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Differential expression of domain III neutralizing epitopes on the envelope proteins of West Nile virus strains

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Abstract

Neutralization of flaviviruses by antibody is primarily mediated via epitopes in the viral envelope (E) protein. Comparative studies using neutralizing monoclonal antibodies revealed differential expression of epitopes within the E protein domain III of ten naturally occurring West Nile virus strains representing major subtypes of genetic lineages 1 and 2. Residues that defined these subtype-specific determinants were identified by mutational studies and found to be surface exposed in the domain III structure. Mutations of residue 332 had the most significant effects on variation of domain III neutralizing epitopes among strains.

Keywords: Flavivirus; West Nile virus; Envelope protein; Neutralizing epitope

Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis serocomplex of the family *Flaviviridae*, genus *Flavivirus*. Molecular phylogenetic analyses have identified two major WNV lineages, designated 1 and 2 (Lanciotti et al., 1999; Scherret et al., 2002), which included genetic subgroups that correlated well with antigenic subtypes that had been defined by cross-neutralization studies using polyclonal sera and monoclonal antibodies (MAbs) (reviewed by Scherret et al., 2002). The flavivirus envelope (E) protein is the major virion surface protein and an important target for virus neutralizing antibodies. Epitope mapping studies with several flaviviruses have identified three major antigenic domains (designated A, B, and C) that correspond to distinct structural subunits of E (designated structural domains II, III, and I, respectively) (Rey et al., 1995).

Many antibodies that bind to antigenic domain B/structural domain III, which is the putative receptor binding domain, are very efficient at neutralizing the infectivity of flaviviruses and also tend to be virus type or sub-type specific (reviewed by Roehrig, 2003). Therefore, epitopes located on domain III of WNV are likely to be highly significant in the development of protective immunity and have also been shown to be important in the specific serological diagnosis of WNV infection (Beasley et al., 2004b).

Previously, we used commercially available neutralizing MAbs to select neutralization-resistant variants of a genetic lineage 1 North American WNV strain, 385-99 (USA99b), that encoded mutations at surface-exposed residues located in domain III (K307R or T330I; Beasley and Barrett, 2002). In addition, a lineage 2 WNV strain that encoded three amino acid differences within domain III compared to USA99b was not neutralized by MAbs, suggesting that significant antigenic differences between WNV strains are encoded within domain III.

In order to assess the extent of antigenic variation within domain III among naturally-occurring strains of WNV, and possibly to identify amino acid residues that define major antigenic subtypes of WNV, we analyzed ten strains (Table 1) representing genetic lineages 1 and 2 using three

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Table 1
Neutralization and Western blot reactivity of West Nile virus strains representative of genetic lineages 1 and 2 with E protein domain III-specific monoclonal antibodies, a polyclonal rabbit antiserum raised against recombinant WNV USA99b E protein domain III, and a polyclonal mouse antiserum raised against a New York 1999 isolate

MAb/serum (Epitope)	Neutralization index[a] (and Western blot reactivity [b]) against WNV strains									
	Lineage 1						Lineage 2			
	USA99b	ETH76a	ISR52	ISR53	AUS60	IND80	MAD78	SA89	MAD88	SA58a
5H10 (B1)	2.3 (++)	2.7 (++)	2.2 (++)	0.9 (−)	1.1 (++)	2.8 (++)	2.5 (++)	1.3 (++)	0.2 (−)	0.2 (−)
5C5 (B2)	2.5 (++)	2.4 (++)	2.4 (++)	1.9 (+/−)	1.1 (++)	2.9 (++)	2.5 (++)	1.2 (++)	−0.2 (−)	0.1 (−)
7H2 (B3)	3.6 (++)	4.2 (++)	3.4 (++)	2.1 (+/−)	1.6 (++)	3.6 (++)	3.1 (++)	1.7 (++)	0.1 (−)	0.1 (−)
Anti-WNV domain III	3.8 (++)	3.9 (++)	3.9 (++)	3.9 (++)	2.0 (++)	≥5.6 (++)	≥4.8 (++)	2.7 (++)	0.3 (++)	0.6 (++)
Anti-WNV	5.1 (++)	5.0 (++)	≥5.3 (++)	4.1 (+)	≥4.9 (++)	n.d.[c] (++)	n.d. (++)	≥4.8 (++)	n.d. (++)	≥4.9 (++)

[a] Neutralization index is $\log_{10}$ reduction in virus titer in the presence of MAb/polyclonal serum compared with culture medium only control.
[b] Values in brackets represent binding of MAb or antiserum to E protein of each WNV strain detected in a non-reducing SDS–PAGE Western blot using Lumigen chemiluminescent substrate (Amersham Biosciences): "++" = binding comparable to that observed with strain USA99b; "+" = reduced binding; "+/−" = binding detected only after >5 min exposure; "−" = no binding detected.
[c] n.d., not determined.

commercially-available WNV-specific neutralizing MAbs–5H10, 5C5, and 7H2 (Bioreliance Corp.)–and a polyclonal rabbit serum raised against a bacterially-expressed recombinant WNV E protein domain III derived from strain USA99b (Beasley et al., 2004b). The three MAbs were raised against the North American prototype strain 382-99 and recognized related but distinct epitopes within domain III (Beasley and Barrett, 2002) that are designated here as epitopes B1, B2, and B3, respectively. Details of the ten WNV strains and their molecular phylogenetic relationships have been described elsewhere (Beasley et al., 2002, 2004a).

Results and discussion

Variable expression of neutralizing epitopes in domain B/III of WNV strains

The expression of epitopes B1, B2, and B3 among wild-type WNV strains was assessed by neutralization assay as well as Western blotting against virus-infected Vero cell lysate antigens using previously published techniques (Beasley and Barrett, 2002; Beasley et al., 2004b). Results of neutralization assays were expressed as "neutralization indices" which represent the $\log_{10}$ reduction in virus titer in the presence of the MAb or polyclonal antiserum. Although differences in neutralization by and/or blot reactivity with the three MAbs did not delineate strains of genetic lineages 1 and 2, variable reactivity with the WNV strains was observed (Table 1).

Most lineage 1 strains were strongly neutralized by all three MAbs (neutralization indices 2.3–4.2) and strong reactivity with their E proteins was detected in Western blots. Two lineage 2 strains, SA58a and MAD88, were not significantly neutralized by any of the MAbs (neutralization indices <1.0), and no reaction with the E proteins of these strains was detected in Western blots, suggesting that neutralization escape was mediated via loss of the B1, B2, and B3 epitopes. Neutralization indices against strains AUS60 and ISR53 (both lineage 1), and strain SA89 (lineage 2), were 10- to 100-fold lower than against USA99b. However, all three MAbs reacted strongly with the E proteins of SA89 and AUS60 in Western blots. Binding to ISR53 E was also detected, although it was markedly reduced compared to USA99b and other lineage 1 strains (Table 1).

The anti-domain III rabbit serum reacted strongly with all E proteins in Western blot assays, but differences in neutralization of the ten WNV strains were observed (Table 1). Similar to results obtained with the MAbs, strains SA58a and MAD88 were not neutralized by the anti-domain III serum, and neutralization of ISR53 and AUS60 was also reduced compared to USA99b and the other strains. These results suggested that the mutation(s) associated with escape from MAb-mediated neutralization for strains SA58a and MAD88 significantly altered all of the epitopes recognized by neutralizing antibodies in the polyclonal anti-domain III serum. Seven of the strains were also compared in neutralization assays using a polyclonal mouse immune ascitic fluid (MIAF) raised against strain USA99b (generously provided from the World Reference Center for Emerging Viruses and Arboviruses [WRCEVA] by Dr. Bob Tesh). All seven strains were strongly neutralized by this MIAF (Table 1), indicating that the E proteins of these WNV strains have other cross-reactive neutralizing epitope(s), presumably located within the other antigenic/structural domains.

Identification of domain III amino acids that contribute to antigenic variation between WNV strains

The domain III coding region of the E gene for each strain was RT-PCR amplified and sequenced, and the derived amino acid sequences were compared (Fig. 1). A maximum of four amino acid differences from strain

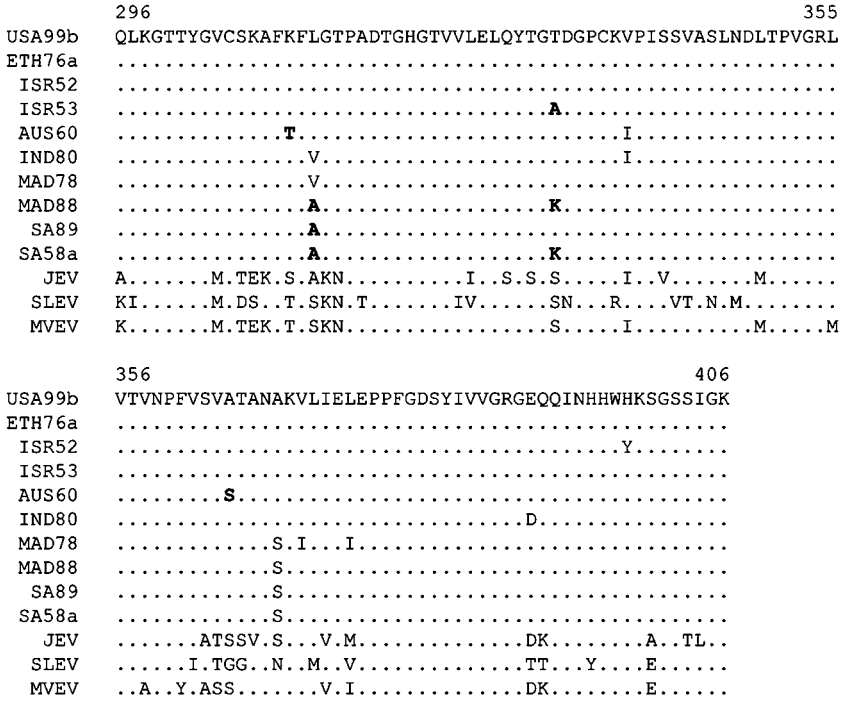

Fig. 1. Alignment of domain III amino acid sequences for ten West Nile virus strains used in this study and representative Japanese encephalitis, St. Louis encephalitis, and Murray Valley encephalitis virus strains (derived from GenBank files U21057, M16614, and M24220, respectively). Conserved residues are indicated by a dot (“·”). Residues that were associated with escape from neutralization by monoclonal antibodies are in bold.

USA99b was identified. Interestingly, the most divergent was lineage 2 strain MAD78, which did not differ appreciably from USA99b in neutralization or Western blot assays (Table 1).

Disregarding mutations that were shared with strongly neutralized strains (e.g., A369S in MAD78, MAD88, SA89, and SA58a), complete or partial escape from neutralization was associated with the following changes: L312A and/or T332K for strains SA89, MAD88, and SA58a; T332A for strain ISR53; K310T and/or A365S for strain AUS60 (Fig. 1). Each of these residues lies on the upper surface of WNV domain III, while the other variable residues were primarily located internal to the domain or on surfaces that would most likely be hidden in the native virion (Fig. 2).

To determine which mutation(s) had the greatest effect on epitopes B1, B2, and B3, domain III MBP fusion protein constructs encoding candidate mutations individually or in combination were generated either by RT-PCR amplification and cloning of the domain III coding region from viral RNA or by site-specific mutagenesis of a USA99b domain III expression plasmid (Beasley et al., 2004b). Fusion proteins were purified on amylose resin and MAb binding to each protein was assessed using an indirect ELISA protocol (Beasley et al., 2004b). Wells of plates were coated with equal quantities of the fusion proteins (~30 ng/well) and the MAbs were each diluted to a concentration approximately 100-fold greater than their Kd (Volk et al., 2004).

Consistent with data from Western blots with virus-derived antigens, no binding of any MAb was detected to domain III derived from strain SA58a (mutations L312A, T332K, A369S) or to a domain III encoding only the T332K mutation, indicating that the loss of epitopes B1, B2, and B3 in SA58a and MAD88 could be primarily attributed to this single mutation (Fig. 3; absorbance values not significantly different to background binding). A T332A mutation (as occurs in strain ISR53) also reduced MAb binding to all three epitopes but, consistent with the results of PRNT and Western blot assays (Table 1), had the greatest effect on epitope B1. A reduction in MAb binding to each epitope was also observed as a result of the L312A mutation, which was consistent with the incomplete neutralization of strain SA89. However, some neutralization-resistant plaques of SA89 (picked in the presence of MAb 7H2) encoded the T332K mutation seen in SA58a and MAD88, indicating that this mutation was present within the SA89 quasispecies and most probably contributed to the incomplete neutralization of this strain by the MAbs (data not shown).

In contrast, mutations K310T and/or A365S, as found in strain AUS60, did not reduce MAb binding to any epitope. Rather, binding of each MAb was actually increased compared to the USA99b control (Fig. 2). Escape from neutralization due to a mutation in domain III without loss or reduction in antibody binding has also been reported for a variant of tick-borne encephalitis virus (Holzmann et al., 1997). Alternatively, low frequency mutations within the AUS60 quasispecies that were not detected by consensus

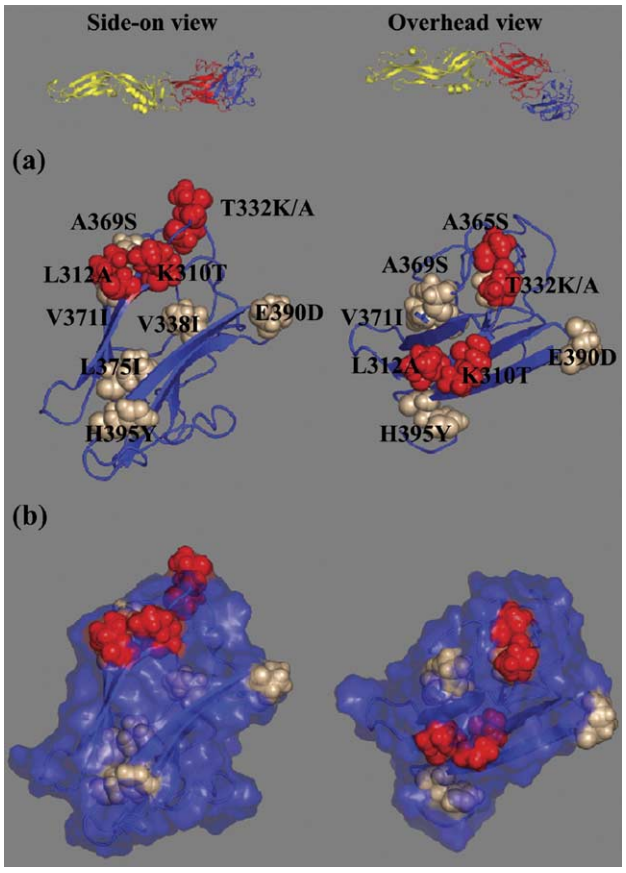

Fig. 2. (a) Locations of residues that differed between West Nile virus strains in a NMR structure of the West Nile virus E protein domain III (PDB file 1S6N; Volk et al., 2004). Side-on and overhead views of domain III are oriented as for the complete E ribbon diagrams shown above the domain III structures. Space-filled residues are those that were associated with neutralization escape (red color) or that varied between WNV strains without affecting neutralization by monoclonal antibodies (cream color). (b) Surface-shaded representation of structures shown in panel a highlighting the exposed locations of residues 310, 312, 332, and 365. Ribbon diagrams were rendered using Pymol v. 0.97 (Delano Scientific LLC, San Carlos, CA).

sequencing or mutations located outside domain III may have played a role in the altered neutralization of AUS60 virions by the MAbs.

Conclusions

Amino acid mutations in domain III of the WNV E protein that were associated with antigenic differences between strains were located at the edges of the solvent-exposed upper surface of the domain (Fig. 2), and were primarily non-conservative in nature. Mutations that were not associated with antigenic differences were generally less surface exposed and/or more conservative. Overall, the data reported here suggest that the biochemical nature of amino acid substitutions as well as their precise location within domain B/III contributes to the degree of antigenic variation between WNV strains. In particular, residue 332 appeared to function as a major antigenic determinant, with different amino acid substitutions at this site (i.e., T in USA99b, K, or A in neutralization-resistant strains) having varying effects on the integrity of epitopes B1, B2, and B3 (Table 1; Fig. 3). The complete loss of all three neutralizing epitopes was mediated solely by the T332K mutation, whereas other mutations (e.g., T332A, L312A) had more subtle effects on the presentation of these epitopes. Strains MAD88 and SA58a, which encoded the T332K and L312A mutations, were not neutralized by MAbs or the anti-WNV domain III rabbit serum. This suggests that these substitutions were sufficient to disrupt all of the epitopes recognized by neutralizing antibodies raised against domain III of New York 1999 lineage 1 WNV.

The observation that antigenic differences in domain III did not clearly discriminate between the major subtypes of WNV was significant, but is consistent with earlier comparisons of WNV strains in which certain MAbs recognized some but not all of the lineage 1 and 2 strains that were compared (Besselaar and Blackburn, 1988; Burt et al., 2002; Morvan et al., 1990; Scherret et al., 2001). Signature E protein amino acids that discriminated between lineage 1 and 2 WNV strains have been identified, but these were not located within domain III (Scherret et al., 2001). We hypothesize that the proposed function of domain III as a receptor-binding determinant places strong constraints on the degree to which residues in this domain can vary and that, as a result, immune pressures can select for only a limited range of surface variations that maintain virus fitness and E protein function. However, the observation that mutation of a single amino acid within domain III can have such dramatic effects on important neutralizing epitopes

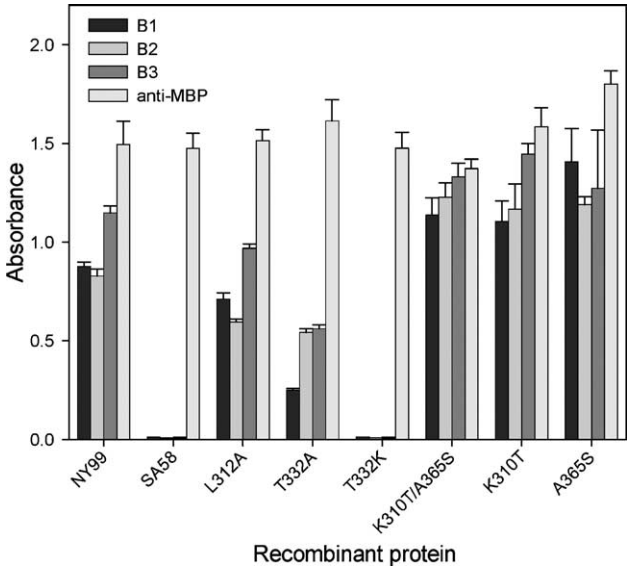

Fig. 3. Presentation of epitopes B1, B2, and B3 on recombinant domain III MBP fusion proteins incorporating mutations associated with escape from neutralization. Effects of specific mutations on these epitopes were determined by reactivity with virus-neutralizing monoclonal antibodies in an indirect ELISA. An anti-MBP serum (New England Biolabs, Beverly, MA) was also used as a control to demonstrate that equivalent quantities of each protein were coated in the wells of ELISA plate.

could have significant implications for WNV vaccines and for serological diagnostic assays that rely on type/subtype specific, domain III-reactive MAbs and/or subunit E protein antigens (e.g., Beasley et al., 2004b; Blitvich et al., 2003).

Model domain III structures incorporating the L312A, T332A/K, K310T, and A365S mutations were generated from the Swiss-Model structure prediction server (Schwede et al., 2003; accessed at "www.expasy.ch") using a WNV strain USA99b domain III structure previously determined by our lab and other collaborators at UTMB as a template (PDB accession 1S6N; Volk et al., 2004). None of these models revealed any changes to the predicted folding of WNV domain III (data not shown), suggesting that the observed effects on epitopes B1, B2, and B3 were probably mediated directly via biochemical changes at the domain III surface caused by mutation of those residues.

In a series of reports, Roehrig and others have described a comparable type-specific "critical neutralizing epitope", designated $E1^C$, in the E proteins of SLEV and MVEV (Hawkes et al., 1988; Mathews and Roehrig, 1984; Roehrig et al., 1983; Vorndam et al., 1993). Although those studies did not identify the specific location of the epitope, the strong neutralizing activity and high degree of specificity of the MAbs that recognized $E1^C$ in SLEV or MVEV suggest that it is probably equivalent to WNV epitope B3, recognized by MAb 7H2, which was disrupted by a T332K mutation and significantly affected by a T332A mutation. Residue 332 is located in close proximity to residues 330 and 307 which were previously associated with neutralization escape variants of USA99b selected with MAbs 5H10 and 5C5, respectively (Beasley and Barrett, 2002). However, unlike the T332K mutation, neither a T330I or K307R mutation prevented binding of MAb 7H2 to domain III (Volk et al., 2004). Mutations of the corresponding residue of Japanese encephalitis virus (JEV) domain III had similar effects on the binding of a JEV-specific neutralizing MAb (Lin and Wu, 2003; Wu et al., 1997). The apparent importance of residue T332 as a critical antigenic determinant for WNV suggests that this uncharged, polar residue may participate directly in antibody binding interactions at the surface of domain III, although this remains to be confirmed experimentally by structural analysis.

Materials and methods

Virus strains, monoclonal antibodies, and antisera

Ten WNV strains representative of major subtypes of genetic lineages 1 and 2 (listed in Table 1) were obtained from WRCEVA at UTMB. The propagation and nucleotide sequencing characterization of the ten WNV strains have been described elsewhere (Beasley et al., 2002, 2004a). All working stocks of virus were grown and plaque titrated in Vero cells.

MAbs 5H10, 5C5, and 7H2 were obtained from Bioreliance Corporation (Rockville, MD) and their properties and the results of preliminary epitope mapping studies have been described elsewhere (Beasley and Barrett, 2002). Rabbit antiserum against the purified, recombinant WNV domain III protein was prepared by Harlan Bioproducts for Science (Indianapolis, IN) as described elsewhere (Beasley et al., 2004b). Polyclonal anti-WNV strain USA99b mouse serum was obtained from WRCEVA.

Neutralization assays

Ten-fold dilutions of virus were prepared in MEM tissue culture medium (Sigma, St. Louis, MO) containing 2% fetal bovine serum (FBS) and mixed with equal volumes of anti-WNV MAb (diluted 1:200), or polyclonal anti-WNV E-III rabbit serum (1:20), or anti-WNV MIAF (1:20), or MEM media only. Virus–antibody mixtures were incubated at room temperature for 60 min before inoculation onto monolayers of Vero cells in 6-well tissue culture plates (Nunc). Plates were incubated at room temperature for 30 min to allow virus adsorption, then overlayed with 5 mL per well of MEM medium containing 1% agarose (MEM/agarose). After incubation at 37 °C in a 5% CO_2 atmosphere for 2 days, the wells were overlayed with an additional 2 mL of MEM/agarose containing 2% v/v neutral red solution (Sigma). Plaques were counted the following day and neutralization indices determined as the $\log_{10}$ reduction in virus titer in the presence of either MAb, or polyclonal anti-WNV E-III rabbit serum or anti-WNV MIAF compared with the medium only control.

Nucleotide sequencing

RNA was extracted from WNV-infected Vero cell supernatants using the QiaAmp viral RNA extraction kit (Qiagen Inc., Valencia, CA) and reverse transcribed using AMV reverse transcriptase (Roche, Indianapolis, IN). A fragment that included the E-III coding sequence was RT-PCR amplified using primers WN1751 ($5'$-$_{1751}$TGCATCAAGCTTTGGCTGGA$_{1770}$) and WN2504A ($5'$-$_{2504}$TCTTGCCGGCTGATGTCTAT$_{2485}$) for lineage 1 strains, or WN1739 ($5'$-$_{1751}$TGCACCAAGCTCTGGCCGGA$_{1770}$) and WN2498A ($5'$-$_{2510}$CGGAGCTCTTGCCTGCCAAT$_{2491}$) for lineage 2 strains. Primer pairs were designed based on GenBank sequences AF196835 and M12294, respectively, and are numbered according to residues in the AF196835 sequence. PCR products of the appropriate sizes were gel purified and directly sequenced using the ABI PRISM Big Dye v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's protocols. Sequence analyses were performed using the Vector NTI Suite package (Informax Inc., Bethesda, MD).

Recombinant protein expression and purification

Site-directed mutagenesis of the WNV strain USA99b domain III gene fragment cloned in the MBP fusion protein expression vector pMAL-c2x (New England Biolabs [NEB], Beverley, MA; Beasley et al., 2004b) was performed using the Quikchange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. In addition, the domain III coding regions from strains ISR53 and SA58a were RT-PCR amplified and cloned into pMAL-c2x in DH5α *E. coli* as described previously (Beasley et al., 2004b).

For each protein, a 20-mL culture of bacteria in LB medium containing ampicillin was grown to an OD_{600} ~0.6 and then induced with 1 mM ITPG at 37 °C for 120 min. Bacterial cells were pelleted by centrifugation and stored overnight at −20 °C before being lysed in 1 mL of MBP column buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA) by freeze–thaw using liquid nitrogen. Fusion proteins were purified from these whole cell lysates using a protocol similar to that recommended by the manufacturer (NEB). Briefly, the lysates were microfuged (12,000 rpm/4 °C/30 min) and the clarified supernatants were then mixed with a small quantity of amylose resin (NEB) in a 1.5 mL eppendorf tube and incubated on ice for 15 min. The tubes were microfuged at low speed (3000 rpm/60 s) and the supernatant removed. The resin was washed twice with 1 mL of native lysis buffer and the bound protein was then eluted in four washes of 0.25 mL of MBP column buffer containing 10 mM maltose. Concentrations of the purified proteins were determined by commercial Bradford assay (Sigma) according to the manufacturer's protocol and by spectrophotometric analysis.

Indirect ELISA

Binding of MAbs to purified MBP domain III fusion proteins was assessed using an indirect ELISA protocol as described elsewhere (Beasley et al., 2004b). Briefly, wells of 96-well ELISA plates (Corning Inc., Corning, NY) were coated overnight with 30 ng of purified proteins diluted in borate saline (pH 9.0). Coated plates were blocked for 60 min at room temperature with a solution of phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). As outlined in the Results above, MAbs were diluted in PBS containing 0.5% Tween 20 (PBS/Tween) to a concentration of 10–20 nM, which was approximately 100-fold greater than their Kd values (Volk et al., 2004). To confirm that equivalent quantities of each fusion protein had been coated in the wells of ELISA plates, an anti-MBP serum (NEB) diluted according to the manufacturer's recommendations was included in each assay as a control. ELISA reactions were performed in triplicate wells for each MAb–protein combination. Plates were incubated at room temperature for 45 min, washed three times with PBS/Tween, and peroxidase-labeled anti-mouse IgG antibody (Sigma) diluted in PBS/Tween was added to each well. After a further 45-min incubation, plates were again washed and reactions visualized by addition of 3,3′,5,5′-tetramethylbenzidene substrate (Sigma). After 10 min, reactions were stopped by addition of 3 M HCl and the absorbance values read on a model 3550-UV plate reader (Bio-Rad, Hercules, CA) at 450 nm with a reference of 595 nm.

Acknowledgments

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References

Beasley, D.W.C., Barrett, A.D.T., 2002. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J. Virol. 76, 13097–13100.

Beasley, D.W.C., Li, L., Suderman, M.T., Barrett, A.D.T., 2002. Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype. Virology 296, 17–23.

Beasley, D.W.C., Davis, C.T., Whiteman, M.C., Granwehr, B., Kinney, R.M., Barrett, A.D.T., 2004a. Molecular determinants of virulence of West Nile virus in North America. Arch. Virol., Suppl. 18, 35–41.

Beasley, D.W.C., Holbrook, M.R., Travassos Da Rosa, A.P.A., Coffey, L., Carrara, A.S., Phillippi-Falkenstein, K., Bohm Jr., R.P., Ratterree, M.S., Lillibridge, K.M., Ludwig, G.V., Estrada-Franco, J., Weaver, S.C., Tesh, R.B., Shope, R.E., Barrett, A.D.T., 2004b. Use of a recombinant envelope protein subunit antigen for specific serological diagnosis of West Nile virus infection. J. Clin. Microbiol. 42, 2759–2765.

Besselaar, T.G., Blackburn, N.K., 1988. Antigenic analysis of West Nile virus strains using monoclonal antibodies. Arch. Virol. 99, 75–88.

Blitvich, B.J., Bowen, R.A., Marlenee, N.L., Hall, R.A., Bunning, M.L., Beaty, B.J., 2003. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. J. Clin. Microbiol. 41, 2676–2679.

Burt, F.J., Grobbelaar, A.A., Leman, P.A., Anthony, F.S., Gibson, G.V., Swanepoel, R., 2002. Phylogenetic relationships of southern African West Nile virus isolates. Emerg. Infect. Dis. 8, 820–826.

Hawkes, R.A., Roehrig, J.T., Hunt, A.R., Moore, G.A., 1988. Antigenic structure of the Murray Valley encephalitis virus E glycoprotein. J. Gen. Virol. 69, 1105–1109.

Holzmann, H., Stiasny, K., Ecker, M., Kunz, C., Heinz, F.X., 1997. Characterization of monoclonal antibody-escape mutants of tick-borne encephalitis virus with reduced neuroinvasiveness in mice. J. Gen. Virol. 78, 31–37.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286, 2333–2337.

Lin, C.-W., Wu, S.-C., 2003. A functional epitope determinant on domain III of the Japanese encephalitis virus envelope protein interacted with neutralizing-antibody combining sites. J. Virol. 77, 2600–2606.

Mathews, J.H., Roehrig, J.T., 1984. Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of Saint

Louis encephalitis virus by passive transfer with monoclonal antibodies. J. Immunol. 132, 1533–1537.

Morvan, J., Besselaar, T., Fontenille, D., Coulanges, P., 1990. Antigenic variations in West Nile virus strains isolated in Madagascar since 1978. Res. Virol. 141, 667–676.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. Nature 375, 291–298.

Roehrig, J.T., 2003. Antigenic structure of flavivirus proteins. Adv. Virus Res. 59, 141–175.

Roehrig, J.T., Mathews, J.H., Trent, D.W., 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology 128, 118–126.

Scherret, J.H., Mackenzie, J.S., Hall, R.A., Deubel, V.S., Gould, E.A., 2002. Phylogeny and molecular epidemiology of West Nile and Kunjin viruses. Curr. Top. Microbiol. Immunol. 267, 373–390.

Scherret, J.H., Poidinger, M., Mackenzie, J.S., Broom, A.K., Deubel, V., Lipkin, W.I., Briese, T., Gould, E.A., Hall, R.A., 2001. The relationships between West Nile and Kunjin viruses. Emerg. Infect. Dis. 7, 697–705.

Schwede, T., Kopp, J., Guex, N., Peitsch, M.C., 2003. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. 31, 3381–3385.

Vorndam, V., Mathews, J.H., Barrett, A.D.T., Roehrig, J.T., Trent, D.W., 1993. Molecular and biological characterization of a non-glycosylated isolate of St Louis encephalitis virus. J. Gen. Virol. 74, 2653–2660.

Volk, D.E., Beasley, D.W.C., Kallick, D.A., Holbrook, M.R., Barrett, A.D.T., Gorenstein, D.G., 2004. Solution structure and antibody binding studies of the envelope protein domain III from the New York strain of West Nile virus. J. Biol. Chem. 279, 38755–38761.

Wu, S.C., Lian, W.C., Hsu, L.C., Liau, M.Y., 1997. Japanese encephalitis virus antigenic variants with characteristic differences in neutralization resistance and mouse virulence. Virus Res. 51, 173–181.