

# 18. The Human Antibody Response Against WNV

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## Abstract

Experimental evidence has shown that antibody responses to West Nile virus (WNV) are critical for protection from WNV-mediated disease. Antibody responses are also an important immune correlate of protection for the clinical evaluation of WNV vaccines. However, little direct study has been carried out on the characteristics of the human antibody response to natural WNV infection. Preliminary evidence suggests that there are important differences in the way humans and experimental animals mount humoral responses to WNV. In humans, IgM is remarkably persistent in the serum and specific IgG is slow to appear. In addition, mapping of the IgG response to the functionally relevant E-protein suggests that it is directed away from critical protective epitopes and towards weakly neutralizing immunodominant epitopes. These findings have important implications for vaccine design and testing.

## Keywords

antibody, flaviviruses repertoire human vaccine

## 1 Introduction

In experimental models, humoral immunity is critical to protection from WNV-mediated disease (Ben-Nathan et al., 2003; Camenga et al., 1974; Diamond et al., 2003a,b; Engle and Diamond, 2003; Gould et al., 2005; Tesh et al., 2002; Wang et al., 2001). B cell-deficient mice are more susceptible to disease (Diamond et al., 2003a; Halevy et al., 1994), and passive transfer of antibodies to these mice restores immunity (Engle and Diamond, 2003). Clinical case reports describing the use of human intravenous immunoglobulin (Ig) containing WNV antibodies in patients with neurological disease suggest some therapeutic benefit (Haley

et al., 2003; Hamdan et al., 2002; Shimoni et al., 2001). Placebo-controlled clinical trials are in progress to determine the validity of these observations (<http://clinicaltrials.gov.htm>).

Analysis of humoral responses to WNV have mainly been carried out after experimental infection of rodents (Oliphant et al., 2005; Sanchez et al., 2005). Isolation of monoclonal antibody (mAb) panels has been used to determine the antigenic variability of WNV, and challenge studies have been performed to test vaccines for clinical development. Classically, the human antibody response to WNV infection has been studied by looking at serological responses to infection. Recently, more advanced protein engineering approaches and display technologies have been utilized to probe the human repertoire against WNV. Collectively, these studies have identified important differences in the humoral response against natural WNV infection in humans compared to experimental WNV infection in rodents. In particular, the persistence of WNV-specific IgM after infection and the immunodominance of weakly neutralizing epitopes of the domain II region of E-protein appear to be unique to the human immune response to WNV. These differences potentially have important consequences for the design and testing of preventative vaccines.

## 2 Natural Infections in Humans

The source of human WNV infections is almost invariably through the bite of an infected mosquito, although transmissions through other routes such as transplantation or blood transfusion have been reported (Iwamoto et al., 2003). The initial replication of WNV after inoculation is thought to occur in dendritic cells in the skin, which migrate to secondary lymphoid tissues and the replicating virus enters the circulation (Byrne et al., 2001). Viremia is transient and of low titer. Virus can typically be detected by reverse transcriptase polymerase chain reaction (RT-PCR)-based assays 4–9 days post infection (Lanciotti et al., 2000). Symptoms generally appear at the time viremia disappears, and the virus becomes detectable in the cerebral spinal fluid (CSF). The period in which WNV is detectable in the CSF is variable, but can last several weeks in some patients often correlating with the severity of symptoms. The clinical course ranges from a self-limited febrile disease to a severe neuroinvasive disease that can lead to permanent disability or death (Klee et al., 2004).

Serological surveillance data from the New York epidemic of 1999 showed that one in five people infected with WNV develop fever and 1 in 150 develop neurological symptoms (Mostashari et al., 2001). These data are supported by subsequent seroprevalence studies which suggest

that most human WNV infections do not result in significant symptoms (Busch et al., 2006). Risk factors for severe disease have not been well defined apart from age. Individuals over the age of 50 have up to a 20-fold increased risk to develop severe disease. Hypertension and diabetes have also been causally associated with serious WNV disease (Granwehr et al., 2004).

### 3 Serology

Patients suspected of WNV infection are tested for the presence of IgM and IgG antibodies to WNV using a commercially available IgM-capture enzyme-linked immunosorbent assay (ELISA) and indirect IgG ELISA, respectively. In some cases, a diagnosis of WNV infection is confirmed by a definitive plaque reduction neutralization assay (PRNT). However, while the PRNT assay is more specific for WNV, it requires specialized laboratory facilities and is not routinely performed. For this reason, most serological data relating to human WNV infections is based on ELISA experiments alone, which give no direct information on the functional activity of detected antibodies.

The first antibody responses detected in humans after primary infection with WNV are of the decavalent, pentameric IgM isotype. Clinical case studies report that serum IgM levels appear 10–14 days after infection (Gea-Banacloche et al., 2004). This correlates well with an analysis of WNV-positive blood donations where the time between infectious mosquito bite and index donation was calculated using the measured viral load and models of virus doubling time (Prince et al., 2005). The peak IgM serum titers are reached 18 days after infection and can persist for more than 12 months. On the basis of regression analysis, IgM levels were estimated to persist above baseline on average 7–8 months post infection (Prince et al., 2005). In one study of patients with meningitis and encephalitis, specific IgM against WNV was measured 500 days after infection (Roehrig et al., 2003). The serum persistence of IgM against WNV is unusual particularly given that viremia is transient and, in general, the titer of WNV antibodies is low. However, it is consistent with reported serology to other flaviviruses such as dengue and St. Louis encephalitis viruses (Han et al., 1988; Summers et al., 1984). Somewhat surprisingly, WNV-specific IgM can appear in the CSF before detection in serum, soon after the virus is detected in the CSF (Gea-Banacloche et al., 2004). IgM is too large to diffuse across the blood–brain barrier (BBB), and therefore may be produced locally by B cells that have trafficked into the CSF (Binder and Griffin, 2003) or through inflamed vessels when the BBB integrity has been compromised

(Wang et al., 2004). In addition, consistent with the persistence of IgM in the serum, IgM can be measured in the CSF 199 days post infection (Kapoor et al., 2004).

Extended maintenance of serological responses have been proposed to result from the presence of long-lived plasma cells in the bone marrow that secrete antibody, from boosting of memory responses through interaction with antigen retained on follicular dendritic cells, or through bystander stimulation of memory B cells by heterologous vaccination or infection (Bernasconi et al., 2002). These responses are generally associated with isotype-switched and affinity-matured immunoglobulin (e.g. IgG). The short serum half-life of human IgM (2 days) requires a larger number of plasma cells to maintain serum levels in contrast to switched isotypes (e.g. IgG1 has a serum half-life ~21 days). Although there is a limited capacity in the bone marrow and spleen to accommodate plasma cells (Cassese et al., 2003; Manz and Radbruch, 2002), there may be a benefit in retaining the IgM isotype compared to switched isotypes at the cost of efficiency in maintaining serological surveillance. This could be due to specific functionality of the IgM constant region (e.g., efficient complement fixation) or detrimental functionality of a switch isotype constant region. Alternatively, viral elements could block isotype switching by interfering with T-cell help to reduce the potential effectiveness of the immune response. Longitudinal studies analyzing the neutralizing potency of serum IgM, and investigation into the nature of the persistent IgM-secreting plasma cells in humans, are required to better understand the relationship between IgM levels, WNV pathogenesis, and clinical outcome.

The serum persistence of IgM has diagnostic implications. WNV-reactive IgM in serum samples is generally regarded as confirmation of WNV infection when taken with the seasonal and clinical information. However, if IgM levels persist over 1 year, they can overlap WNV seasons of infectivity, creating the possibility of false positives. The IgM assay can be additionally confounded, as it fails to distinguish WNV infection from other closely related flaviviruses such as the Japanese or St. Louis encephalitis virus (Martin et al., 2002).

Isotype-switched isoforms of immunoglobulin generally follow the initial detection of IgM in a primary immune response. Potent virus-neutralizing antibodies are generally of the IgG isotype and reflect a process of affinity maturation (Hangartner et al., 2006). In humans, measurable IgG against WNV does not appear in the serum until after the second or third week of infection, a time during which most symptoms would have disappeared (Prince et al., 2005). In most patients,

serum IgG titers are low, a finding that is also observed with live attenuated viral vaccines that have been tested in clinical trials (Monath et al., 2006). The avidity of IgG increases over time and in response to a secondary infection (Fox et al., 2006), consistent with somatic hypermutation. IgG can sometimes be found in the CSF but this is highly variable and does not necessarily follow an IgM response even when IgG is measurable in the serum (Gea-Banacloche et al., 2004).

#### 4 Antibody Repertoire Analysis

The antigen-binding site of human Ig is derived from the translated product of two rearranged genes VH and VL coupled via constant regions. The VH gene is made up of the V–D–J regions that are chosen from pools of V (~50), D (25), and J (6). Similarly the VL gene is made up of two segments V (~70) and J (9) (V-base <http://vbase.mrc-cpe.cam.ac.uk/>). The rearrangement of these gene segments theoretically gives rise to more than 10 million combinations, which is certain to be an underestimation given the extra variability introduced at junctions and through processes such as receptor editing (Neuberger, 2002). The process of somatic hypermutation (SHM), which occurs in B cells specifically activated by T-cell interaction, can further modify the antigen-binding site to increase its binding affinity to cognate antigen (Di Noia and Neuberger, 2007).

Analyses of antibody repertoires generated against infectious pathogens have revealed diverse strategies to elicit protective immunity. In some cases, a single V gene combination binding to a conserved carbohydrate epitope dominates the immune response. This kind of restriction usually results from surface exposure of a low-complexity antigen, such as the repetitive capsule polysaccharide of *Haemophilus influenza* (Lucas and Reason, 1999). In cases where the pathogen displays a more complex antigenic surface, greater repertoire diversity is observed. In one study analyzing the antibody repertoire in two donors after boosting with tetanus vaccine, ~100 unique VH–VL combinations were calculated to comprise the complete response to the complex protein target tetanus toxoid (Poulsen et al., 2007).

Two studies have analyzed the human antibody repertoire against WNV using display approaches. In one approach, scFv phage libraries were constructed from B cells of donors following a symptomatic infection with WNV (Throsby et al., 2006). Blood samples were taken from convalescent patients 1, 2, and 3 months after clinical presentation. Serum IgG ELISA titers were maximum at 1 month after infection. The

ELISA titers from the different patients did not correlate with neutralization, nor did neutralization titers increase over the sampled time points. The IgG VH and VL genes were amplified from these patients and pooled in combinatorial libraries (Kramer et al., 2005). VH and VL gene representation in clones sequenced from the unselected libraries were in accordance with previously published analysis of naïve human repertoires (de Wildt et al., 1999; Poulsen et al., 2007). These libraries were screened for antibodies binding to WNV antigenic preparations that included either purified inactivated virus, virus-like particles (VLPs) consisting of prM and E-protein, or soluble recombinant E-protein. A total of 72 different unique VH genes defined at the level of HCDR3 and VH gene alignment use were identified. Sequencing of the V genes demonstrated ample diversity among the selected scFv VH-VL combinations; but in contrast to the unselected library, the selection of anti-WNV clones revealed a bias to VH1 (32% vs. 21%) and VH3 genes (39% vs. 23%) and to V $\lambda$  1–3 (67% vs. 28%). The diversity in the repertoire is consistent with antigenic complexity displayed on the WNV surface. Structural analysis of the flavivirus envelope protein has revealed that a relatively large proportion of the envelope protein is solvent exposed (Rey et al., 1995) compared to the dense packing of spike glycoproteins such as on the vesicular stomatitis virus (VSV) or the SARS coronavirus. In addition, the structural proteins of WNV are arranged in a complex pattern on the viral surface that potentially creates further antigenic diversity (Kaufmann et al., 2006). From the unique human mAbs identified from immune libraries, ~90% bound to E-protein, a single mAb was reactive with prM, and the remaining small number was bound to unidentified targets. Heterogeneity was observed among the panel in terms of ELISA, immunoprecipitation, and western blot reactivity to E-protein, indicating that the WNV mAb binds to a diverse number of epitopes. In contrast, only nine of the mAbs in the panel demonstrated activity in a microneutralization assay against WNV. This observation was not due to affinity, as some of the highest affinity antibodies in the panel had poor or no neutralizing activity, but rather suggests that only a restricted number of the epitopes displayed by WNV are targets of neutralizing mAb.

Unfortunately, in this study the repertoire of IgM expressing B cells was not examined. It would be valuable to compare the IgM to the IgG repertoire in convalescent patients after WNV infection given the important protective role of anti-WNV IgM in animal models and its persistence in serum.

In a complementary set of experiments, two large combinatorial libraries built from naive donors were extensively screened for antibodies

binding to recombinant WNV-E-protein (Gould et al., 2005). Although not formally tested, it is assumed that none of the 57 donors used to generate the library was seropositive for WNV. Only five unique scFv against WNV were isolated, of which two had neutralizing activity when converted into a bivalent IgG-like format. Although the poor recovery of WNV-specific binders from these libraries could be related to technical issues (library panning was only carried out on recombinant E-protein, which may have restricted the diversity), high-affinity scFv have been isolated against a variety of targets in previous experiments using these libraries (<http://research.dcfi.harvard.edu/nfcr-ctae/research/mehta.php>), including a potent neutralizing mAb against SARS coronavirus with nanomolar affinity (Sui et al., 2004). This may indicate that in the normal repertoire of individuals not infected with WNV, rearranged immunoglobulins with specificity to WNV are rare.

## 5 Epitope Mapping

The antigenic structure of various flaviviruses including WNV has been extensively studied. Effective development of vaccines against WNV depends on a sound knowledge of the key neutralizing and protective epitopes on WNV. However, a detailed understanding of how the human immune system responds to the presentation of these epitopes is currently lacking. In experimental WNV infections, antibodies develop against E, prM, NS1, NS3, and NS5 but only antibodies against E-protein have been identified as neutralizing and protective. E-protein mediates two important functions in flavivirus replication: virus attachment to susceptible host cells and the fusion of the viral and the cellular membranes (Mukhopadhyay et al., 2005). The only other target of protective antibodies that has been described is the NS1 protein, which is secreted from infected cells and not present on the virus surface (Chung et al., 2006; Henchal et al., 1988; Roehrig, 2003). The mechanism of protection by these NS1-specific antibodies has not been formally elucidated but is likely due to antibody-dependent cellular cytotoxicity of infected cells expressing NS1 on their surface (Chung et al., 2006).

Fine mapping of neutralizing epitopes on E-protein domains using a novel yeast display system (Oliphant et al., 2005), (escape) mutant generation (Beasley and Barrett, 2002; Sanchez et al., 2005), NMR (Wu et al., 2003), and strain comparison (Li et al., 2005) have identified several critical residues required for neutralization activity. Residues consistently identified as important for potent neutralization cluster

around E-protein amino acid positions 305–312, 330–333, and 365. The recent co-crystallization of WNV E-protein domain III in complex with the potent neutralizing mAb E16 showed that these residues, located on adjacent exposed loops of the domain III, form a discontinuous epitope referred to as the domain III lateral ridge (DIII-lr) (Nybakken et al., 2005). When sequences of these loops from other flaviviruses such as Japanese encephalitis and dengue viruses are aligned, considerable variation is observed compared to the E-protein as a whole (Nybakken et al., 2005). This is consistent with the observation that E-protein DIII binding neutralizing antibodies are virus-type specific (Roehrig, 2003). Several studies have mapped cross-reactive, neutralizing mAbs to regions outside of DIII. Most of these bind in or around the fusion loop at the distal tip of domain II (DII-fl) (Crill and Chang, 2004; Goncalvez et al., 2004; Oliphant et al., 2006; Stiasny et al., 2006). Antibodies directed against this region are not as potent *in vitro* or protective *in vivo* as the DIII-binding antibodies. Recent evidence suggests that many of these epitopes are partially occluded on the surface of the infectious virus, in part explaining their lack of efficient neutralizing activity (Oliphant et al., 2006; Stiasny et al., 2006).

Mapping studies were carried out with a representative panel of human mAbs isolated from the WNV-immune libraries described above (Throsby et al., 2006). In an ELISA competition assay, 47% of WNV antibodies from the panel competed for binding with the DII-fl-binding mAb 6B6C, while in contrast only 8% competed with DIII-lr-binding mAb 7H2. Results from *in vitro* and *in vivo* functional testing of the human mAb panel were consistent with experimental systems: potently neutralizing and protective mAbs were exclusively directed to domain III of E-protein, whereas mAbs that recognized domain II had weak, if any, *in vitro* neutralizing activity and no *in vivo* protective activity (Throsby et al., 2006). CR4374 was the most potent inhibitory human anti-WNV mAb identified from the panel. Fine mapping using yeast display demonstrated that the binding activity of CR4374 was lost by mutation at residue E307, indicating that its epitope is located in the DIII-lr (Oliphant et al., 2007). However, in contrast to E16, 7H2, and other potent mAbs binding in this region, mutation at position 332 did not affect binding, suggesting it may bind a novel overlapping epitope. Taken together, the data from these experiments indicate that in humans the immunodominant epitopes on WNV are situated predominantly in E-protein domain II and generate only weak neutralizing activity, while the weakly immunogenic E-protein domain III is associated with the most potent antiviral activity.



Experimental evidence has suggested that antibodies against E-protein domain II of flaviviruses can, under certain conditions, enhance infectivity of WNV *in vitro* (Nybakken et al., 2005; Pierson et al., 2007). In animal models, sub-neutralizing concentrations of antibodies against a related flaviviruses resulted in lower levels of survival compared to animals that were treated with a control antibody (Hawkes and Lafferty, 1967), and the administration of nonprotective YF mAb at the time of YF or JEV virus challenge was shown to enhance neurovirulence (Gould and Buckley, 1989). In humans, a serious hemorrhagic disease is associated with previous exposure to a heterologous subtype of dengue (Cardosa, 1998; Halstead, 1988). This process called antibody-dependent enhancement (ADE) is thought to result from the internalization and productive infection of macrophages and other myeloid cells through Fc- $\gamma$  receptors (Cardosa et al., 1986; Iankov et al., 2006; Takada and Kawaoka, 2003; Tirado and Yoon, 2003). In a similar way, complement fixation and viral uptake through complement receptors on macrophages have also been associated with enhanced infectivity (Cardosa et al., 1986; Tirado and Yoon, 2003). A significant proportion of non-neutralizing mAbs (~30%) isolated from the WNV-immune repertoire above bound to an epitope depending on the homodimeric form of the E-protein and were particularly active in fixing the complement (Throsby et al., 2006). Thus, the most commonly identified antibodies from the human antibody repertoire could, in theory, enhance infection via mechanisms that allow the virus to be internalized by a larger number of cell types. It is important to note that enhancement of WNV infection has also been demonstrated *in vitro* for the very potent anti-E-protein domain III-neutralizing antibodies at low concentration and that neutralization and enhancement activity overlap (Pierson et al., 2006, 2007); however, this finding has not been reproduced experimentally *in vivo* (Engle and Diamond, 2003).

Two studies, one screening the naive human B cell repertoire before infection (Gould et al., 2005) and the other looking at the B cell repertoire after infection (Throsby et al., 2006), indicate that the human immune response is skewed toward E-protein domain II of WNV, and the potent neutralizing E-protein domain III antibodies are rare in the human B cell repertoire. To address this point further, analysis of human serum samples has been carried out with gain or loss of function WNV E-protein mutants (Oliphant et al., 2007). Convalescent serum samples screened at various time points after clinical disease demonstrated a skewing toward DII epitopes compared to DIII-binding epitopes. Although substantial variation was observed, on average, only 7.3%

(range, 0.6–50.5%) of the total IgG response was directed to DIII and an even smaller fraction (1.6%) to the potent neutralizing epitope DIII-lr. These percentages are remarkably close to those derived from repertoire analysis described above (8% for DIII and 2% for DIII-lr). In contrast, 61% (range 8.8–91%) of total IgG was directed to a single epitope at the tip of domain II. Again, this is consistent with repertoire analysis, where 47% of antibodies were shown to bind in the same region (Throsby et al., 2006).

A second observation to emerge was the clear difference in the response of experimental animals and humans to WNV infections. In mice, the WNV E-protein-specific IgM appeared early after infection, peaked by 8 days, and declined rapidly. Interestingly, and in contrast to the situation in humans, a specific IgM response against DIII was already present in naïve serum and increased after infection. Pre-infection immunoreactivity to viral proteins is frequently observed (Avrameas, 1991) and is generally ascribed to natural IgM (Casali and Schettino, 1996; Ochsenbein and Zinkernagel, 2000). In mice, natural IgM is produced in a T-independent fashion by B1 cells that are defined by surface expression of CD5 and localization to the peritoneum (Baumgarth et al., 2000; Casali and Notkins, 1989). Natural antibodies are characterized by polyspecific low-affinity binding and generally encoded by germline V genes without much evidence of somatic mutation. Natural antibodies are proposed to form a first line of defense against infection (Ochsenbein and Zinkernagel, 2000). In humans, the existence of a specific lineage of B cells producing natural antibodies is controversial and, to date, has not been conclusively demonstrated. T-independent antibody responses to carbohydrate antigens are mediated by marginal zone B cells which harbor mutated V genes (Lucas and Reason, 1999) that may be prediversified (Weller et al., 2004). However, most human polyreactive immunoreactivity is ascribed to naïve B cells that have not undergone antigen-driven proliferation and maturation (Tsuiji et al., 2006).

Although there was a prompt IgM response directed toward DIII-lr in mice, IgG against this critical protective epitope was not detected until days 10–15. Sequence analysis of several strongly neutralizing murine mAb binding the DIII-lr epitope showed that their V regions were in germline configuration (Oliphant et al., 2007). Under these circumstances, a rapid protective IgG response would be expected as observed for other acute cytopathic viruses (Bachmann et al., 1997). A possible explanation is that during the explosive B-cell proliferation and expansion in germinal centers, antibody specificities directed against

the multiple immunodominant DI and DII epitopes simply outcompete the more restricted DIII-Ir epitope.

Although delayed, a robust murine IgG response was observed against the DIII-Ir epitope, indicating that there is nothing intrinsic in its molecular structure that makes DIII-Ir nonimmunogenic. Rather, the failure in humans to generate IgM and consistent IgG responses against DIII-Ir may result from a hole in the human antibody germline repertoire, which makes it difficult to develop high-affinity antibodies to the DIII-Ir epitope. This phenomenon has been reported in the immune response to lymphocytic choriomeningitis virus (LCMV) in mice. LCMV was shown to be an effective immunogen when transgenic mice already bearing high-affinity cognate receptors were infected, but in wild-type mice infection results in a very poor antibody response against the principal neutralizing epitope (Hangartner et al., 2003). It is speculated that the coevolution of LCMV with its host has selected a neutralizing epitope not recognized with high avidity by antibodies in the murine germline repertoire (Hangartner et al., 2006). It is unlikely that WNV has evolved in an analogous manner. Most mammals, including humans, are dead-end hosts for the virus (Hayes and Gubler, 2006). B-cell immunoglobulin receptors against DIII-Ir may simply be absent in the repertoire of many individuals, or are so rare that they are out-competed for antigen by antibodies against immunodominant epitopes. Alternatively, the conformational epitope on DIII-Ir may mimic antigenic structures on human self-proteins. If this were the case, then B cells expressing high-avidity Ig receptors to these cross-reactive epitopes would be purged by negative selection during B-cell differentiation and not contribute to the humoral immune response against WNV.

## 6 Conclusion

Healthy individuals appear to be able to mount a robust protective response against WNV infection. Only a relatively small number of those infected display clinical signs and an even smaller number develop serious symptoms. Innate resistance mechanisms such as interferon secretion and signaling likely explain much of this protective activity. In experimental models, humoral immunity plays an important role in protection from lethal infection; however, the protective role antibodies play in humans is less clear. The studies performed to date suggest that there are several deficits in the human antibody response to natural WNV infection when compared to experimental models. In particular, the antibody response to a critical potent neutralizing epitope on

E-protein domain III of the envelope protein only develops in a minority of individuals compared to immunodominant but poorly neutralizing epitopes. Another anomalous observation is the persistence of IgM serum levels that could result from viral interference in isotype switching. Deficits in the human antibody response to WNV infection may impact protection from disease when the innate response is weakened owing to age or immunosuppression. Further analysis of the human antibody response to WNV will likely help direct development of effective and safe vaccines that can be targeted to high-risk groups such as the elderly. The current findings suggest that vaccine developmental efforts should focus on increasing the immunogenicity of E-protein domain III while maintaining the broad coverage of WNV strains and preventing escape within an individual due to the formation of quasispecies.

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