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West Nile Virus: Biology, Transmission, and Human Infection

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INTRODUCTION

West Nile virus (WNV) is a neurotropic human pathogen that is the causative agent of West Nile fever and encephalitis. WNV was introduced into the Western Hemisphere during the late summer of 1999, when infected individuals were diagnosed in New York State (104, 125). In 2000, the epizootic expanded to 12 states and the District of Columbia (125), and WNV can now be found in many avian and mosquito species throughout North America (72, 73). From 1999 to 2010, more than 2.5 million people were infected, with over 12,000 reported cases of encephalitis or meningitis and over 1,300 deaths (93).

The purpose of this review is to present and summarize recent discoveries about the acquisition and transmission of WNV by mosquitoes as well as insights into human infection. We discuss and review data collected and presented over the last decade, and we present future directions of research.

BIOLOGY

Flaviviridae

The family *Flaviviridae* contain 3 genera: the flaviviruses, which include WNV, dengue virus (DENV), and yellow fever virus (YFV); the hepaciviruses, which include hepatitis B and C viruses; and the pestiviruses, which affect hoofed mammals. Within the *Flavivirus* genus, which contains more than 70 viruses, viruses can be further classified into tick-borne and mosquito-borne virus groups. The mosquito-borne viruses may be roughly sorted into the encephalitic clade, or the JE serocomplex, which includes WNV and Japanese encephalitis virus (JEV), and the nonencephalitic or hemorrhagic fever clade, which includes DENV and YFV, and there are 10 serologic/genetic complexes (30, 101, 118). The geographic distribution of the mosquito-borne flaviviruses largely depends on the habitat of the preferred mosquito vector, with *Culex* mosquitoes transmitting encephalitic flaviviruses mainly in the Northern Hemisphere.

Structure and Proteins

WNV is an enveloped virion containing a single-stranded, positive-sense RNA genome. The genome consists of a single open reading frame of approximately 11 kb with no polyadenylation tail at the 3′ end. Both the 5′ and 3′ noncoding regions of the genome form stem-loop structures that aid in replication, transcription, translation, and packaging (63, 92, 196). The viral RNA is translated as a single polyprotein that is post- and cotranslationally cleaved by both host and viral proteases, resulting in three structural (capsid, envelope, and premembrane) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (174). The 5′ end of the genome encodes the structural proteins, which are necessary for virus entry and fusion as well as encapsidation of the viral genome during assembly (118). The nonstructural proteins have many diverse functions, which is understandable as the virus has a very limited number of proteins and they must each serve multiple purposes during infection. NS1 has both a "cellular" form and a secreted form and is highly immunogenic

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doi:10.1128/CMR.00045-12

but has no described role in virion assembly, though it has been suggested to play a role in replication (234). NS3 is the viral protease responsible for cleaving other nonstructural proteins from the viral polyprotein and encodes enzyme activities, and these functions have been widely characterized (118). The NS5 protein serves as the viral polymerase and encodes a methyltransferase, and it is necessary for viral replication (117, 174). Several of the nonstructural proteins, including NS2A, NS2B, NS4A, and NS4B, have been shown to inhibit one or more components of the innate immune response against viral infection (116, 121, 122, 139).

The West Nile virus virion is an icosahedral particle with the capsid protein associating with the RNA genome to form the nucleocapsid, which is surrounded by a lipid bilayer. A high proportion of capsid protein localizes to the nucleus, while viral assembly takes place in the cytoplasm, with budding in the endoplasmic reticulum (ER) (17, 41, 183). Although the nuclear functions of capsid are not fully understood, recent evidence suggests a role in gene regulation through binding with histone proteins (41). During virus assembly, the envelope protein embeds in the lipid bilayer of the virus and is exposed to the virion surface. The envelope protein is responsible for binding the receptor on the cell surface for viral entry (134). The prM protein is also known to embed in the lipid bilayer and is thought to protect E from undergoing premature fusion upon virus exocytosis to the cell surface. During infection, the virus population contains both mature and immature virus particles containing a varying number of immature prM protein molecules on the surface (57, 239).

Life Cycle

Entry of WNV is through receptor-mediated endocytosis after virus attachment to the cell surface. Several molecules have been implicated as receptors for West Nile virus, including DC-SIGN, mannose receptor, and several glycosaminoglycans (52, 110, 211). The virus-containing endosome matures during internalization from the cell surface, with the pH dropping from neutral to slightly acidic in the early endosome and becoming more acidic during maturation to the late endosome. Within the late endosome, the envelope protein will undergo a conformational change resulting in fusion of the viral lipid membrane with the endocytic membrane and the release of the viral RNA genome into the cell cytoplasm (134). Following capsid disassociation, the RNA genome is replicated and virus assembly is initiated following a well-documented program (118). The viral polyprotein is translated and processed on intracellular membranes, resulting in the expression of the 10 viral proteins. The original viral RNA is replicated by viral and cellular proteins into multiple copies to be used in the production of new virions. The structural proteins assemble onto membranes in the endoplasmic reticulum, associate with the nucleocapsid, and bud into the cytoplasm via the Golgi network. The virus travels to the cell surface in an exocytic vesicle and matures as cellular enzymes cleave the prM, resulting in the release of mature virus from the cell surface (174).

There has been a recent spike in interest in the role of partially or fully immature flavivirus particles during infection. These immature flavivirus particles form when there is inefficient cleavage of the prM protein from the virion surface during maturation and budding (237). Immature or partially mature flavivirus particles of both DENV and WNV have been shown to account for up to as much as 40% of the total virus population in a given infection (135). While they were traditionally thought to be noninfectious, several recent studies have shown that immature WNV particles can be highly immunogenic and infectious *in vitro* and *in vivo* when bound by antibodies against the E or prM protein (43, 51, 179). These antibody-bound immature virus particles enter immune cells via the Fc receptor, resulting in productive infection. Further work remains to be done to determine the role that immature particles play in viral pathogenesis and disease in both vector and mammalian hosts.

VECTOR-VIRUS RELATIONSHIP

Vector Preference

The ability of different mosquito species to acquire and transmit WNV is highly variable. *Culex* mosquitoes are accepted as the primary global transmission vector; *C. tarsalis* is a main mosquito vector of WNV in the western United States and can feed on a variety of avian and mammalian species (95, 163). Other vectors shown to have competence for both infection and transmission of West Nile virus are *C. quinquefasciatus*, *C. stigmatosoma*, *C. thriambus*, *C. pipiens*, and *C. nigripalpus*; to date, over 65 mosquito species have been shown to be infected by WNV (79, 164, 222). There is evidence that *C. pipiens* in the eastern United States may feed on mammals and humans instead of birds during the late summer and early fall, and this "host switching" has also been reported with *C. tarsalis* in the western United States (96, 212). There are several reports of WNV in *Aedes* mosquitoes, though they are not considered a primary vector in nature (46, 58, 83, 184, 216, 221). WNV has also been detected in field-collected male *A. triseriatus* and *C. salinarius* (219), which not only points to vertical transmission of virus, as only females feed on animal blood, but also further supports that WNV has the ability to infect *Aedes* mosquitoes in nature. The ability to infect various mosquito species, the geographic range of mosquito species, and the ability of mosquitoes to feed on and transmit virus to particular hosts all play a role in WNV vector preference.

Host Reservoirs

WNV is maintained in nature in a cycle between mosquitoes and animal hosts (Fig. 1 shows a schematic of mosquito-mammal transmission), with the predominant and preferred reservoir being birds (3, 75, 136, 162). Birds of some species become ill, show symptoms of disease, and may die, while others become infected and serve as carriers without showing signs of disease. Although house sparrows and crows are highly susceptible to WNV, they make up a small fraction of analyzed mosquito blood meals and may be of minor importance in transmission. The American robin is instead thought to be the main host species responsible for the maintenance and transmission of WNV in the United States due to the feeding preference for robins by the dominant viral vectors (80, 91, 94). Bird-bird transmission has been demonstrated in the laboratory, with several species proving to be capable of contact transmission (99). Humans are considered "dead-end" hosts for WNV, as the low level of viremia in mammals is usually not sufficient to be transmitted to mosquitoes, thereby ending the transmission cycle (20). The ability of mammals to act as hosts could change, though, should *Aedes* mosquitoes, which feed primarily on humans, become primary transmission vectors for WNV.

Vector Acquisition

Mosquitoes acquire WNV after taking a blood meal from a viremic animal. The stages of infection and replication in the mos-

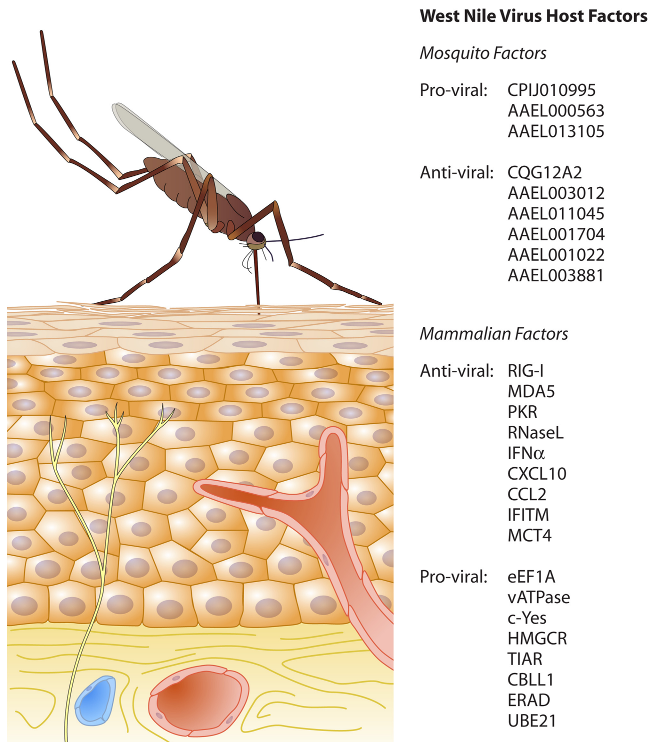

FIG 1 Schematic of West Nile virus transmission from mosquito to mammal and host factors known to be involved in infection.

quito have been well described (68, 126, 175). The virus must then infect and replicate in cells of the mosquito midgut as the blood meal is being processed. After replication in the midgut epithelia, the virus travels through the mosquito hemolymph to the salivary glands. Accumulation of virus in the salivary glands will eventually result in high viremia in the saliva, from where it can then be transmitted to mammalian hosts during feeding. The mosquito midgut can serve as a barrier to infection due to the presence of certain chitins and other proteins as well as a strong immune response to the virus (194). The peritrophic matrix, which consists of chitin microfibrils embedded in a proteoglycan matrix, has been shown to play a role in reducing pathogen invasion of the midgut epithelium, though its role in flavivirus infection is not entirely understood (89). A recent study looking at alteration in midgut gene expression in *C. pipiens quinquefasciatus* during WNV infection found 21 genes to be upregulated and 5 genes downregulated after mosquitoes fed on infected blood. Most of the genes were not canonical immune genes, though a putative Toll-like receptor (TLR) with increased expression during infection was identified (201). Proteins that have significantly increased or reduced levels in the mosquito midgut during WNV infection may play a role in disease acquisition or viral spread throughout the mosquito, and many are under active investigation as virulence factors. For example, a recent study found that a C-type lectin from mosquitoes facilitated WNV entry into mosquito cells by directly binding the virion and aiding interaction with a mosquito CD45 receptor homolog on the cell surface (36). These molecules may prove to be important for virus acquisition in the mosquito midgut. In mosquitoes that are refractory to infection, apoptosis in infected midgut epithelial cells has been proposed to limit the dissemination of WNV throughout the mosquito body (220). There is also evidence of a midgut barrier to secondary flavivirus infection, where mosquitoes which acquired more than one virus showed no evidence of dissemination of the second virus, which would prevent transmission (151). Research supports the existence of both physical and immune midgut barriers to WNV infection, and the list of genes both required for and inhibitory to acquisition is sure to increase with further experimentation.

Vector Response to Infection

There have been many recent studies aimed at elucidating the transcriptomic and proteomic response to flavivirus infection in the mosquito vector. Although WNV establishes a persistent infection in mosquito cells *in vitro* and in live mosquitoes, there is growing evidence that the mosquito does mount some immune response to virus infection. Most of what is known about the insect immune system comes from experiments with *Drosophila melanogaster*, though current examination of the mosquito immune response is starting to reveal corresponding proteins and pathways. The mosquito antiviral response is thought to consist of two pathways: the innate immune pathway and the RNA interference (RNAi) pathway (7). The innate immune response is comprised of three signaling pathways: Toll, JAK-STAT, and IMD. The Toll and IMD pathways both culminate in NF-κB-mediated expression of antimicrobial peptides (AMPs), and IMD signaling has been shown to control RNA virus infection in *Drosophila* (44). Not much is known regarding the role of mosquito AMPs in antiviral immunity, though their expression is often induced by viral infection. Both Toll signaling and the JAK-STAT pathways have been shown to play a role in the control of DENV infection in *Aedes aegypti* (161, 202) and may also be significant during infection of *Culex* with WNV. The RNAi pathway in mosquitoes is activated by viral double-stranded RNA and has been shown to be crucial for controlling alphavirus infection in both *Aedes* and *Anopheles* (32, 90). The RNAi pathway is known to be induced during WNV infection of *Culex pipiens* (21), and another RNAi pathway, PIWI, may participate in the mosquito response to virus, as it was shown to be involved in limiting WNV infection in *Drosophila* (39). Infection with dengue virus was also found to actively suppress mosquito immune responses *in vitro* (200).

Evidence for a transcriptomic signature of flavivirus infection was found during a comprehensive study of *Aedes aegypti* infected with WNV, DENV, and YFV (42). Genes involved in transcription and ion binding were found to be downregulated, and genes coding for proteases and cuticle proteins were found to be upregulated, during infection with all three viruses (42). Serine proteases had previously been shown to be important for viral propagation and blood digestion, though there have been varying reports regarding their impact on flaviviral infection in the mosquito (22, 138). Another global study of flaviviral infection in *Drosophila* identified many insect host factors relevant during dengue virus infection of the mosquito, including a putative NADH dehydrogenase and proteins involved in vesicular transport and endocytosis (193). Adding to our knowledge of the mosquito response to WNV infection, a recent transcriptomic analysis of *Culex quinquefasciatus* revealed that many genes involved in metabolism and transport are upregulated during infection (14). Given that the virus must infect a variety of cell types and organs in the mosquito vector, as well as optimize the cellular environment to benefit its

life cycle, there are likely a large number of differentially regulated genes, proteins, and other host factors important to WNV infection of the mosquito that have yet to be discovered.

Transmission to Vertebrate Host

WNV is transmitted to its vertebrate hosts by an infected mosquito vector during the probing process of blood feeding. Mosquitoes probe host skin using their proboscis in order to inject pharmacologically active saliva proteins and to locate a blood source (84, 171, 172). Although many hematophagous insects can obtain a blood meal without functional salivary glands, the efficiency of blood feeding is severely limited (84, 171, 172). In order to combat the host's hemostatic system, all hematophagous insects inject at least one vasodilator, one coagulation inhibitor, and one platelet inhibitor, and often the saliva includes immunomodulatory, digestive, and antimicrobial proteins as well (167, 169, 170, 186). While numerous proteins in the saliva of hematophagous insects have been described, many remain that have not been characterized, especially with respect to viral infection.

During probing, mosquito saliva is injected mostly extravascularly in the skin's dermal layer (205). Dermal blood vessels are the targets for hematophagous insects. In order to locate these structures, the proboscis must navigate through a very elastic environment that has a high tensile strength. To efficiently move through this environment, mosquito saliva may contain components that liquefy the bite site. A salivary endonuclease with a proposed function to facilitate probing in host skin has been identified in *C. quinquefasciatus* (31).

Host skin acts as an important barrier to many infections, though WNV antigen has been detected in skin at multiple phases of infection. WNV replication was observed in skin tissue at the inoculation site at 1 and 3 days postinfection (189), and WNV has also been shown to spread to areas of skin contralateral to the site of inoculation (27). Infectious WNV has been shown to persist in skin at the inoculation site for at least 14 days postinfection (5). Many reports document that both keratinocytes and fibroblasts are permissive to WNV infection *in vitro* and *in vivo* (8, 37, 38, 55, 60, 62, 86, 87, 102, 109, 115, 165, 185, 195, 233). By immunohistochemistry and fluorescence-activated cell sorter (FACS) analysis, WNV antigen was detected in keratinocytes at 4 and 5 days postinfection, and virus presence in a small subset of skin cells that lacked the keratin marker K10 suggests that skin cells other than keratinocytes may also be important early reservoirs (115).

Mosquito Saliva Factors

Saliva from hematophagous insects has been shown to alter the transmissibility of many pathogens (1, 50, 160, 178, 187, 206, 223, 231). Saliva from both *A. aegypti* and *C. tarsalis* has been shown to alter transmissibility in a WNV mouse model (189, 206). Specifically, when mice were fed on by uninfected *A. aegypti* prior to intradermal inoculation with WNV, more progressive infection, higher viremia, and accelerated neuroinvasion occurred. Even at a low dose of infection, mice that were previously fed on by mosquitoes had a lower survival rate after WNV infection (189). Similar experiments with *C. tarsalis* showed that mice infected with WNV through the bite of a single mosquito had viremia and tissue titers that were 5- to 10-fold higher postinoculation and showed faster neuroinvasion than those in animals infected by syringe inoculation (206). Enhanced early infection was also observed when mice were inoculated with WNV mixed with mosquito salivary gland extract (SGE). Importantly, enhanced viremia was not observed when SGE was inoculated in a distal site, supporting that mosquito saliva exerts its effect locally (206).

Due to the complex nature of mosquito saliva, multiple activities may lead to the enhancement of early virus infection. Further, due to the intense selective pressures exerted on mosquito saliva proteins by the host immune systems, successful viruses likely coevolve with their mosquito vectors in order to coopt unique saliva protein activities. For example, *A. aegypti* SGE reduced murine splenocyte proliferation and production of both Th1 and Th2 cytokines while *C. quinquefasciatus* SGE did not have this activity (224). These data suggest that the reduction of splenocyte proliferation and Th1/Th2 cytokine production may be critical for virus transmission and predict that *C. quinquefasciatus* would be less efficient at transmitting virus. The adaptation that has taken place between a virus and its vector's saliva proteins may contribute to vector competence, although these mechanisms remain poorly defined.

Multiple reports have suggested that immunomodulatory activities in mosquito saliva could result in enhanced early infection (45, 188, 190, 224, 231). These reports suggest that saliva modulates skin-resident immune cells. In one report, *A. aegypti* saliva was able to decrease beta interferon (IFN-β) and inducible nitric oxide synthase in macrophages *ex vivo* (188). Recruitment of T cells was also reduced when WNV was inoculated during mosquito feeding, rather than by syringe, suggesting that saliva hinders infiltration of these cells into the inoculation site (188). These effects correlated with enhanced expression of interleukin-10 (IL-10), which has anti-inflammatory activities, including the down-regulation of Th1 cytokines, major histocompatibility complex (MHC) class II molecules, and costimulatory molecules on macrophages (188). While this study is limited by the use of *A. aegypti* SGE, which is not the primary vector for WNV, it is likely that some *Culex* salivary proteins act to enhance WNV infection.

It is unknown whether *Culex* sp. SGEs have similar immunomodulatory activities; however, *C. pipiens* SGE was able to enhance Cache Valley fever virus infection, and *C. tarsalis* saliva was able to enhance WNV infection in a mouse model (56, 206). Additionally, saliva from *C. tarsalis* and *C. pipiens* was able to enhance WNV infection in chickens (204). The fact that saliva from multiple species in both the *Aedes* and *Culex* genera was able to enhance virus infectivity would suggest either that the relevant saliva proteins are highly conserved or that a similar activity has convergently evolved in multiple mosquito vectors. If all *Culex* spp. modulate a specific component of the host immune system to facilitate blood feeding, WNV may have evolved to benefit from this universal mosquito saliva activity. In addition, differences in salivary gland protein activities could alter the ability of a mosquito species to enhance pathogen transmission. Multiple activities that differ between *Aedes* and *Culex* mosquitoes have been noted (166, 169, 170, 173, 223). Since such dramatically different saliva activities exist between *Aedes* and *Culex* spp., direct comparisons of mosquito saliva activities that are responsible for the enhancement of WNV transmission need to be performed for each *Culex* sp. that is able to vector WNV.

Though mosquito saliva has been shown to enhance WNV infection, the precise mechanisms as well as the specific saliva proteins involved remain to be investigated. In one example, hyaluronidase from sand fly saliva was found to be important for the enhancement of *Leishmania* infectivity in mice (223).

Saliva hyaluronidase may enlarge the feeding lesion and serve as a spreading factor for other pharmacologically active factors present in saliva (223). This activity was also found in *C. quinquefasciatus* saliva and may also affect the spread of WNV and other saliva components as well as influence the local host immune response (168, 223). In another example, Salp15 from tick saliva was able to directly interact with the surface of *Borrelia burgdorferi* and facilitated evasion from host B cell-mediated immunity (160), and immunization against Salp15 protected mice from Lyme disease (50). Another study identified two tick saliva proteins that functioned to inhibit polymorphonuclear leukocyte recruitment during infection of mice with *Borrelia burgdorferi*, likely increasing the spirochete burden and enhancing infection (77). Identification of proteins in mosquito saliva that are responsible for the enhancement of WNV transmission is under way, and these investigations may provide novel nonvirus targets for vaccine design.

Multiple negative salivary gland factors that limit flavivirus transmission have been identified (42, 124). In one example, microarray analysis of DENV-infected and uninfected salivary gland mRNAs showed an upregulation of a putative antibacterial, cecropin-like peptide (i.e., AAEL000598), which showed antiviral activity against both DENV and Chikungunya virus (124). A recent comparative microarray analysis of mRNAs from DENV-, YFV-, and WNV-infected and uninfected whole *A. aegypti* identified multiple genes that were downregulated by all three viruses (42). Genes downregulated by day 14 postinfection likely play a role in salivary gland invasion or virus transmission. Among those, a recombinant pupal cuticle protein was able to directly interact with WNV envelope protein and inhibit infection *in vitro* and prevent lethal WNV encephalitis in mice (42). Although these proteins were expressed in salivary glands, they have yet to be formally identified in saliva.

Transgenic traits and introduced factors can also alter the transmission of vector-borne pathogens and may play a role in the future control of virus-infected mosquito populations. Transgenic mosquito populations that can be selected to either block transmission, block acquisition, decrease host seeking, decrease probing and biting, increase background mortality, or increase mosquito infection-induced mortality are in development (1, 59, 98, 128, 147, 148). To date, most studies have focused on producing transgenic mosquitoes that block transmission. For example, experimental strains of *A. aegypti* that inhibit flavivirus replication in the midgut and consequent migration to the salivary gland have been engineered (59, 98, 147, 148). Another gene that is responsible for host seeking behavior has been identified (203). Many strategies that lead to increased background mortality have been implemented, and field trials have already begun to test the effectiveness of these transgenic mosquitoes in reducing wild mosquito populations (9, 64, 65, 214). Laboratory infection with *Wolbachia* bacteria also reduces the life span of mosquitoes (127). This strategy has also been tested in field trials to reduce wild mosquito populations (82). The release of insect-specific densoviruses also shows high mortality in mosquito populations and may be used as a control strategy (34). The advantage of using *Wolbachia* or *Densovirus* infection as opposed to insecticide treatment is that these pathogens are expected to replicate and spread through the wild mosquito populations (128).

MAMMALIAN INFECTION

Epidemiology and Clinical Features

The emergence of WNV in North America was first documented in the fall of 1999 in New York City following an outbreak of mosquito-borne encephalitis responsible for the death of humans, birds, and horses (3, 26, 104, 145, 232). Over the next decade, WNV spread throughout the United States and into Canada, Mexico, and the Caribbean (75). From 2005 to 2009, 12,975 cases were reported to the CDC, including 496 fatalities, and 35% of reported cases were the more severe forms of neuroinvasive disease, including encephalitis (119). As detailed above, in most cases the virus is transmitted by the *Culex* mosquito vector (4), but transmission may occur through blood transfusion, organ transplantation, breast-feeding, or intrauterine exposure, and laboratory-acquired infection has also been reported (35a, 81, 85, 103, 177).

Infections in humans are predominantly subclinical, but reported infection manifestations may range from fever and myalgias to meningoencephalitis and death (152). Encephalitis occurs in only a small subset of patients; progression to severe neurological illness may induce acute flaccid paralysis after meningitis or encephalitis, with rapidly progressing symptoms that may involve all four limbs (111). Severe poliomyelitis-like syndrome can occur and has a poor long-term outcome (191). Elderly individuals are more susceptible to neurological involvement that may result in death, and among those older adults who survive, as many as 50% may have significant postillness morbidity for at least a year following infection (33) and may have an increased risk of death for up to 3 years after acute illness (120). Among individuals over 70 years of age, the case-fatality rate ranges from 15% to 29% (152). Higher fatality is also seen in infected infants and immunocompromised patients (73). Risk factors for encephalitis and death include being homeless, a history of cardiovascular disease or chronic renal disease, hepatitis C virus infection, and immunosuppression (140, 192). In addition, in some cases convalescent patients may have persistent or chronic infection detected through PCR of the urine, which suggested ongoing viral replication in renal tissue (141, 143). Although persistence of WNV has also been noted in several animal models (156, 199, 213), it has not been uniformly evident in assays of urine (66).

Diagnostics

The diagnosis of WNV infection is based largely on clinical criteria and testing for antibody responses (28). The incubation period for WNV infection is thought to range from about 2 to 14 days (143). The presence of anti-WNV IgM, particularly from cerebrospinal fluid (CSF), is used for diagnosis. Cross-reactivity with related flaviviruses (Japanese encephalitis virus, St. Louis encephalitis virus, YFV, and DENV), if suspected, can be assessed through plaque neutralization assays (143). Replication of WNV has been documented in human monocytes *in vitro* and with even higher efficiency in polymorphonuclear leukocytes; this could lead to transmission via transfusion of blood (10, 177). Thus, several rapid tests have been developed for blood donor screening using nucleic acid testing (NAT), an amplification-based transcription technique, which identifies WNV-infected individuals before they become symptomatic and may be used to safeguard the blood supply (238). Of note, 45% of NAT-positive subjects were subsequently not confirmed, and in one study, only 4 to 5% of the patients received a diagnosis of WNV infection (238).

TABLE 1 *In vivo* function of murine genes in WNV infection

Gene	Survival[a]	Viremia	Brain viral load	Remarks	Reference(s)
Myd88$^{-/-}$	S	Up	Up	Reduced leukocytes in CNS	209, 218
Tlr3$^{-/-}$	R	Up	Up	Reduced viral entry into CNS	230
Tlr7$^{-/-}$	S	Up	Up	Defective leukocyte homing	218
Il10$^{-/-}$	R	Down	Down	Enhanced antiviral response	9a
Irf7$^{-/-}$	S	Up	Up	Defective type I IFN production	48
Casp12$^{-/-}$	S	Up	Up	Defective type I IFN production	226
*Ifnα*β*R*$^{-/-}$	S	Up	Up		181
*Ifn*β$^{-/-}$	S	Up	Up		109
*Ifn*γ*R*$^{-/-}$ or *Ifn*γ$^{-/-}$	S	Up	Up		53a
Ips1$^{-/-}$	S	Up	Up	Lack of regulatory T cells	208
Pkr$^{-/-}$ or *RnaseL*$^{-/-}$	S	Up	Up		182, 185
Mmp9$^{-/-}$	R	Equivalent	Down	Reduced viral entry into CNS	227
Irf3$^{-/-}$	S	Up	Up	Impaired IFN-stimulated gene expression in macrophages	47
C3$^{-/-}$	S	Up	Up	Impaired CD8^{+} T/antibodies	131
Compl R1/2$^{-/-}$	S	Up	Up	Impaired protective antibodies	129
Ccr5$^{-/-}$	S	Equivalent	Up	Reduced T cells, NK cells, macrophages in CNS	69
Cxcr2$^{-/-}$	R	Down	Down		10
Cxcr3$^{-/-}$	S	Equivalent	Up	Impaired CD8^{+} T cell recruitment to brain	97a
Cxcl10$^{-/-}$	S	Equivalent	Up	Impaired CD8^{+} T cell recruitment to brain	114a
Ccr2$^{-/-}$	S	Equivalent	Up	Fewer inflammatory monocytes in CNS	197a
sIgM$^{-/-}$	S	Up	Up	Reduced WNV-specific IgG, no IgM	197b
Casp3$^{-/-}$	R	Equivalent	Equivalent	Reduced neuron apoptosis	182
Icam1$^{-/-}$	R	Equivalent	Down	Reduced viral entry into CNS	50
Cd8a$^{-/-}$ *MHCclass1a*$^{-/-}$	S	Equivalent	Up	Increased viral loads (spleen), persistent infection in surviving mice	197
Cd4$^{-/-}$ *MHCclass2*$^{-/-}$	S	Equivalent	Up	Impaired WNV-specific IgM and IgG production, persistent infection	200a
Cd40$^{-/-}$	S	Equivalent	Up	Impaired WNV-specific IgM/IgG production, reduced CD8^{+} cells in CNS	200b
Il22$^{-/-}$	R	Equivalent	Down	Reduced viral entry into the CNS	225
Dhx58$^{-/-}$	S	Equivalent	Up	Reduced CD8^{+} T cell expansion	208a
TRAIL$^{-/-}$	S	Equivalent	Equivalent	CD8^{+} T cells use TRAIL to limit infection	236a

[a] R and S, mice are more resistant or susceptible, respectively, to lethal WNV infection than their wild-type controls.

Antibody testing in patients follows an expected timetable of median times of 3.9 days from RNA detection to IgM seroconversion and 7.7 days from RNA detection to IgG seroconversion (28). RNA generally became undetectable after 13.2 days, although it rarely was found to persist for >40 days. IgM and IgA antibodies fell significantly, although not universally, while the IgG level remained elevated for >1 year after detection of viremia (28, 141, 149, 180). Antibody to WNV NS5 persists *in vivo*, and thus NS5 antibody cannot be used to distinguish recent from past WNV infection (157).

Immune Response

Control of WNV infection by the human and murine hosts has been investigated for both innate and adaptive immune responses. Through integrating these results, a picture of critical elements in immune responses to WNV is emerging (Tables 1 and 2). Sensing

TABLE 2 Genes and corresponding SNPs important in human WNV infection

Gene(s)	SNP(s)	Comparison groups (*n*)	Study results	Reference
OASL	rs3213545	WNV^{+} cases (33) vs healthy controls (16)	Associated with increased susceptibility to WNV infection	236
CCR5	Δ32 deletion	WNV^{+} cases (395) vs WNV^{-} (1,463)	Increased risk of symptomatic WNV infection	69
		WNV^{+} cases (224) vs healthy controls (1,318)	Increased risk of symptomatic WNV infection	113
		WNV^{+} cases (634) vs WNV^{-} (422)	Not a risk factor for WNV initial infection; associated with symptomatic WNV infection	114
OAS1	rs10774671	WNV^{+} cases (501) vs healthy controls (552)	A risk factor for initial infection with WNV	112
IRF3, MX1, OAS1	rs2304207, rs7280422, rs34137742	Symptomatic cases (422) vs asymptomatic cases (331)	Associated with symptomatic WNV infection	19
RFC1, SCN1A, ANPEP	rs2066786, rs2298771, rs25651	Severe WNV cases (560) vs mild WNV cases (950)	Associated with neuroinvasive disease in patients infected with WNV	123

WNV pathogen-associated molecular patterns through pathogen recognition receptors such as Toll-like receptors (TLRs) and cytoplasmic RNA helicases is critical for early detection and activation of innate immune pathways that facilitate early control of viral replication (48, 61, 208–210, 218, 226, 230). This early response is mediated largely by macrophages; WNV infection of macrophage-depleted mice results in increased mortality, higher and extended viremia, and substantially shortened survival. Moreover, in mice, even a nonneurotrophic WNV strain may cross the blood-brain barrier (BBB) in the absence of macrophage clearance of virus (16). Macrophages express TLRs, mediate clearance of opsonized viral particles, produce proinflammatory cytokines, and upregulate costimulatory proteins that link innate to adaptive immune responses (114a, 215). Macrophages are also a major component in inflamed central nervous system (CNS) tissues and are considered protective against WNV infection. The control of WNV by macrophages has been linked both to constitutive expression of innate immune genes, such as those for RIG-I, MDA5, PKR, and RNase L, and to direct effector mechanisms such as the production of radical oxygen species and type I IFN (47, 49, 61, 67, 181, 182, 208a, 226, 229).

Although cellular immune mechanisms remain incompletely explained, innate immunity and in particular interferon responses have been shown to be critical in resistance to WNV (7, 9a, 181, 197b). Patients who mount a robust IFN-α response show lower viral loads, even before IgM seroconversion, concomitant with significant upregulation of IFN-γ during the viremic phase (217). Permeability of the blood-brain barrier (BBB), which is enhanced by cytokine responses, has been shown in murine models to be critical to resistance to WNV infection (230), and elements which decrease the integrity of the BBB contribute to susceptibility to infection with WNV (7, 226, 227). Entry to the CNS may be afforded by trafficking of infected $CD45^+$ leukocytes and CD11b macrophages (218), T cells (228), or neutrophils (225). Mice lacking TLR3 show improved survival over wild-type animals due to a lower cytokine response and protection from BBB permeability (100, 230). Human studies show a role for CXCL10 and CCL2 in control of early infection and an important role for IFN-mediated innate immunity in resolving acute WNV infection (217). RNAi studies in human cell lines have indicated that interferon-inducible transmembrane protein (IFITM) inhibits the early replication of WNV (23).

Infection with flaviviruses leads to upregulation of MHC class I, MHC class II, and adhesion molecules, which may enhance infection through reducing NK cell activity, or enhance a transient autoimmunity in early infection (97). It is clear that $CD8^+$ T cells are critical in the response to flavivirus infections. Overall T cell responses in humans revealed that multiple peptide regions of WNV proteins are recognized by T cells, with a subset of 8 peptides predominating, and the highest magnitude of specific T cell responses was from $CD8^+$ cells (105). The immunodominant T cell epitopes which elicited both highest-frequency and highest-magnitude responses included sequences from WNV M, E, NS3, and NS4 proteins and, furthermore, were equivalent between symptomatic and asymptomatic subjects in this cohort (105). During infection with WNV, $CD8^+$ T cells expand dramatically and migrate to the site of CNS infection (97, 236a). Examination of immune responses from WNV patients shows that memory T cell responses to WNV are mainly due to $CD8^+$ T cells with a defined set of epitopes; these were quite constant over 12 months of observation and were not apparently related to disease severity (150). Examination of memory T cells from 40 patients months after infection showed persistence of the memory phenotype and WNV-specific polyfunctional $CD8^+$ T cell responses. More cytolytic memory T cells were found in patients with neurological disease (154). Indeed, $CD8^+$ T cells have been shown to be important for control of viral load in mouse models of WNV infection, at least in part due to a role for perforin (97a, 197, 197a, 200a). WNV-specific murine CD4 T cells produced IFN-γ and IL-2 and also showed direct antiviral activity (25, 197b, 200b). Tregs play an important role in protecting against severe disease, and it has been shown in both human patients and animal models that symptomatic patients show a lower frequency of Tregs despite having similar systemic T cell responses (108).

Complement has also been indicated as an important component of the host innate immune response to flavivirus infection. However, while complement traditionally limits the spread of many pathogens, it appears to have both protective and pathogenic roles during flavivirus infection. Whether or not complement is protective or pathogenic depends on a variety of factors, including the specific virus, the phase of infection, and the underlying immune status of the host (40, 130, 131).

A paradoxical role for polymorphonuclear cells (PMNs) in WNV infection has been described, where PMNs are recruited to the site inoculated with WNV (10). It was determined by depleting PMNs prior to WNV infection that recruitment of PMNs to the inoculation site was associated with enhanced WNV replication. However, if PMNs were depleted after WNV infection, mice developed higher viremia and mortality. Thus, infiltrating PMNs may serve as an early reservoir of WNV replication (10). Dendritic cells express DC-SIGN, suggesting that they may also be early cellular targets in host skin (211). WNV infection of dendritic cells leads to production of IFN. Interestingly, studies with dendritic cells from human donors showed that type I IFN expression in response to WNV *in vivo* is lower in cells from older donors than in those from younger donors, which may contribute to older individuals being more susceptible to WNV disease (159).

These innate pathways are critical not only for immediate antiviral defense pathways such as the upregulation of type I interferons but also for the generation of an effective adaptive T and B cell-mediated sustained immune response (24, 53a, 129, 131, 155, 181, 198). The γδ T cell population rapidly expands after WNV infection. Mice that lack γδ T cells have higher viremia and increased mortality (229). Soon after infection, γδ T cells produce IFN-γ, which correlates with an increase in perforin expression in splenic T cells. Bone marrow chimera reconstitution experiments in mice support that IFN-γ production by γδ T cells is critical for the early control of WNV infection (229). γδ T cells also promote a protective adaptive immune response by facilitating dendritic cell maturation, providing an important link between the innate and adaptive immune responses against WNV infection (229).

Genetic Determinants of Disease

Specific human genetic factors that influence the severity of infection with WNV and the antiviral innate immune response have been identified (Table 2). Certain HLA types appear to be associated with risk of a more severe outcome (HLA-A*68 and C*08) or better resistance to infection (B*40 and C*03) (107). Single nucleotide polymorphism (SNP) studies have detected SNPs in key regulators of immune function, including interferon pathway elements. In particular, polymorphisms in IRF3 and MX-1 were

associated with symptomatic infection, and an SNP in the oligoadenylate synthetase 1b (OAS-1) gene, an interferon-regulated gene involved in RNA degradation, was associated with an increased risk for initial infection with WNV and severe neurological disease (>750 subjects) (19, 112). Notably, the 2′,5′-oligoadenylate synthase (2′-5′-OAS) gene has also been identified as a susceptibility factor in WNV in horses and as a contributing factor for severity of neurological disease in tick-borne encephalitis virus (13, 176, 236). A dominant negative splice variant of RNase L, which functions in the antiproliferative roles of interferon, was detected more often in WNV patients than in control patients (236). Another genomic study investigated >1,500 symptomatic subjects (with severe versus mild disease) and showed more severe neurological disease to be associated with SNPs in the genes for RFC1 (a replication factor), SCN1a (a sodium channel), and ANPEP (an aminopeptidase), although even more differences might have been revealed when comparing asymptomatic and symptomatic cases (123). In addition, a deletion in CCR5, which is known to be protective in infection with HIV, while not associated with susceptibility to WNV, did correspond to severity of infection, presumably due to reduced function of CCR5 pathways in infected hosts (69, 113, 114). As more host factors are identified, there are sure to be a number of new determinants of WNV infection.

Therapeutics

Current therapeutic options against WNV are mainly supportive; there are no FDA-approved vaccines or treatments available (54). Investigations to identify individual susceptibility markers, recombinant antibodies, peptides, RNA interference, and small molecules with the ability to directly or indirectly neutralize WNV have been reported; however, an effective drug is still lacking (6, 12, 70, 71, 74, 146, 158). There are currently four USDA-licensed vaccines available for equines (two are inactivated whole WNV, one is a nonreplicating live canary pox recombinant vector vaccine, and one is an inactivated flavivirus chimeric vaccine). Though passive immunization has been used in a few cases, it has serious limitations, such as inadvertent transfer of blood-borne pathogens, inconsistent quality of the donor antisera, cost, and allergic reactions (78). A case study of two WNV encephalitis patients treated with alpha interferon, the standard of care for infection with the related flavivirus hepatitis C virus, showed substantial improvement and an improved convalescence course (88).

Several approaches are being pursued for the development of a vaccine in humans that may prove valuable for use by targeted populations. Investigations include live attenuated vaccines, recombinant subunit vaccines, vectorized vaccines, DNA vaccines with constructs that express the WNV E protein, live recombinant vaccines, and an attenuated strain based on nonglycosylated E and mutant NS1 proteins (15, 235). A neutralizing, WNV-specific monoclonal antibody, E16 (MGAWN1), which penetrates the CNS in animal models, produced neutralizing antibodies in phase I trials (15). Very promising results were seen with a chimeric vaccine based on the WNV prM and E proteins inserted into the yellow fever 17D vaccine moiety (ChimeriVax-WN02). It was shown to be safe and immunogenic in phase II clinical trials, with high seroconversion rates, but it is no longer available (18).

CONCLUSIONS AND FUTURE DIRECTIONS

WNV has now persisted and become established in North America. Of particular significance is the expansion of the mosquito vectors harboring WNV to include *Aedes albopictus*, a common mammal-biting mosquito (2, 73, 136). It is hoped that the increase in our knowledge of the interactions of WNV with the mosquito vector will lead to new avenues for therapeutics and preventative measures. Mosquito responses at the levels of protein and gene expression as well as a more complete understanding of viral pathogenesis in the vector, especially with regard to the immune response, may point to novel targets to focus our efforts to inhibit or block WNV infection in both mosquitoes and mammals.

For example, a single-chain human monoclonal antibody developed through phage display directed against the fusion loop of the envelope protein showed both pan-flaviviral protection and therapeutic efficacy when tested in the murine model (71, 207). Recent advances in nanoparticle technology have also been employed in vaccination studies of murine WNV infection and show promising efficacy of TLR9-targeted biodegradable nanoparticles, which produce a high number of circulating effector T cells and antigen-specific lymphocytes (53). Potential relevant viral susceptibility mechanisms, including host antagonism of chemokine responses as has been noted in infection with the related flavivirus hepatitis C virus (35), may reveal infectious mechanisms used by WNV and other mosquito-borne flaviviruses. The pace of discovery of vector, virus, and host components of pathogenesis continues to provide critical insights for the successful development of controls and treatments for WNV.

ACKNOWLEDGMENTS

We are grateful to our long-standing colleague John F. Anderson of the Connecticut Agricultural Experiment Station and to members of the Yale IMAGIN and HIPC teams for valuable discussions.

This work was supported in part by the NIH (grants HHS N272201100019C, U19AI089992, and U01AI 070343). E.F. is an Investigator of the Howard Hughes Medical Institute. T.M.C. is supported by grant 2T32HL007974-11, and M.J.C is supported by grant 5T32AI07019-35.

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Ruth R. Montgomery received a B.A. from the University of Pennsylvania and a Ph.D. from Rockefeller University, where she studied with Zanvil Cohn and Carl Nathan. After postdoctoral work on macrophage endocytosis with Ira Mellman, she remained at Yale, where she is now Associate Professor of Medicine. The focus of her lab is human innate immunity, specifically the interaction of macrophages, neutrophils, and dendritic cells with pathogens such as West Nile virus and the agent of Lyme disease, *Borrelia burgdorferi*, including elucidating effects of vector saliva on phagocyte function. In studies of the pathogenesis of West Nile virus, the Montgomery lab has described inhibition of macrophage function, an unexpected biphasic role for PMNs in infection, and effects of aging on innate immunity, including dysregulation of TLR3 responses in macrophages and reduced responses of dendritic cells to infection with West Nile virus.

Erol Fikrig received a B.A. in chemistry from Cornell University and an M.D. from Cornell University Medical College. Dr. Fikrig did a residency in internal medicine at Vanderbilt University School of Medicine and was a fellow in infectious diseases and immunobiology at Yale University School of Medicine. He is currently a Professor of Medicine, Microbial Pathogenesis, and Epidemiology and Public Health, a Waldemar Von Zedtwitz Professor of Medicine, Chief of Infectious Diseases, and an investigator with the Howard Hughes Medical Institute. He currently leads a research group studying the immunopathogenesis of arthropod-borne diseases. Lyme disease, human granulocytic anaplasmosis, and West Nile encephalitis are areas of particular interest. Studies are directed at understanding the interactions between pathogen, host, and vector that result in virulence and transmission and the molecular basis of disease in animal models and patient populations.