Human monoclonal antibodies to West Nile virus identify epitopes on the prM protein

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Abstract

Hybridoma cell lines (2E8, 8G8 and 5G12) producing fully human monoclonal antibodies (hMAbs) specific for the pre-membrane (prM) protein of West Nile virus (WNV) were prepared using a human fusion partner cell line, MFP-2, and human peripheral blood lymphocytes from a blood donor diagnosed with WNV fever in 2004. Using site-directed mutagenesis of a WNV-like particle (VLP) we identified 4 amino acid residues in the prM protein unique to WNV and important in the binding of these hMAbs to the VLP. Residues V19 and L33 are important epitopes for the binding of all three hMAbs. Mutations at residue, T20 and T24 affected the binding of 8G8 and 5G12 only. These hMAbs did not significantly protect AG129 interferon-deficient mice or Swiss Webster outbred mice from WNV infection.

Introduction

West Nile virus (WNV) is an emerging global pathogen causing WN fever and meningoencephalitis. Since its introduction into the Western Hemisphere in 1999 it has spread throughout North and Central America and the Caribbean and currently is the leading cause of mosquito-borne human encephalitis in the region (Mackenzie et al., 2004). WNV is a member of the virus family, Flaviviridae, genus, Flavivirus. It is maintained in an enzootic cycle between mosquitoes and birds with humans and other vertebrate animals as incidental hosts (Mackenzie et al., 2004). Other medically important flaviviruses include Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus, St. Louis encephalitis virus (SLEV) and the four serotypes of dengue virus (DENV).

WNV has a single-stranded positive sense, 11 kb RNA genome that encodes 3 structural proteins at its 5'-end. The envelope (E) protein is the major virion glycoprotein responsible for viral cell membrane attachment and fusion. The capsid (C) protein binds the genomic RNA to form the nucleocapsid. The pre-membrane (prM) protein is a chaperone protein that assists in the maturation of the E protein, and occurs as a prM/E heterodimer. The pr peptide is the amino terminal part of the prM protein that is cleaved from prM by the multibasic protease, furin. The pr stays associated with the virion to protect the fusion peptide of the E protein from premature fusion until reaching a neutral pH environment. Once the virion reaches a neutral pH, the pr peptide dissociates resulting in the formation of a fusion competent particle (Yu et al., 2009).

Many technologies have been developed to produce humanized or fully human monoclonal antibodies (hMAbs). These include phage display for selection of hFAbs and humanization of murine MAbs (mMAbs) (Tsurushita et al., 2005), direct re-engineering of mMAbs (Thibodeaux and Roehrig, 2009), using transgenic mice that have had the murine genes for antibody production replaced with human genes (Lonberg, 2008), and transformation of activated B cells with Epstein Barr virus to immortalize B-cells (Traggiai et al., 2004).

For arthropod-borne viruses (arboviruses), Hunt et al. (2006) humanized the highly neutralizing Venezuelan equine encephalitis virus (VEEV)-reactive mMAb, 3B4C-4, using combinatorial antibody libraries and phage-display technology. The humanized version of 3B4C-4 has been shown to be protective in mice when given prophylactically (Hunt et al., 2006). Subsequently, hMAbs were prepared against VEEV using phage display to produce VEEV-reactive hFabs, and their protective and therapeutic capacity is currently being investigated (Hunt et al., 2010). The humanized anti-WNV antibody, Hu-E16, was also developed using phage display technology. This MAb binds to a highly conserved epitope on domain III of the WNV envelope protein and has been shown to protect mice from lethal virus challenge (Oliphant et al., 2005). The Hu-E16 MAb is in
commercial clinical trials, and has been shown to be safe for humans (Beigel et al., 2010). By immortalizing the peripheral blood lymphocytes (PBLs) with Epstein Barr virus from a person recently infected with dengue virus (DENV), Schieffelin et al. (2010) were able to show that this technology is a viable option for the production of MAbs against DENV (Schieffelin et al., 2010). Beltramello et al. (2010) have recently published their work isolating anti-DENV hMAbs in the same way (Beltramello et al., 2010). Direct humanization of the flavivirus group-reactive mMAb 686C-1 converted this mouse IgG MAb to human IgM and IgG (Thibodeaux and Roehrig, 2009). These humanized antibodies can be used as positive control antibodies for all flaviviruses in human antibody-specific diagnostic assays.

We have used a new technology to develop hMAbs that are produced by fusing a human cell line, MFP-2, with human B cells from PBLs. The MFP-2 cell line was developed by first generating the heteromyeloma (B6B11) by fusing a murine myeloma cell line with a human myeloma cell cell. B6B11 was then subsequently fused with human lymph node lymphocytes to produce the trionma cell line, MFP-2 (Kalantarov et al., 2002). The MFP-2 cell line was fused with the PBLs from a person who had been infected with WNV in 2004, and three hybridomas producing hMAbs reactive to WNV were isolated and characterized.

To investigate the protein specificities of these hMAbs we used virus-like particles (VLPs) as antigen in ELISA. These VLPs have been used previously to identify binding sites of E protein-specific mMAbs for DENV, WNV, and SLEV (Crill and Chang, 2004; Crill et al., 2007; Trainor et al., 2007). For DENV, JEV, and SLEV the prM→M processing does not occur, and only prM and E occur in VLPs (Hunt et al., 2001; Purdy and Chang, 2005; Purdy et al., 2004). The prM→M cleavage of the WNV VLPs is inefficient, so the WNV VLP contains E, prM, and M proteins (Davis et al., 2001). Interestingly, only TBEV-derived VLPs contain only E and M protein (Schalich et al., 1996).

These hMAbs reacted only with WNV, and reacted with the WNV prM protein. Using site-directed mutagenesis of the WNV VLP system we identified amino acids (a.a.) important in hMAb binding. Residues V19 and L33 of the prM protein were determined to be the important sites for binding of all 3 hMAbs. Mutations at residues T20 and T24 had some affect on binding of two hMAbs. When tested in mice for their ability to inhibit lethal WNV infection, hMAbs, 8G8 and 5G12 slightly increased the median survival time (MST) of WNV-infected AG129 and Swiss Webster (SW) mice, respectively; however, neither hMAb fully protected animals from lethal infection.

Results

Production of hMAbs to WNV

We previously described the construction of a unique fusion partner cell line, MFP-2, and its use for the immortalization of human PBLs (Kalantarov et al., 2002). Three independent, fully hMAbs, 2E8, 8G8 and 5G12, were generated using this protocol with PBLs from a donor diagnosed with WN fever in 2004. These hMAbs were found to be IgG1 λ-chain isotype by ELISA.

Characterization of hMAbs

To determine the protein specificities of these hMAbs, reduced and non-reduced purified WNV NY99 was separated on a 4–12% Bis/Tris polyacrylamide gel and used as antigen in immunoblots. All three hMAbs reacted specifically with non-reduced prM protein which is approximately 20 kDa in mass (Fig. 1, Table 1). The three hMAbs failed to react with reduced prM protein (data not shown) indicating that the reactive prM epitopes were conformational in nature. The hMAbs did not react with the M protein (approximately 6 kDa) suggesting that they recognize the pr portion of the prM. Interestingly, serum from the donor of the PBLs used to make the hMAbs was also tested in the immunoblot and only reacted with the E and C proteins of WNV.

A panel of 7 flavivirus VLP antigens (DENV 1–4, JEV, WNV, and SLEV) was used to test for virus cross-reactivity of the hMAbs (Davis et al., 2001; Hunt et al., 2001; Purdy and Chang, 2005; Purdy et al., 2004). The VLPs were derived from transformed COS-1 cells (Davis et al., 2001). The VLPs were concentrated by ultracentrifugation (Purdy et al., 2004), and the concentration of VLP bound to a well was standardized by VLP-capture using the broadly flavivirus-reactive mMAb, 4G2. Flavivirus group-reactive human serum was used as a positive control antibody. An OD of two times the background was considered a positive result and endpoints are expressed in μl/ml of hMAb. All three hMAbs reacted only with WNV antigen indicating that these hMAbs are most likely WNV specific (Table 1). To assess whether these hMAbs could neutralize virus hybridoma supernatants were tested in a plaque reduction neutralization test (PRNT) with WNV NY99. None of the hMAbs neutralized virus at a 90% plaque reduction level (Table 1). The PRNT50 titer of the donor serum was determined to be 1:80.

prM hMAb binding site analysis

To identify the specific binding sites of these hMAbs we prepared VLPs with changes in the prM a.a. sequence using site-directed mutagenesis of the pVAXWN plasmid. Since these hMAbs were found to be specific for WNV, we compared the a.a. sequence similarity of 10 flaviviruses in the pr portion of the prM protein using GeneDoc (version 2.6.002) software. Nine WNV-specific a.a. residues were identified (Fig. 2). Corresponding residues were identified on the crystal structure of the prM-E heterodimer of DENV2 virus at neutral pH using Swiss Pdb Viewer (version 4.0.1) software to predict surface accessible sites of the protein (Fig. 3). Five of the WNV specific pr a.a.s were found to occur in surface accessible sites (V19, T20, T24, K31 and L33), while the other 4 sites (T70, S72, K77 and K83) were predicted to be internally located in the protein. We focused our mutagenesis efforts on the 5 surface accessible sites, and produced 18 mutant plasmids (Table 2). The mutant plasmids were transfected into COS-1 cells, and the resulting VLPs were used as antigens in ELISA as
immunofluorescence. All of the mutant transfected cells bound well with the anti-prM hMAbs, with no significant increase or decrease in reactivity (data not shown).

Animal protection studies

We tested the ability of these hMAbs as a prophylactic treatment for WNV infection in both Swiss Webster (SW) outbred mice and interferon deficient AG129 mice. Three groups (n = 10) of AG129 or SW mice were treated with 500 μg of hMAb 8G8, 500 μg of hMAb 5G12, or 500 μg of a hMAb to tetanus toxoid (hMAb TT) as a negative control antibody (Table 4). AG129 and SW mice inoculated with PBS (n = 3 and n = 6, respectively) served as an additional negative control group. Since the hMAbs were non-neutralizing it was unknown whether they would protect mice from a lethal WNV infection, therefore we used a high dose of hMAb to test their protective capacity. Twenty-four hours after inoculation with hMAb, mice were challenged with 100 PFU of WNV NY99 a dose previously determined to be 100% lethal for AG129 mice and 83% lethal for SW mice (Calvert et al., 2006; Yamshchikov et al., 2004). Mice were monitored for signs of morbidity, including weight loss, a change in body temperature, and neurological signs of infection. While no AG129 mice survived WNV challenge, MSTS of the mice treated with hMAb 8G8 were significantly longer than those treated with the hMAb TT (P = 0.0007). Relative to the control group treated with the hMAb TT, treatment with 8G8 followed by WNV challenge increased mouse MST by 1 day. The mouse group treated with 5G12 did not have an increase in MST (4 days) following WNV challenge compared to those mice treated with hMAb TT (P = 0.6; 4 days) (Table 4). When SW mice were treated with 5G12, 3 out of 10 mice survived challenge compared to 1 out of 10 mice treated with 8G8, and none survived challenge when treated with hMAb TT. The MST for mice treated with 5G12 was 9 days, a significantly longer than those treated with the hMAb TT (P = 0.24).
revealed that the region for binding of these pr-specific hMAbs to WNV prM protein. Not surprisingly, evaluation of the high-resolution epitopes they recognize mapped to the most variable region of the antigenic structure of WNV prM, and its contribution to protective immunity. These hMAbs were found to be WNV type-specific for the prM protein of WNV, since the donor serum was most reactive with the E and C proteins. While much is known about the structure and composition of flaviruses grown in cell culture, nothing is known about the structure and composition of the virus as it replicates in either vertebrate or mosquito hosts. We know that the prM→M cleavage is dependent on both the prM→M cleavage motif and the cells in which the virus grows (Fischl et al., 2008; Keelapang et al., 2004; Randolph et al., 1990). It is conceivable that flaviruses circulating in the vertebrate host might contain a significant amount of prM-containing immature particles. If this is the case, a robust anti-prM antibody response might be expected.

While little is known about the protective capacity of antibodies to prM, some studies have shown that prM is able to induce protective antibody responses in mice. Kaufman et al. (1989) isolated 5 anti-prM mMAbs to DENV3 and DENV4. These were shown to passively protect mice against homologous as well as heterologous DENV challenge.

### Table 2
Nucleotide sequences of primers used for mutagenesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Mutagenic primer sequence (5’–3’)</th>
<th>Nucleotide mutation</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>V19A</td>
<td>GGTAAACTCTACATCGCCAATGTCATCAAG</td>
<td>GTC-GCC</td>
<td>Val-Ala</td>
</tr>
<tr>
<td>V19N</td>
<td>GGTAAACTCTACATCGCCAATGTCATCAAG</td>
<td>GTC-ACC</td>
<td>Val-Arg</td>
</tr>
<tr>
<td>V19R</td>
<td>GGTAAACTCTACATCGCCAATGTCATCAAG</td>
<td>GTC-ACC</td>
<td>Val-Thr</td>
</tr>
<tr>
<td>V19T</td>
<td>GGTAAACTCTACATCGCCAATGTCATCAAG</td>
<td>ACA-GCA</td>
<td>Thr-Ala</td>
</tr>
<tr>
<td>T20A</td>
<td>GTAATTACTCTGAGCTGAAGCTGACAGTCA</td>
<td>ACA-GCA</td>
<td>Thr-Gly</td>
</tr>
<tr>
<td>T20D</td>
<td>GTAATTACTCTGAGCTGAAGCTGACAGTCA</td>
<td>ACA-GCA</td>
<td>Thr-Gly</td>
</tr>
<tr>
<td>T20Q</td>
<td>GTAATTACTCTGAGCTGAAGCTGACAGTCA</td>
<td>ACA-GCA</td>
<td>Thr-Gly</td>
</tr>
<tr>
<td>T24A</td>
<td>CGTCACTACGGGTCAACACACACACACACAG</td>
<td>ACG-GCC</td>
<td>Thr-Ala</td>
</tr>
<tr>
<td>T24L</td>
<td>CGTCACTACGGGTCAACACACACACACACAG</td>
<td>ACG-CTG</td>
<td>Thr-Leu</td>
</tr>
<tr>
<td>T24R</td>
<td>CGTCACTACGGGTCAACACACACACACACAG</td>
<td>ACG-AGG</td>
<td>Thr-Arg</td>
</tr>
<tr>
<td>K31A</td>
<td>GATTCACAGCGTCCCTAGGGACACTGCTCG</td>
<td>AAG-GCG</td>
<td>Lys-Ala</td>
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<tr>
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<td>AAG-GCG</td>
<td>Lys-Val</td>
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<tr>
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<td>GATTCACAGCGTCCCTAGGGACACTGCTCG</td>
<td>AAG-GCC</td>
<td>Lys-Thr</td>
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<tr>
<td>L33A</td>
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<td>CTA-GCA</td>
<td>Leu-Ala</td>
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<tr>
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<td>AACACGGTCCGAAGAAGACAGGCTGAGCTG</td>
<td>CTA-AAA</td>
<td>Leu-Lys</td>
</tr>
<tr>
<td>L33Q</td>
<td>AACACGGTCCGAAGAAGACAGGCTGAGCTG</td>
<td>CTA-CAC</td>
<td>Leu-Gln</td>
</tr>
<tr>
<td>L33T</td>
<td>AACACGGTCCGAAGAAGACAGGCTGAGCTG</td>
<td>CTA-ACA</td>
<td>Leu-Thr</td>
</tr>
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</table>

*a* The mismatched nucleotides causing the desired substitutions are shown in bold.

### Table 3
Effect of hMAb binding to WN VLP antigen after amino acid substitutions in pr peptide.

<table>
<thead>
<tr>
<th>pr a.a. substitution</th>
<th>Fold change in hMAb titer with pr mutants</th>
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<tr>
<td></td>
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<td>WT prM</td>
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<tr>
<td>V19A</td>
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<tr>
<td>V19N</td>
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<tr>
<td>V19T</td>
<td>9</td>
</tr>
<tr>
<td>T20A</td>
<td>4</td>
</tr>
<tr>
<td>T20Q</td>
<td>4.5</td>
</tr>
<tr>
<td>T24A</td>
<td>2.5</td>
</tr>
<tr>
<td>L33A</td>
<td>10.0</td>
</tr>
<tr>
<td>L33K</td>
<td>&gt;102</td>
</tr>
<tr>
<td>L33Q</td>
<td>412</td>
</tr>
<tr>
<td>L33T</td>
<td>13.6</td>
</tr>
</tbody>
</table>

*a* Amino acid substitution at specific residue in pr portion of the protein.

*b* Four-fold reduction or more (bold) in MAb end-point titer of mutant VLPs compared to wild type VLP. Blank means two-fold or less reduction in titers. Actual end-point titration on wild-type (WT) prM containing VLP with these MAbs were: 2E8 = 0.0161 μg/ml, 8G8 = 0.0256 μg/ml, and 5G12 = 0.0321 μg/ml.

### Discussion

This is the first report of the isolation and characterization of fully hMAbs to WNV with PBLs from a person previously infected with the virus fused to MFP-2 cell lines. Previously, MFP-2 cells have been used to develop fully hMAbs to breast cancer associated antigens (Kalantarov et al., 2002; Kirman et al., 2002; Rudchenko et al., 2008).

While an abundance of information exists on the structure and function of the E protein and its importance in flavivirus immunity in mice and humans, little is known about prM and its role in immunity. This study is the first report of the development and characterization of hMAbs for the WN prM protein, their use in analyzing the antigenic structure of WNV prM, and its contribution to protective immunity. These hMAbs were found to be WNV type-specific and the epitopes they recognize mapped to the most variable region of the prM protein. Not surprisingly, evaluation of the high-resolution crystal structure of the DEN2 prM-E heterodimer at neutral pH, revealed that the region for binding of these pr-specific hMAbs (V19 and L33, highlighted in red in Fig. 3) are localized at the top of the prM protein (Li et al., 2008). The other residues that affected binding of hMAbs, T20 and T24 (highlighted in orange in Fig. 3) are in close proximity to V19 and L33. While these a.a. were important in the binding of hMAbs 8G8 and 5G12, their influence may be due to mutations made in this region that change the epitope only slightly (e.g., T20A or T24A changes). The T20Q mutation may sterically obstruct the ability of the hMAbs to bind to the key epitope at V19.

It is interesting that all three of these hMAbs were found to be specific for the prM protein of WNV, since the donor serum was most reactive with the E and C proteins. While much is known about the structure and composition of flaviruses grown in cell culture, nothing is known about the structure and composition of the virus as it replicates in either vertebrate or mosquito hosts. We know that the prM→M cleavage is dependent on both the prM→M cleavage motif and the cells in which the virus grows (Fischl et al., 2008; Keelapang et al., 2004; Randolph et al., 1990). It is conceivable that flaviruses circulating in the vertebrate host might contain a significant amount of prM-containing immature particles. If this is the case, a robust anti-prM antibody response might be expected.

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**Fig. 3.** DEN2 prM-E heterodimer. The image (PDB ID: 3C6E) shows a top view towards the viral surface of the DEN2 prM protein at neutral pH oriented near the fusion peptide in domain II of the E glycoprotein. In the inset, the E protein structural and functional domains I, II and III are shown in red, yellow and blue, with the fusion peptide in domain II shown in cyan. The prM protein is shown in green, with the WNV epitopes important in the hMAbs’ binding shown in space-filling representations. V19 and L33 are highlighted in red. T20 and T24 are highlighted in orange. Disulfide bonds in the pr-peptide are shown in magenta.
Two out of five of the MAbS were found to have some neutralizing activity while four out of five were also able to fix complement (Kaufman et al., 1989). By constructing recombinant vaccinia viruses expressing prM, M or pr proteins of DENV4 virus, Bray and Lai (1991) found that mice immunized with the recombinant prM or M were protected against homologous virus challenge. However, vaccinating with the recombinant virus expressing pr alone was not protective (Bray and Lai, 1991). Synthetic peptides of the DENV2 prM protein were found to elicit neutralizing antibody that was protective against virus challenge. A synthetic peptide of the DENV2 prM protein containing residues 1–33 (B19-6) was found to elicit neutralizing antibody in mice that allowed for a protective response against DENV2 challenge (Vazquez et al., 2002). It is interesting that this peptide contained all of the a.a that we have defined as critical to the binding of our pr-specific hMAbs.

We found these hMAbs to be minimally protective in outbred mice as well as interferon deficient mice. These anti-prM hMAbs also did not neutralize WNV infectivity in vitro. The non-neutralizing capacity of our hMAbs may be due to a reduced number of epitopes available on the virion to which the antibody can bind after prM/M cleavage. The cleavage of the prM protein by furin in the secretory pathway results in the M protein being present in the mature viral envelope. Once the virion has reached a neutral pH the pr peptide dissociates, resulting in a fusion-compotent particle (Yu et al., 2009). As stated previously, since this cleavage by furin is inefficient not all virions released from cells may contain solely M, and in fact, there might be a mixed population of virions containing only M, only prM or a mixture of prM and M on the virion surface (Cherrier et al., 2009). Consequently, the availability of prM to pr-reactive antibody likely varies from virus to virus, and also from cell type to cell type in which the virus grows.

It has been shown that complete maturation of flavivirions results in a reduction of neutralization with E protein-specific antibodies reactive with epitopes that may be more accessible in immature forms of the virus (Nelson et al., 2008). It is also necessary for antibodies that recognize epitopes that are not surface exposed to have more sites bound in order to neutralize the virus (Pierson et al., 2007). It is possible that the virus used to challenge mice contained more M than prM, and therefore, there was a lack of surface-exposed epitopes for antibodies to bind resulting in an inability of the antibodies to neutralize virus effectively and protect mice from a lethal virus infection.

Four mutations made in the WN VLP (T20D, K31A, K31V, K31T) resulted in failure of transfected cells to release particles. Although VLPs were not secreted with these mutations, prM was identified in the heterodimer, or on the neighboring E protein in particle assembly, but in fact, its placement is at the top of prM, which sits on top of the E protein in the immature particle. More investigation into the mutations at K31 along with the mutation T20D need to be completed, and experiments are ongoing to determine if K31 is a critical element for WNV particle maturation and secretion.

Recent studies have explored the possibility of prM antibodies facilitating the infection of DENV in vitro. Antibodies to the prM protein can enhance DENV infection with non-Fc receptor bearing cells, suggesting a mechanism different from that of anti-E antibodies (Huang et al., 2008; Huang et al., 2006). Specifically, human anti-prM antibodies to the M3 epitope (a.a. 52–67) cross-reacted with epithelial, kidney, endothelial cells and T lymphocytes. They were also found to cross-react with heat shock protein 60 (HSP60) on the surface of BHK and A549 cells. This interaction may cause apoptosis in endothelial cells or may indirectly cause complement mediated cytolysis or antibody dependent cellular cytotoxicity (Huang et al., 2008). It is likely that anti-prM antibodies to DENV assist immature particles in cell entry where they are able to undergo the conformational change and cleavage of the prM protein by furin in the acidic endosomal environment making an otherwise non-infectious virus particle infectious. Therefore, pre-existing heterotypic anti-prM antibodies may assist in increasing the viral load in secondary infections (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010).

The hMAbs described here offer a new tool in understanding the human immune response to WNV. While most studies of prM have been in the context of creating an immunogenic virus particle with the E protein for vaccinations, it should not be assumed that the anti-prM antibody response in humans is not important. Although our results indicate that these anti-prM antibodies did not offer a significant source of protection in mice, the inclusion of anti-prM antibodies in the human antibody repertoire may be more important than previously considered. Further study into the immunogenic capacity of prM in humans or humanized animal models is necessary in order to fully understand the importance of prM in a WNV infection.

Materials and methods

Cells and viruses

C6/36 cells and COS-1 cells were cultured as described previously (Davis et al., 2001; Huang et al., 2000). WNV strain, NY99 35262, originally isolated from a Chilean flamingo at the Bronx Zoo (NY, USA) in 1999, was obtained from the Arbovirus Diseases Branch, Diagnostic and Reference Activity, CDC. The virus was passaged once in suckling mouse brain and twice in C6/36 cells. Stock virus was grown in C6/36 cells to a titer of 1.5 × 10^6 PFU/ml.

Production of hMAbs to WNV

Hybridoma cell lines, secreting 2E8, 8G8 and 5G12 fully hMAbs, were produced as a result of PE-g-fusion between the MFP-2 fusion partner cell line and human PBLS from a donor diagnosed with WN fever (Kalantarov et al., 2002). All three cell lines were maintained in
RPMI 1640 media (Invitrogen) supplemented with 10% FBS, L-glutamine, non-essential amino acids, Na pyruvate, and vitamins. Positive hybridomas were selected in ELISA, using mAbB, D1-4G2-4-15, as a capturing antibody (ATCC cat# HB-112). Plates were coated overnight with 4G2, then washed and blocked with 1% BSA in PBS. Plates were then incubated with WNV-VLP (1:20) and COS-1 (1:20) negative control (Hennessy Research, cat# P120-1) for 1 h, washed, and incubated with hybridoma supernatants, as well as positive and human sera for 1 h. Goat-anti-human IgG-HRP (mouse adsorbed, Invitrogen cat# H10507) was used as the detector antibody. Hybridomas were also adapted for growth in serum-free media (HyQ-CCM1, HyClone, cat# SH30043.03). Hollow fiber cartridges 4300C2011 (Fiber Cell Systems) were used for large-scale production of all three hMAbs.

Purification of virus

WNV NY99 was grown in C6/36 cells and purified on a glycerol tartrate gradient as described by Obijeski et al. (1976). The purified stock was used in the immunoblot (Obijeski et al., 1976).

Purification of hMAbs

For animal studies, hMAbs 2E8, 8G8 and 5G12 were purified from highly concentrated culture media using protein A/G agarose (Pierce, cat# 20422) according to the manufacturer's instructions. hMAbs were further dialyzed against PBS and the concentration was measured in ELISA using purified human IgG (GenScript, cat# A01006) as the calibrator.

Immunoblotting

WNV proteins from purified virus were separated by SDS-PAGE on a reduced or non-reduced 4-12% Bis/Tris polyacrylamide gel (Invitrogen). All procedures were performed at room temperature. Proteins were blotted electrophoretically from the gels onto nitrocellulose membranes and washed for 15 min in PBS/0.1% Tween wash buffer. Non-specific binding sites were blocked with 1% BSA/PBS for 1 h while rocking. Undiluted hybridoma supernatants were incubated with the membrane for 1 h with gentle rocking. Membranes were washed again in PBS/0.1% Tween wash buffer three times for 5 min each. Goat anti-human antibody conjugated to horseradish peroxidase (Invitrogen) was diluted 1:500 and incubated on the membrane for 1 h with gentle rocking. Membranes were washed and Novex HRP chromogenic (TMB) substrate (Invitrogen) was added to the membrane until a color change appeared. The reaction was stopped by the addition of water.

Plaque-reduction neutralization test (PRNT)

Human serum was heat-inactivated at 56 °C for 30 min. One hundred plaque forming units (PFU) of WNV NY99 were incubated with equal amounts of serial two-fold dilutions of hybridoma supernatant or human serum for 1 h at 37 °C. Six-well plates of Vero cells were then inoculated with the virus–antibody mixtures and incubated at 37 °C with 5% CO2 for 1 h after which cells were overlaid with 3 ml of medium containing 1% SeaKem LE agarose (FMC BioProducts) in nutrient medium (0.165% lactalbumin hydrolysate, 0.033% yeast extract [Difco], Earl’s balanced salt solution and 2% FBS). Following incubation at 37 °C for 2 days a second overlay containing additional 80 μg of neutral red vital stain (GIBCO-BRL) per ml was added. Plaques were counted on day 3 and endpoint titers were expressed as the concentration of antibody (mg/ml) that yielded 90% reduction in the number of plaques (PRNT90).

Site-directed mutagenesis

Site-specific mutations were introduced into the prM gene using the QuikChange Site-Directed Mutagenesis kit (Stratagene), with pVAXWN plasmid previously described as a DNA template (Davis et al., 2001). Mutagenic primer sequences used for all constructs are listed in Table 2. After transformation with mutagenic PCR products, colonies were grown in Luria–Bertani broth (5 ml); plasmid was purified and sequenced to determine the correct mutation was present, and then the structural genes and regulatory elements of all purified plasmids were sequenced. Automated DNA sequencing was performed with a 3130X Genetic Analyzer (Applied Biosystems) and sequences were analyzed with Lasergene software (DNASTar). Molecular modeling of the mutations was constructed using Pymol software (v0.99) and the PDB image (3C6E) (Li et al., 2008).

Transient expression of WN VLP in COS-1 cells by electroporation

COS-1 cells were electroporated with the pVAXWN plasmid as previously described (Davis et al., 2001). Electroporated cells were recovered in 6 ml of DMEM supplemented with 10% fetal calf serum (FCS). Cells were seeded into 25-cm² culture flasks for VLP expression, and incubated at 37 °C with 5% CO2. Six hours after electroporation, the growth medium in flasks was replaced with DMEM containing 2% FCS. Tissue culture medium and cells were harvested 5 days after electroporation for antigen characterization by ELISA and immunofluorescence.

ELISAs

All ELISAs were performed in 96-well plates (Maxisorp plates, Nunc). Starting concentrations of hMAbs from serum-free hybridoma supernatant in ELISAs were as follows: 2E8, 6.7 μg/ml, 8G8, 8.0 μg/ml, 5G12, 5.5 μg/ml. Starting concentrations of hMAbs to test for cross-reactivity with WN and SLE VLP were as follows: 2E8, 3.0 μg/ml, 8G8, 5.5 μg/ml, 5G12, 7.5 μg/ml. All flaviviral VLP antigens in ELISAs to determine cross-reactivity were obtained from the Reference Collection of the Diagnostic Lab, DVBID, CDC. Plates were coated with a capture antibody, flavivirus group-reactive mAb 4G2 asfcic fluid diluted 1:1000 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Plates were washed five times with PBS/0.1% Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with Pierce Starting Block (PBS) blocking buffer (100 μl/well). Blocking buffer was immediately removed before flavivirus antigen diluted in PBS was added to the wells (50 μl/well). VLP antigen was incubated on the plates for 2 h at 37 °C, after which the plates were washed as previously described. Hybridoma supernatant was added in 3-fold dilutions (50 μl/well) and incubated for 1 h at 37 °C. Plates were washed five times before the addition of goat anti-human antibody conjugated to horseradish peroxidase (50 μl/well), diluted 1:3000 in 5% skim milk/PBS. After an incubation period of 1 h at 37 °C, plates were washed again 10 times. Enhanced K-blue TMB substrate (Neogen) was added to each well of the plate (100 μl/well) and incubated in the dark at room temperature for 10 min. The reaction was stopped with the addition of 1 N H2SO4 (50 μl/well), and the plates were read at 450 nm.

For the standardization of mutant WN VLPs the protocol remained the same with some exceptions. The capture antibody, 4G2, was replaced with rabbit hyperimmune sera to WN prM and E proteins diluted 1:1000. The antigen produced from transfections of COS cells with the mutated pVAXWN plasmid were diluted two-fold in PBS. Human sera positive for reactivity with all flaviviruses was obtained from the Reference Collection of the Diagnostic Laboratory, ADB, DVBID, CDC, and used as the primary antibody at a dilution of 1:1000 in PBS. To test the reactivity of the mutant VLPs with the hMAbs, VLPs
were diluted appropriately to obtain a standardized amount of antigen for all preparations. HybriDoma supernatant was used as the primary antibody and added to the wells in four-fold dilutions (50 μl/well), and the ELISA was carried out as described above.

**Immunofluorescence**

In order to determine the reactivity of the mAbs with mutant VLP plasmid that was unable to secrete particles, COS-1 cells were harvested 5 days after transfection and fixed to 12-well glass slides. Cells were fixed in 70% acetone in PBS. HybriDoma supernatant was added in doubling dilutions (20 μl/well) to the wells of the slide and incubated at 37 °C for 30 min. Slides were washed three times in PBS and allowed to dry before goat anti-human conjugated to FITC (Invitrogen) diluted 1:200 in PBS was added (20 μl/well) and incubated at 37 °C for 30 min. Slides were washed, dried and mounted with Dabco mounting medium and examined on a Zeiss epifluorescence microscope.

**Mouse experiments**

AG129 mice deficient in IFN-α/β and -γ receptors (van den Broek et al., 1995) and SW mice were bred in-house. The mice were handled as specified by institutional guidelines for care and use in accordance with the Institutional Animal Care and Use Committee recommendations. HMAbs, 8G8 and 5G12, were evaluated for their ability to inhibit WNV infection in 5–8 week old AG129 mice, and 3–4 week old SW mice. For each breed two groups of mice (n = 10) were inoculated intraperitoneally (i.p.) with 500 μg of hMAbs 8G8 or 5G12. One group of mice (n = 10) was inoculated i.p. with a hMAb of the same isotype (lgG1) to tetanus toxoid (TT) as a control (hMAb TT). Another group of mice (n = 3, AG129 mice and n = 6, SW mice) was inoculated i.p. with 100 μl of PBS. Challenge was performed by i.p. inoculation with 100 PFU of WNV NY99 diluted in medium 24 h after the hMAb inoculation (Calvert et al., 2006; Yamshchikov et al., 2004). Challenged mice were monitored for an increase followed by a decrease in IFN-γ and allowed to dry before goat anti-human conjugated to FITC

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**References**


