronal infection is associated with degeneration, a loss of cell architecture, and caspase-3 associated apoptosis (Samuel et al., 2007a); this correlates with the development of microglial nodules that surround infected neurons. Later in the course of infection a mononuclear cell infiltrate appears diffusely throughout infected regions although it is not clear whether these inflammatory cells eradicate infection or contribute to pathogenesis by destroying infected neurons and releasing potentially toxic cytokines (Getts et al., 2008). Of note, Purkinje neurons in the cerebellum (Diamond et al., 2003a; Xiao et al., 2001; Zhang et al., 2008) and motor neurons in the spinal cord are highly vulnerable (Morrey et al., 2008a; Samuel et al., 2007b; Siddharthan et al., 2009). Indeed, the more virulent North American strains of West Nile virus cause a polio-like syndrome in humans that predominantly affects lower motor neuron function (Glass et al., 2002; Leis et al., 2003; Leis et al., 2002). Infection of neurons causes vacuolization, a proliferation of rough endoplasmic reticulum and Golgi-derived membranes, and apoptosis. The expression of WNV proteins may directly induce apoptotic cell death of neurons (Samuel et al., 2007a; Shrestha et

al., 2003), possibly due to activation of the unfolded protein response pathway (Medigeshi et al., 2007): accumulation of West Nile virus capsid (Yang et al., 2002; Yang et al., 2008a) and NS3 (Ramanathan et al., 2006) proteins cause apoptosis through activation of caspases 3, 8 and 9.

D. Immune Control

Although an immunocompromised status predisposes to more severe disease in humans, the individual risk factors, beyond the aforementioned *CCR5* and *OAS1* genes, are not fully characterized. The severity of WNV infection is increased in immunosuppressed patients (Bode et al., 2006; Chan-Tack and Forrest, 2006; Kumar et al., 2004a; Kumar et al., 2004b; Murray et al., 2006) suggesting an essential role for immune control mechanisms. The high incidence of WNV neuroinvasive disease in patients on anti-T cell therapies (Kleinschmidt-DeMasters et al., 2004) and in mice with CD4 or CD8 T cell deficiencies (Brien et al., 2007; Brien et al., 2008; Purtha et al., 2007; Shrestha and Diamond, 2004; Sitati and Diamond, 2006; Wang et al., 2003b) indicate that the integrity of adaptive cellular immune responses is essential for clearance of WNV. B cells also protect against disseminated infection as *SCID, RAG1* mice and B cell deficient mice uniformly succumb to WNV infection (Chambers et al., 2008; Diamond et al., 2003a; Diamond et al., 2003b; Halevy et al., 1994). Humoral immunity has been linked to peripheral clearance of WNV whereas T cells appear more critical for clearance within the CNS. For example, in CD4 or CD8 T cell deficient mice, which exhibit increased WNV encephalitis, serum viral loads and IgM levels were no different from wild-type animals but T cell trafficking and/or function in the CNS was impaired (Sitati and Diamond, 2006), indicating that survival is not solely a function of preventing CNS dissemination but also of clearing it.

The past five years has provided new perspective as to how different components of the innate immune response restrict WNV infection. Host cells recognize and respond to RNA virus infection through endosomal the nucleic acid sensors, Toll-like receptor 3 (TLR-3) and 7 (TLR-7), and the cytoplasmic dsRNA sensors, retinoic acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) (Colonna, 2007; Kawai and Akira, 2006). Binding of RNA to these pathogen recognition receptors (PRRs) results in downstream activation of transcription factors, such as interferon regulatory factors 3 and 7 (IRF-3 and IRF-7), and the expression of IFN and IFN-stimulated genes (ISG). Each of these PRRs demonstrate specificity for different RNA virus families with RIG-I, MDA5, and TLR-3 essential for IFN responses in response to flavivirus infections (Kato et al., 2006). Cultured fibroblasts deficient in RIG-I, MDA5, or IPS-1 demonstrate delayed induction of host responses, decreased IRF-3 activation, and augmented WNV replication (Fredericksen and Gale, 2006; Fredericksen et al., 2008; Fredericksen et al., 2004). In vivo, however, MDA5 may be less essential for cellular recognition and host response as IFN production by $MDA5^{-/-}$ myeloid dendritic cells remains largely intact after WNV infection (Gitlin et al., 2006). Systemic IFN-α production in mice appears largely independent of the transcription factor, IRF-3 (Bourne et al., 2007; Daffis et al., 2007) but is dependent on IRF-7 (Daffis et al., 2008b). Individual cell types (myeloid, fibroblast, and neuronal) use IRF-3-depedent responses to protect against WNV infection through IFN-dependent and independent pathways (Daffis et al., 2007). In cells that generate robust IFN responses after WNV infection in the absence of IRF-3, alternate sets of PRRs and transcriptional regulators are likely used, including TLR-7/8 and IRF-7.

Recognition of WNV by TLR is mediated by TLR3, which likely binds to viral dsRNA, and TLR7/8, which bind ssRNA, including uridine-rich RNA motifs. Activation of both TLR3 and TLR7/8 in response to viral infection induces production of type I IFN (Alexopoulou et al., 2001; Diebold et al., 2004). However, the signaling pathways that TLR3 and TLR7/8 utilize

differ. TLR7/8 recruits the adaptor protein MyD88, which forms a complex with TRAF3, TRAF6, IRAK1 and IRAK4. This complex recruits TAK1, a kinase that activates NF-κB, or TBK1 and IKKε, kinases that activate IRF-3 and/or IRF-7. TLR3 recruits TRIF, which stimulates the IRF-3/IRF-7-dependent induction of type I IFN genes via interactions with TRAF3, TBK1 and IKKε.

Despite several in vitro studies showing that binding of TLR3 by dsRNA in vitro regulates IFN and other cytokine responses, its role in protection against viral infection in vivo remains less clear. Conflicting results have been observed during WNV infection in mice. Two studies using same TLR3$^{-/-}$ mice reported somewhat opposing phenotypes: Wang and colleagues showed a detrimental role of TLR3 as deficient mice had improved survival rates after WNV infection. This was associated with a mildly increased WNV burden in peripheral tissues yet a decreased pro-inflammatory cytokine response. The diminished inflammatory response reduced blood-brain barrier permeability and direct entry of WNV into the brain (Wang et al., 2004). A contrasting study showed a protective role with decreased survival of TLR3$^{-/-}$ mice after WNV infection, mildly elevated viral titers in peripheral tissues, and early viral entry in the CNS (Daffis et al., 2008a). At present, it remains unclear why the results are discordant although the disparate route of inoculation and passage history of the virus could impact cytokine responses. TLR3 appears to have an independent role in the CNS, potentially by restricting WNV replication in neurons. TLR3$^{-/-}$ cortical neurons sustained enhanced WNV viral replication, although type I IFN responses were normal. TLR3$^{-/-}$ microglia and astrocytes showed reduced activation and production of proinflammatory cytokines (TNF-α, IL-6 and IL-12 p40) after poly (I:C) challenge (Kim et al., 2008; Town et al., 2006). Thus, the exact contribution of TLR3 for WNV protection requires further study but likely involves direct effects in the CNS.

TLR7 was initially identified as a trigger of the IFN-α response after exposure to ssRNA from influenza or other viruses (Hornung et al., 2008). TLR7 was also characterized as the primary PRR responsible for systemic IFN production by plasmacytoid DC through a MyD88-dependent pathway (Asselin-Paturel and Trinchieri, 2005). The contribution of TLR7 in protecting from WNV infection in vivo was recently examined (Town et al., 2009). TLR7$^{-/-}$ mice were more vulnerable to WNV infection and sustained increased viremia after infection. These mice showed a defect of immune cell homing to WNV-infected tissues via a novel IL-23-dependent mechanism. Interestingly, systemic levels of proinflammatory cytokines (IL-6, TNF-α, and IL-12) and type I IFN were higher in TLR7$^{-/-}$ mice when compared to wild type animals. This result suggests that abrogation of the TLR7 pathway has little systemic impact on IFN production after WNV infection.

The complement system is a family of serum proteins and cell surface molecules that participate in pathogen recognition and clearance. Complement contributes to host protection through direct oposnization and/or cytolysis, chemotaxis, immune clearance, and modulation of B and T cell functions (Carroll, 2004). Complement is required for protection from lethal WNV infection in mice. WNV activates complement in vivo, and mice lacking in the central complement component C3 or complement receptors (CR)1 and 2 showed enhanced lethality after WNV infection (Mehlhop and Diamond, 2006; Mehlhop et al., 2005). All three complement activation pathways coordinate control against WNV, as mice deficient in molecules of the alternative, classical, or lectin pathway exhibit increased mortality. Interestingly, the activation pathways modulated WNV infection through distinct mechanisms. Alternative pathway deficient mice demonstrated normal B cell function but impaired CD8^{+} T cell responses, whereas classical and lectin pathway deficient mice had defects both in WNV-specific antibody production and T cell responsiveness (Mehlhop and Diamond, 2006).

Complement also augments the efficacy of IgG antibodies against WNV. Whereas initial studies with anti-WNV IgM antibodies suggested that complement could efficiently enhance WNV infection in macrophages in vitro (Cardosa et al., 1986; Cardosa et al., 1983), more recent investigations indicate that the complement component C1q augments the potency of neutralizing antibody against WNV in an IgG subclass-specific manner (E. Mehlhop, S. Nelson, T. Pierson, and M. Diamond, manuscript submitted), analogous to that observed for other viruses including measles (Iankov et al., 2006), influenza (Feng et al., 2002; Mozdzanowska et al., 2006), vesicular stomatitis (Beebe and Cooper, 1981), and human immunodeficiency (Aasa-Chapman et al., 2005; Spruth et al., 1999) viruses. C1q also restricts antibody-dependent enhancement of WNV infection in vitro and in vivo (Mehlhop et al., 2007).

While few studies have directly addressed the function of cellular innate immunity in WNV infection, macrophages and dendritic cells likely inhibit WNV though direct viral clearance, enhanced antigen presentation, and cytokine and chemokine secretion. Consistent with this, depletion of myeloid cells systemically or in the draining lymph nodes enhanced lethality in mice after WNV infection (Ben-Nathan et al., 1996; Purtha et al., 2008). Macrophages basally express key host defense molecules, including RIG-I, MDA5, ISG54, and ISG56, and thus, restrict WNV infection by inducing type I IFN (Daffis et al., 2007) and other inhibitory cytokines. Macrophages may also control flaviviruses through the production of nitric oxide (NO) intermediates (Kreil and Eibl, 1996; Lin et al., 1997), although the role of NO in WNV infection has not been established.

γδ T cells also function in early immune responses and directly limit WNV infection. As they lack MHC restriction, γδ T cells can react with viral antigens in the absence of conventional antigen processing (Steele et al., 2000). γδ T cells expand following WNV infection (Welte et al., 2008), and increased viral burden and mortality and delayed priming of adaptive immune responses were observed in mice deficient in γδ T cells (Wang et al., 2006; Wang et al., 2003a). Bone marrow chimera reconstitution experiments demonstrated that γδ T cells require IFN-γ to limit WNV infection (Shrestha et al., 2006b). Natural killer (NK) cells also have the potential to control WNV infection through recognition and elimination of virus-infected cells. NK cell activity was transiently activated and then suppressed following flavivirus infection in mice (Shresta et al., 2004; Vargin and Semenov, 1986). As WNV infection increases surface expression of class I MHC molecules by enhancing the transport activity of TAP and by NF-κB-dependent transcriptional activation of MHC class I genes (Douglas et al., 1994; King and Kesson, 1988; Liu et al., 1988), natural killing may be inhibited (Diamond, 2003; King and Kesson, 2003; King et al., 1989). Notably, antibody depletion of NK cells in mice did not alter morbidity or mortality after WNV infection (Chung et al., 2007; Shrestha et al., 2006a).

II. Candidate anti-WNV Therapeutics

At present, no specific therapy has been approved for use in humans with WNV infection as current treatment is supportive. Tissue culture and animal model studies have applied multiple strategies for the generation of novel therapies against WNV, and possibly other flaviviruses. Nonetheless, the development of therapeutics that mitigate or abort disease is challenging as patients with the most severe disease often have underlying immune deficits and present to clinical attention relatively late in their course (Granwehr et al., 2004; Jackson, 2004). Among the additional impediments will be developing therapeutics that efficiently cross into the central nervous system and clear virus from infected neurons. Finally, once a candidate agent is identified, regulatory hurdles will be encountered in the design and implementation of multi-center trials given the sporadic temporal and spatial occurrence of WNV infections (Jester et al., 2006).

A. Ribavirin and Mycophenolic acid

Ribavirin is a broad-spectrum antiviral agent and has been used clinically to treat respiratory syncytial (Hall et al., 1983), hepatitis C (Davis et al., 1998), Lassa (McCormick et al., 1986), Hantaan (Huggins et al., 1991) and La Crosse (McJunkin et al., 1997) viruses. It acts as a guanosine analogue and competitively inhibits inosine monophosphate dehydrogenase (IMP), resulting in depleted intracellular guanosine pools (Leyssen et al., 2005). This may interfere with the guanylylation step of RNA capping, inhibit viral polymerases or compromise the integrity of the viral genome by being incorporated directly into the nascent RNA strand and serving as a template for both cytidine and uridine (Crotty et al., 2000; Day et al., 2005). Ribavirin has inhibitory activity against WNV infection in cell culture (Anderson and Rahal, 2002; Day et al., 2005; Jordan et al., 2000) at high doses (EC_{50} of 60 to 100 μM). Limited animal studies have been performed with less than promising results. Treatment of WNV-infected hamsters with ribavirin increased mortality (Morrey et al., 2004). Moreover, during a WNV outbreak in Israel in 2000, 37 patients received ribavirin and a high mortality rate (41%) was observed in this group (Chowers et al., 2001).

Mycophenolic acid (MPA) is a non-nucleoside inhibitor of IMP dehydrogenase that is used clinically to prevent rejection of transplanted organs. The immunosuppressive properties of MPA are attributed to its anti-proliferative effect on lymphocytes in vitro (Allison and Eugui, 1993; Nagy et al., 1993). MPA inhibits to varying degrees the replication of a number of DNA, RNA, and retroviruses in vitro including arenaviruses, Sindbis virus, reovirus, parainfluenza virus, coxsackie virus, Epstein-Barr virus, hepatitis B virus, and HIV (Gong et al., 1999; Ichimura and Levy, 1995; Neyts and De Clercq, 1998). Four studies have demonstrated that MPA inhibits flavivirus infection including WNV in cells by limiting viral RNA replication (Diamond et al., 2002; Morrey et al., 2002; Ng et al., 2007; Takhampunya et al., 2006). Although MPA blocked WNV infection efficiently in cell culture, in vivo its inhibitory properties were overshadowed by its immunosuppressive effects. Increased mortality after WNV infection was observed in mice treated with several different doses of MPA (B. Geiss and M. Diamond, unpublished results). Thus, the preclinical data suggests that inhibitors of guanosine biosynthesis are not therapeutic candidates against WNV infection, likely because of their effects on immune system function.

B. Interferon-α

Type I IFNs (IFN-α and β) comprise an important innate immune system control against viral infections. IFNs induce an antiviral state within cells through the induction of antiviral proteins and by modulating adaptive immune responses (Samuel, 1991). Pretreatment of cells *in vitro* with type I IFN potently inhibits flaviviruses including WNV (Anderson and Rahal, 2002; Best et al., 2005; Crance et al., 2003; Diamond and Harris, 2001; Diamond et al., 2000; Fredericksen et al., 2004; Samuel et al., 2006). However, the inhibitory effect of IFN is markedly attenuated after viral replication has begun (Diamond et al., 2000; Lin et al., 2004) as flavivirus non-structural proteins antagonize type I IFN effects by preventing JAK1 and Tyk2 phosphorylation, STAT1 and STAT2 signaling, and IFN-β gene transcription (Ashour et al., 2009; Best et al., 2005; Evans and Seeger, 2007; Jones et al., 2005; Lin et al., 2008; Lin et al., 2004; Liu et al., 2004; Liu et al., 2006; Liu et al., 2005; Munoz-Jordan et al., 2005; Munoz-Jordan et al., 2003). Nonetheless, IFN may still have therapeutic potential. Mice that were deficient in IFN-α and β receptors were acutely vulnerable to WNV infection with 100% mortality and a mean time to death of ~4 days after subcutaneous inoculation with 1 PFU of virus (Samuel and Diamond, 2005). Pretreatment of rodents with IFN-α inhibited St. Louis encephalitis virus infection and resulted in decreased WNV viral loads and mortality (Brooks and Phillpotts, 1999; Morrey et al., 2004). Treatment with IFN-α reduced complications in human St. Louis encephalitis virus cases and has been used in an uncontrolled manner to treat small numbers of human cases of WNV encephalitis (Kalil et al., 2005; Lewis and Amsden,

2007; Rahal et al., 2004; Sayao et al., 2004). Nonetheless, in Vietnam, a double-blinded, randomized placebo controlled clinical trial was performed on 1112 children with suspected or documented Japanese encephalitis virus infection; treatment with IFN α_{2a} failed to improve outcome (Solomon et al., 2003).

C. Antibodies

Although antibody has been utilized as a therapeutic against several viral infections (Sawyer, 2000; Zeitlin et al., 1999), with the exception of its prophylactic use against tick-borne encephalitis virus, it has not been used extensively against flavivirus infections in humans. Most neutralizing antibodies recognize the structural E protein, although a subset also have been described against another virion-associated protein, the prM or membrane protein (Colombage et al., 1998; Falconar, 1999; Pincus et al., 1992; Vazquez et al., 2002). Several groups also have generated non-neutralizing, yet protective mAbs against NS1 (Chung et al., 2006; Chung et al., 2007; Despres et al., 1991; Falgout et al., 1990; Henchal et al., 1988; Putnak and Schlesinger, 1990; Schlesinger et al., 1986; Schlesinger et al., 1990; Schlesinger et al., 1987; Schlesinger and Chapman, 1995), a protein that is absent from the virion. Thus, protection against flavivirus infections in vivo does not always correlate with neutralizing activity in vitro (Brandriss et al., 1986; Roehrig et al., 1983; Schlesinger et al., 1985). The ability to cure rodents of flavivirus infection with immune serum or monoclonal antibodies depends on the dosage and time of administration (Camenga et al., 1974; Chiba et al., 1999; Kimura-Kuroda and Yasui, 1988; Oliphant et al., 2005; Phillpotts et al., 1987; Roehrig et al., 2001), and polyclonal antibodies that prevent infection against one flavivirus do not provide durable cross-protection against heterologous flaviviruses (Broom et al., 2000; Roehrig et al., 2001).

Although these studies suggest that antibodies could have a potential therapeutic role, there are at least theoretical concerns that treatment could exacerbate disease. Sub-neutralizing concentrations of antibody enhance flavivirus replication in myeloid cells in vitro (Cardosa et al., 1986; Cardosa et al., 1983; Gollins and Porterfield, 1984; Gollins and Porterfield, 1985; Peiris and Porterfield, 1979; Peiris et al., 1981; Peiris et al., 1982; Pierson et al., 2007) and in vivo (Goncalvez et al., 2007; Mehlhop et al., 2007), and thus could complicate the antibody therapy. This phenomenon of antibody-dependent enhancement of infection (ADE) may cause the pathologic cytokine cascade that occurs during secondary dengue virus infection (Halstead, 1989; Halstead et al., 1980; Kurane and Ennis, 1992; Morens, 1994); despite its extensive characterization in vitro, the significance of ADE in vivo with WNV or other flaviviruses remains uncertain. Apart from or perhaps related to ADE, an “early-death” phenomenon (Morens, 1994) has been reported that could also limit the utility of antibody therapy. According to this model, animals that have pre-existing humoral immunity but do not respond well to viral challenge may succumb to infection more rapidly than animals without pre-existing immunity. Although it has been described after passive acquisition of antibodies against yellow fever and Langat encephalitis viruses (Barrett and Gould, 1986; Gould et al., 1987; Gould and Buckley, 1989; Webb et al., 1968), this phenomenon was not observed after transfer of monoclonal or polyclonal antibodies against Japanese (Kimura-Kuroda and Yasui, 1988) or tick-borne (Kreil and Eibl, 1997) encephalitis viruses.

Passive administration of anti-WNV antibodies is both protective and therapeutic and does not cause adverse effects related to immune enhancement. Transfer of immune serum prior to WNV infection protected wild type, B cell-deficient (*μMT*), and T and B-cell deficient (*RAG1*) mice from infection (Diamond et al., 2003a) and no increased mortality was observed even when sub-neutralizing concentrations of antibodies were used. Similarly, passive administration of immune serum (Tesh et al., 2002) or antiserum that recognized WNV E protein (Wang et al., 2001) protected hamsters and mice against lethal WNV infection. In

therapeutic trials, immune human γ-globulin protected mice against WNV-induced mortality (Ben-Nathan et al., 2009; Ben-Nathan et al., 2003; Engle and Diamond, 2003; Julander et al., 2005). Therapeutic intervention even five days after infection reduced mortality; this time point is significant because WNV spreads to the brain and spinal by day 4. Thus, passive transfer of immune antibody improved clinical outcome even after WNV had disseminated into the CNS.

Small numbers of human patients have received immunotherapy against WNV infection. Prophylaxis and therapy with neutralizing anti-WNV antibodies may be a possible intervention in the elderly and immunocompromised. Case reports (Haley et al., 2003; Hamdan et al., 2002; Saquib et al., 2008; Shimoni et al., 2001) have documented improvement in humans with neuroinvasive WNV infection after receiving immune γ-globulin from Israeli donors. Given the endemic nature of WNV in the Middle East, pooled human immunoglobulin from Israeli donors was shown to contain significant neutralizing titers of antibodies against WNV (Ben-Nathan et al., 2009; Ben-Nathan et al., 2003; Engle and Diamond, 2003). Although promising, γ-globulin immunotherapy against WNV infection in humans has limitations: (a) batch variability may affect the quantitative titer, functional activity, and therapeutic efficacy of specific antibody preparations; (b) it is purified from human blood plasma, and has an inherent risk of transmitting known and unknown infectious agents; and (c) it requires a large volume of administration, which can increase adverse events in patients with cardiac or renal co-morbidities.

To overcome these limitations, humanized or human monoclonal antibodies or antibody fragments with therapeutic activity against WNV infection (Gould et al., 2005; Oliphant et al., 2005; Throsby et al., 2006; Vogt et al., 2009) have been developed by several groups. These human or humanized antibody fragments have high neutralizing activity in vitro and provide excellent protection in vivo in mice. If mAbs are to be an effective therapy for WNV encephalitis they should function after the onset of symptoms and ideally, after infection in the central nervous system. When mouse or humanized mAbs were given as a single dose five or six days after infection 90% of mice or hamsters were protected (Morrey et al., 2006; Morrey et al., 2007; Oliphant et al., 2005). Acute flaccid paralysis in hamsters also was blocked by treatment with one neutralizing mAb, E16 several days after infection (Samuel et al., 2007b). MacroGenics has initiated a phase I/II randomized, double-blinded clinical trial to evaluate safety and efficacy of the E16 antibody (also termed MGAWN1) against severe WNV infection (http://clinicaltrials.gov/ct2/show/NCT00515385). Thus, neutralizing antibody therapeutics show promise as they directly inhibit transneuronal spread of WNV infection and prevent the development of paralysis in vivo. Future use of a combination of monoclonal antibodies that bind distinct epitopes and neutralize by independent mechanisms could diminish the potential risk of selecting escape variants in vivo (Zhang et al., 2009a), especially in immunocompromised individuals who generate high-grade viremia and tissue viral burden.

D. Nucleic Acids

(1) RNA Interference—RNA interference (RNAi) is a cellular process that specifically degrades RNA within the cytoplasm of cells in a sequence-specific manner (Meister et al., 2004). RNAi occurs in plants, nematodes, parasites, insects, and mammalian cells and is believed to function as a regulator of cellular gene expression and possibly as an innate defense against RNA viruses (Voinnet, 2005; Waterhouse et al., 2001). RNAi uses double stranded RNA (dsRNA) to target and degrade sequence-specific single-stranded RNA. The cytoplasmic ribonuclease DICER recognizes and cleaves long dsRNA molecules into 21 to 30 base pair small interfering RNA (siRNA) molecules; these associate with the RNA Induced Silencing Complex (RISC) to target and degrade complementary single-stranded RNA molecules (Sontheimer, 2005).

RNAi is now widely used to transiently disrupt various gene products to study their function in cells. Many mammalian viruses appear susceptible to treatment with exogenous siRNA. Cells that express virus-specific siRNA are resistant to infection by WNV (Anthony et al., 2009; Bai et al., 2005; Geiss et al., 2005; McCown et al., 2003; Ong et al., 2008; Yang et al., 2008b) in vitro. The sequence specific activity of siRNA against viruses has led to great interest in its potential as a new class of antiviral therapy. Two studies have shown that administration of siRNA to mice reduces WNV load and affords partial protection against lethal challenge (Bai et al., 2005; Kumar et al., 2006). Studies were also performed to determine whether WNV-specific siRNA could act efficiently as a therapeutic by administering it after viral challenge. Although siRNA could protect against lethal infection when given within 6 hours of infection (Kumar et al., 2006), no significant difference in survival was observed when siRNA was delivered 24 hours after infection (Bai et al., 2005). In vitro studies may explain some of the attenuated therapeutic effect of siRNA as pre-treatment but not post-treatment of cells with siRNA greatly reduced WNV replication and infection (Geiss et al., 2005). Because flaviviruses replicate in a specialized membranous compartment (Welsch et al., 2009), its genome may not be exposed to the cytoplasmic RNAi machinery. RNAi based therapeutics against WNV may await the development of enhanced delivery systems that allow siRNA to efficiently cross intracellular membranes and inhibit actively replicating viruses.

(2) Antisense Technology—Antisense oligomers have been used to modulate gene expression of pathogenic viruses, and several are in clinical development or trials (Kinney et al., 2005; Ma et al., 2000). This class of compounds inhibits viruses by binding to RNA in a sequence specific manner, effectively blocking access to a particular region of the viral genome. The development of phosphorodiamidate morpholino oligomers (PMO) has overcome prior limitations by enhancing water solubility and nuclease resistance (Summerton et al., 1997). The conjugation of arginine-rich peptides to PMOs has facilitated cellular uptake and inhibitory activity in cell culture systems (Neuman et al., 2004). Sequence-specific antisense oligomers have inhibitory activity against several flaviviruses, including WNV in cell culture (Deas et al., 2005; Kinney et al., 2005; Raviprakash et al., 1995; Stein and Shi, 2008). Low micromolar (5 to 20 μM) concentrations of arginine rich peptide-conjugated PMOs that targeted the 5’ untranslated or 3’ cyclization sequences inhibited WNV by 5 to 6 $\log_{10}$ PFU/ml (Deas et al., 2005; Kinney et al., 2005). However, effective suppression of viral replication in vitro required PMO to be present before or soon after infection, as administration at either 2 or 4 days after infection had little or no antiviral effect. PMO directed against the 5’ and 3’ conserved sequences partially protected mice from WNV disease without causing appreciable toxicity, although selection of resistant mutants was observed (Deas et al., 2007). Some clinical improvement was observed even when PPO was administered to mice at day 5 after infection although statistically significant differences were not achieved. AVI Biopharma has initiated a phase I/II human clinical study for treatment of WNV infection (http://www.clinicaltrials.gov/ct/show/NCT00091845) with AVI-4020. This trial is a randomized, double-blinded study that is focused on determining safety, tolerability, pharmacokinetics, and potential efficacy.

E. Peptides

The hemagglutinin of influenza virus is a prototypical class I viral envelope fusion protein. In response to receptor engagement and acid pH, the α helices of the viral hemagglutinin rearrange and expose an N-terminal fusion peptide that facilitates fusion of two lipid membranes and viral entry (Carr and Kim, 1993). Importantly, peptide mimics of the class I fusion proteins of HIV (Sodroski, 1999), Sendai (Rapaport et al., 1995), Newcastle (Young et al., 1999), and herpes (Okazaki and Kida, 2004) viruses efficiently inhibit entry and infection. Indeed, Fuzeon™ is a fusion inhibitor approved for clinical use in HIV-infected patients. The flavivirus E proteins are structurally distinct from the class I fusion proteins, and together with the

envelope proteins of alphaviruses comprise a second class of viral fusion proteins. Class II fusion proteins facilitate viral entry and nucleocapsid release after undergoing an analogous series of pH-dependent conformational changes (Bressanelli et al., 2004; Kuhn et al., 2002; Lescar et al., 2001; Modis et al., 2004). Using an algorithm that predicted peptide inhibitors of class I fusion proteins, one group identified inhibitory peptides in WNV and dengue virus E protein that correspond to the proposed fusion and stem anchor domains. Low micromolar concentrations of these peptides inhibited WNV and dengue virus infection in cell culture in a sequence-specific manner (Hrobowski et al., 2005). As an alternative approach, another group identified two E protein peptides that could inhibit WNV infection with EC50 values as low as ~3 μM. Mice challenged with WNV that had been administered these inhibitory peptides showed reduced viremia and lethality (Bai et al., 2006).

F. Imino sugars

Flavivirus assembly takes place within the endoplasmic reticulum (ER). The structural glycoproteins prM and E localize to the luminal side of the ER and encapsidate as an immature particle with prM and E in a heterodimeric complex (Chambers et al., 1990; Zhang et al., 2003b). In flavivirus-infected mammalian cells, a 14-residue oligosaccharide $(Glc)_3(Man)_9(GlcNAc)_2$ is added in the ER to specific asparagine residues specific on the prM and E proteins. This high mannose carbohydrate is sequentially modified in the ER and Golgi by resident glucosidases to generate N-linked glycans that lack the terminal α(1,2) and α(1,3) glucose residues (Hebell et al., 1991). Trimming of N-linked carbohydrates in the ER is required for proper assembly or secretion of flaviviruses (Courageot et al., 2000; Wu et al., 2002). Imino sugar derivatives, such as deoxynorjirimycin or castanospermine, inhibit endoplasmic reticulum α-glucosidases I and II. This prevents processing of high mannose N-linked glycans from nascent glycoproteins, a step that is required for interaction with the ER chaperones, calnexin and calreticulin. Several flaviviruses are strongly inhibited by α-glucosidase inhibitors in vitro and in vivo (Chang et al., 2009; Courageot et al., 2000; Gu et al., 2007; Schul et al., 2007; Whitby et al., 2005; Wu et al., 2002). More recently, a family of imino sugar derivatives was synthesized with superior antiviral activity (EC50 of ~0.1 to 1 mM) and low toxicity (selectivity index ~ 100) against several flaviviruses, including WNV (Chang et al., 2009). One possible advantage of α-glucosidase inhibitors is that they target a host enzyme that is an essential step in virus secretion rather than the virus directly, and are thus, less likely to select for resistant variants.

G. High-throughput Screens with Small Molecules

Over the last five years, high-throughput screens with small molecule libraries have been performed by several groups and identified classes of “druggable” compounds that inhibit WNV. Inhibitors have been identified that attenuate WNV translation, protease activity, and replication (Borowski et al., 2002; Goodell et al., 2006; Gu et al., 2006; Johnston et al., 2007; Nouiery et al., 2007; Puig-Basagoiti et al., 2006). Gu et al. (Gu et al., 2006) used a cell-based WNV subgenomic replicon to screen 35,000 compounds and identify pyrozolopyrimidine compounds with anti-WNV activity. Puig-Basagoiti et al. (Puig-Basagoiti et al., 2006) used a full length WNV that expressed a luciferase reporter gene to identify a triaryl pyrazoline compound that inhibits flavivirus RNA replication with an EC_{50} of ~15 μM. Noueiry et al. (Nouiery et al., 2007) evaluated a chemical library of 80,000 compounds for their ability to inhibit reporter gene expression from a WNV replicon; they identified inhibitory secondary sulfonamides and cyclopenta pyridines with EC50 values of ~3 μM. Johnston et al. (Johnston et al., 2007) screened a 65,000 compound library for the ability to inhibit NS2B-NS3 protease; they identified a common 5-amino-1H-pyrazoyl-3-yl scaffold as non-competitive inhibitors of WNV protease activity. Analogously, Mueller et al. (Mueller et al., 2008) utilized a high-throughput assay to screen 32,000 compounds for inhibition of the WNV

protease; lead compounds in the 8-hydroxyquinolone family bound in the substrate cleft and inhibited the protease.

Fewer studies have been performed with small molecules in animals to assess their therapeutic potential. One oral pyrazine derivative with broad-spectrum antiviral activity, T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was protective in rodents when administered twice daily beginning two days after WNV infection (Morrey et al., 2008b). However, administration of T-705 at days 3 or 4 after infection showed little apparent efficacy. Another preliminary study showed that oral active hexose could protect against lethal WNV infection in young and old mice by augmenting early antibody and γδ T cell responses (Wang et al., 2009). Although no post-exposure therapeutic trials were performed, they raise the possibility that dietary supplementation with oral active forms of hexose could improve antiviral immune responses and decrease the risk of severe neuroinvasive WNV disease.

III. Conclusions

Given the lack of existing therapies and its continued global emergence, the development of antiviral agents against WNV is essential. At present, several candidate therapies that act through distinct mechanisms are moving through various stages of pre-clinical development. Based on the epidemiology and pathogenesis of severe WNV infection effective antiviral agents against WNV must have minimal detrimental effects on immune system function. Even with the identification of new classes of anti-WNV agents, a major hurdle remains as to whether they can be administered in a timely manner before extensive and irreversible neuronal injury occurs. Technical challenges will include creating inhibitors that efficiently cross the blood-brain-barrier to allow for control of WNV replication within neurons. Regulatory hurdles will be encountered in implementing multi-center trials. It may be difficult to define referral sites that can recruit adequate numbers of patients so that statistically meaningful data can be acquired and analyzed. Unlike other diseases with high incidence, it may take years to complete a WNV clinical trial. With the introduction of several classes of candidate antiviral agents, there may be competition for patient cohorts. Because of this, extensive pre-clinical experiments in small animals, horses, and non-human primates may be useful to define whether a candidate therapeutic against WNV reaches human clinical trials.

Ongoing pathogenesis and infection studies undoubtedly will inform novel drug design strategies that target individual viral proteins (Dong et al., 2008). Experiments in animals should continue to define the essential components of the protective immune response, and the immunologic risk factors that predispose to severe neurological disease. Ultimately, a combination drug strategy that blocks viral replication, boosts protective immune responses, minimizes neuronal injury, and limits the development of resistant variants will likely be more effective than single agents.

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