A glycosylated peptide in the West Nile virus envelope protein is immunogenic during equine infection

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Using a monoclonal antibody directed to domain I of the West Nile virus (WNV) envelope (E) protein, we identified a continuous (linear) epitope that was immunogenic during WNV infection of horses. Using synthetic peptides, this epitope was mapped to a 19 aa sequence (WN19: E147–165) encompassing the WNV NY99 E protein glycosylation site at position 154. The inability of WNV-positive horse and mouse sera to bind the synthetic peptides indicated that glycosylation was required for recognition of peptide WN19 by WNV-specific antibodies in sera. N-linked glycosylation of WN19 was achieved through expression of the peptide as a C-terminal fusion protein in mammalian cells and specific reactivity of WNV-positive horse sera to the glycosylated WN19 fusion protein was shown by Western blot. Additional sera collected from horses infected with Murray Valley encephalitis virus (MVEV), which is similarly glycosylated at position E154 and exhibits high sequence identity to WNV NY99 in this region, also recognized the recombinant peptide. Failure of most WNV- and MVEV-positive horse sera to recognize the epitope as a deglycosylated fusion protein confirmed that the N-linked glycan was important for antibody recognition of the peptide. Together, these results suggest that the induction of antibodies to the WN19 epitope during WNV infection of horses is generally associated with E protein glycosylation of the infecting viral strain.

INTRODUCTION

West Nile virus (WNV) is a globally significant pathogen of the genus Flavivirus. Of recent concern is the rapid spread of a particularly virulent strain of this virus through North, Central and South America (Gubler, 2007). This mosquito-borne virus can cause a fatal form of encephalitis in humans, birds and horses (van der Meulen et al., 2005; Sejvar & Marfin, 2006) and since its introduction to America in 1999, has caused tens of thousands of clinical cases and thousands of deaths in humans and horses (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#maps; http://www.aphis.usda.gov/hs/animal_health/diseases/westnile/wnv_distribution_maps.htm).

Diagnosis of human and equine WNV infection is commonly achieved using serological assays (Castillo-Olivares & Wood, 2004; Prince & Hogrefe, 2005). While plaque reduction neutralization tests are still considered the gold standard for specific diagnosis, ELISA is now routinely used (Dauphin & Zientara, 2007), as it is less laborious and more suited to high-throughput screening. The antigenic similarity of WNV with other members of the Japanese encephalitis serocomplex, such as Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), Kunjin virus...
(KUNV) and Murray Valley encephalitis virus (MVEV), can make definitive diagnosis difficult due to the cross-reactive nature of the immune response (Calisher et al., 1989; Kuno, 2003). There is a need to improve the specificity of WNV diagnostic assays, especially to differentiate infections caused by virus strains such as NY99 and weakly pathogenic strains such as KUNV.

One approach to developing a rapid, WNV-specific diagnostic assay is to identify immunogenic peptides of viral proteins that are significantly different between WNV subtypes and closely related flaviviruses. Small peptides are ideal for incorporation into rapid platforms, due to comparatively easy synthetic or recombinant production and greater specificity, due to the decreased likelihood of encoding cross-reactive epitopes.

The viral envelope (E) protein is the major viral immunogen during infection and is generally the antigen of choice for serological diagnostic assays (Prince & Hogrefe, 2005). However, some E protein epitopes induce flavivirus cross-reactive antibodies (Roehrig, 2003; Sitasny et al., 2006) and recent attempts to improve the specificity of E protein-based antigens have focused on using peptides or individual domains of the E protein (Beasley et al., 2004b; Herrmann et al., 2007; Roberson et al., 2007). While E-subunit antigens significantly improved diagnostic specificity, allowing differentiation between infections caused by WNV and other flaviviruses, their ability to differentiate infections caused by different subtypes of WNV has not been explored.

Defined epitope-blocking ELISAs have also been used to increase the specificity of WNV serodiagnosis and have been useful for differentiating WNV infections from those caused by SLEV and MVEV (Blitvich et al., 2003; Hall et al., 1995). However, they are unable to differentiate infections caused by different subtypes of WNV.

Sanchez et al. (2005) developed and characterized a WNV-specific, domain I (DI)-reactive, monoclonal antibody (mAb17D7), recognizing an epitope with desirable diagnostic characteristics. Analysis in a competitive ELISA (Sanchez et al., 2007) showed that WNV-positive horse sera specifically blocked the binding of this antibody, indicating that it bound to, or adjacent to, an immunogenic epitope. Furthermore, the inability of mAb17D7 to cross-react with SLEV, IEV or dengue virus, indicated that the epitope was unique to WNV.

In this investigation, we describe the mapping of the epitope of mAb17D7 to a 10 aa region, encompassing the WNV NY99 E protein glycosylation site. Using sera from WNV-infected horses, we further demonstrate the diagnostic potential of a mammalian-expressed 19 aa peptide (WN19), which incorporates the mAb17D7 epitope. Additional analysis using glycosidase digestion revealed that N-linked glycosylation was important for the recognition of peptide WN19 by WNV-positive horse sera.

**METHODS**

**Cell culture.** Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 2% fetal bovine serum (FBS) and C6/36 cells were maintained in RPMI 1640 (Gibco) with 10% FBS. COS-7L cells (Gibco) were grown in RPMI 1640 with 10% FBS, while N50 mouse myeloma cells (European Collection of Cell Cultures) were grown in either RPMI 1640 containing 10% FBS or HyQ CD4M5N0 (Hyclone, Perbio Science) supplemented with 10 μg insulin ml⁻¹ and 6 mM l-glutamine. All media, except CD4M5N0, were supplemented with 50 U penicillin ml⁻¹, 50 μg streptomycin ml⁻¹ and 2 mM GlutaMAX−1 (Gibco). All mammalian cells were incubated at 37 °C with 5% CO₂, while the mosquito cell line (C6/36) was grown at 28 °C.

Virus stocks were prepared from the cell culture supernatant of virus-infected Vero or C6/36 cells.

**Horse serum samples.** One set of sera (Table 2, samples 1–5) was obtained from horses involved in WNV experimental infection studies (Richard Bowen, Colorado State University, Fort Collins, CO, USA, personal communication). Horses were infected using WNV NY99 and bled on the day of infection (pre) and about 21–26 days post-infection (post). A second set of WNV-positive sera (samples 7–14) was obtained from naturally infected horses in North and Central America. These horses were not known to have clinical symptoms of WNV disease, or to have been vaccinated against WNV infection (Richard Bowen; Carolina Arévalo, Universidad Nacional, Heredia, Costa Rica, personal communication). Sample 6 was collected from an uninfected horse living in Central America. All of the American samples were exposed to gamma irradiation (50 kGy) upon importation into Australia. Samples 15–22 were collected from horses living in the Northern Territory of Australia, for which detailed clinical information was not available. All sera had been heated at 56 °C for 30 min. For the microsphere assay, gamma-irradiated serum from a naturally WNV-infected horse demonstrating clinical symptoms of disease (WN horse AAHL) was used (Ross Lunt, Australian Animal Health Laboratory, Geelong, Victoria, Australia, personal communication). Pooled serum from WNV-naive horses was used as an additional negative control.

**Virus neutralization tests (VNT).** VNT were performed (with some modifications) as described previously (Hall et al., 1995). In this study, the lowest dilution of horse serum was 1:40 and the assay was performed in Vero cells.

**mAb17D7 epitope characterization.** The epitope conformation of mAb17D7 was confirmed by reactivity of the mAb to reduced and carboxymethylated WNV antigen, essentially as described previously (Clark et al., 2007). The reactivity of mAb17D7 to various viruses was assessed in ELISA using fixed, virus-infected cells, as described by Clark et al. (2007). The virus identity was confirmed using the binding of strain-specific mAbs in ELISA (data not shown). The strains used in this study, and the sequence GenBank accession numbers of these were as follows: WNV NY99 strain (AF196835); KUNV RM661C strain (BA001176); KUNV Hu6774 strain (AF196493); WNV Sarafend strain (AF196533); KUNV Sarawak MP502-66 strain (AF196534); WNV Wengler strain (NC_001563); Koutango virus DakKad 5443 strain (AF196532); MVEV 1-51 strain (AF161266); and Alfuy virus MRM3929 strain (AY989809).

The reactivity of mAb17D7 to KUNV MRM61C strain E protein (AF196505) and recombinant KUNV MRM61C antigen was assessed using Western blot. Lysates of KUNV MRM61C or WNV NY99-infected C6/36 cells were prepared as described by Clark et al. (2007), while recombinant KUNV MRM61C and WNV NY99 E protein in cell lysates were prepared by transiently transfecting COS-7L cells with plasmids pCDNA3-prME (Chang et al., 2008) and pCBWN (Davis et al., 2001), respectively, using the methods described below for the expression of the single-chain Fv fragment (scFv) fusion proteins.
Peptide ELISA. Peptide sWN10 (N–C; SHGNYSTQVG, 81% purity) (Sigma-Genosys) was dissolved in a solution of 25% glacial acetic acid, while sWN19 (N–C; CTYVESHGNYSTQVGAQAG, 36% purity, Sigma-Genosys) was dissolved in dimethylformamide (DMF). Peptide sHIV35, which comprises 35 aa of human immunodeficiency virus type 1 (HIV-1) glycoprotein 41L (N–C; RILAVERYLDQQLGIWGCSDKLICTTAVPNAS, 95% purity, United Biomedical) was dissolved in 5% glacial acetic acid.

ELISAs were performed in 96-well ELISA plates (Greiner Bio One). The plates were coated with serial, twofold dilutions of the synthetic peptides in coating buffer (50 mM NaHCO₃, 50 mM Na₂CO₃, pH 9.6) from 1 μg per well (sWN10 and sHIV35) or 2.25 μg per well (sWN19, to allow for differences in purity). After incubation for 1 h at room temperature with gentle agitation, the plates were washed three times with PBS/0.05% Tween 20 (PBS/T). mAb17D7 ascitic fluid (1 : 500) or affinity-purified anti-HIV-1 mAb1B111/14 [AGEN Biomedical, 4 μg ml⁻¹], diluted in PBS/T, was added (50 μl) for 1 h. Following four washes with PBS/T, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins (Dako) (1 : 1000) and incubated for a further 1 h. After a final six washes, 100 μl ABTS substrate solution [2 mM 3,3′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 0.03% H₂O₂ (v/v), 25 mM citric acid, 25 mM trisodium citrate (pH 4.4)] was added per well and incubated for 30 min. The reaction was stopped with 50 μl per well 3.9% oxalic acid and the plate was read on an automated plate-reader at 405 nm. The OD readings represent the average of readings from two wells.

Microsphere assay. sWN10 and sWN19 were dissolved at 1 mg ml⁻¹ in Ca²⁺/Mg²⁺-free PBS (PBSA) containing 0.1% SDS. Each peptide (30 μg) was coupled to 1 × 10⁶ carboxylated (COOH) microspheres [bead size #46 (Fisher Biotec)] and 30 μg soluble Nipah virus glycoprotein (NipV G) was coupled to microspheres [bead size #42 (Fisher Biotec)] as described previously (Bosassart et al., 2007). The microsphere assay was performed essentially as described by Bosassart et al. (2007), using a mAb or mouse sera dilution of 1 : 25 in PBSA, or horse sera diluted 1 : 100. WNV-immune mouse serum was generated upon experimental infection with WNV NY99.

Cloning and expression of scFv fusion proteins. scFv fusion protein plasmids were constructed by subcloning the extended scFv sequence from pGC038C2 (Goia et al., 1996) into the mammalian expression vector pcDNA 3.1 (+) (Invitrogen). Murine immunoglobulin heavy chain signal peptide and signal peptide intron sequences were amplified by PCR from a proprietary plasmid (AGEN Biomedical), and inserted upstream of the scFv sequence to create pCDscFv. scFv–WN19 and scFv–WN67-encoding plasmids were constructed by inserting the sequences corresponding to amino acids E147–165 and E127–193, respectively, of WNV NY99, amplified by PCR from pCBWN (Davis et al., 2001), downstream of the scFv sequence (Fig. 1).

Initial assessment of the recombinant proteins was performed using lysates of transiently transfected COS-7L cells. COS-7L cells were transfected using lipofectamine 2000 (Invitrogen) and incubated for 40–48 h. The cells were gently washed twice with PBS and harvested with a cell scraper into PBS. In some cases, the cells were lysed in situ by incubation of the monolayer in a solution of 0.1% SDS, 1% Triton X-100, 10.5 μg aprotinin ml⁻¹, and the supernatant was recovered following centrifugation.

Further studies used recombinant fusion proteins (scFv and scFv–WN19) secreted from stably transfected NS0 cells. Transfections were performed as described above and transfected cells were selected using 0.5 mg Geneticin (Gibco) ml⁻¹. Geneticin was removed once off all of the cells in a transfection control (no plasmid) were dead. Clones secreting glycoporin-reactive fusion proteins were selected using an ELISA and converted to growth in a serum-free medium (CDM4N50) in roller bottles. Cells were removed from the harvested medium by centrifugation at 1000 r.p.m. for 5 min in a Cenntafast Orbital 310. The clarified supernatant was passed through a 0.45 μm filter and concentrated 2.5-fold using a 10 000 molecular mass cut-off centrifugal concentrator (Sartorius).

Fusion protein Western blots. In initial experiments to assess the binding of mAb17D7 and WNV-positive horse sera to the recombinant scFv–WN19 fusion protein, lysates of transfected COS-7L cells were mixed with 1 × sample loading buffer (NuPage LDS sample buffer; Invitrogen) and 25 mM 2-mercaptoethanol. The samples were heated at 90 °C for 10 min and then gently sheared by repeated aspiration through a 29-gauge insulin needle to fragment the COs cell genomic DNA. The antigens were separated by SDS-PAGE (4–12% gradient polyacrylamide gels; Invitrogen) and transferred to nitrocellulose (Invitrogen). The blots were blocked with 5% skim milk in PBS for 1 h at room temperature, or at 4 °C overnight and then probed with the relevant antibody, diluted in 1% skimmed milk for 1 h (mAb17D7: 1 : 500, mAb1B111/14: 4 μg ml⁻¹), horse sera (1 : 100) or 30 min (goat anti-mouse immunoglobulins; (GAM; Dako) 0.01 mg ml⁻¹). The blots were washed with PBS/T and probed for 30 min with the relevant anti-immunoglobulin–HRP conjugate (1 : 2000). After a final wash, the blots were developed with metal-enhanced diaminobenzidine (DAB; Pierce).

Reactivity of an additional panel of WNV-positive, KUNV and/or MVEV-positive and negative control sera to scFv–WN19 was assessed using the N60-secreted fusion proteins, except for samples 12 and 22 (diluted 1 : 25), which were assessed using the COS-7L lysates prepared during PNGase F cleavage studies. The concentrated cell culture supernatants were transferred to nitrocellulose as described for the COs lysates above. Each serum (diluted 1 : 50) was assessed for reactivity with the secreted scFv and scFv–WN19 antigens (reduced), as well as lysates of COS-7L cells transiently transfected with pcDNA3.1+ (empty vector) and pCBWN (encoding WNV NY99 prM and E antigens; Davis et al., 2001), which were assessed in non-reduced form. However, in this instance, a 1 : 5000 dilution of HRP-conjugated rabbit anti-horse IgG (Sigma) was used. Initial quality control was performed on all antigens. To ensure similar loading concentrations of the scFv and scFv–WN19 antigens, their relative concentrations were assessed visually by Western blot using GAM, as described above. The presence of the WN19 peptide as a C-terminal fusion protein was confirmed by the binding of the mAb17D7, and the presence of E antigen in the pCBWN transient COS-7L lysate was confirmed by the binding of anti-E mAbs 3.91D and 2B2 (Hall et al., 1991; Adams et al., 1995). Upon optimization of this Western blot, it was observed that there was competition between the scFv–WN19 fusion protein and the recombinant E protein for binding of the anti-WN19 antibodies in the horse sera, due to the assessment of the antigens on a single blot. Thus, the amount of E protein was reduced, to decrease this competition.
**N-glycosidase F (PNGase F) digestion.** scFv–WN19 COS-7L cell lysates were digested with PNGase F (NEB) according to manufacturer’s instructions. Alternatively, the lysate was boiled for 3 min in the presence of 3% SDS, followed by the addition of EDTA to 24 mM, n-octylglucoside to 3% and 12 U PNGase F (Boehringer-Mannheim) and incubated at 37 °C overnight.

Western blots were performed, as described above. Digestion by PNGase F was confirmed by increased mobility of the scFv–WN19 protein, as detected by probing with mAb17D7 and GAM.

**RESULTS**

**Epitope mapping of mAb17D7**

The binding of mAb17D7 to a linear epitope was confirmed by reactivity to reduced and carboxymethylated WNV antigen (data not shown). Sanchez et al. (2005) previously mapped the epitope of this mAb to DI of the WNV NY99 E protein, specifically to a region encompassing residues 146–193. Amino acid sequence alignments of this region, coupled with the reactivity of mAb17D7 to various strains of WNV and closely related flaviviruses using ELISA (Table 1), suggested the involvement of the residues including, and adjacent to, the E protein glycosylation site (E154–156) (Lanciotti et al., 1999). For example, mAb17D7 did not bind to WNV Wengler, which has a deletion of four amino acids in this region. mAb17D7 also failed to bind Koutango virus antigen, which differs from WNV NY99 at four amino acids within the E151–160 region. The significantly reduced binding to KUNV MRM61C, WNV Sarawak and Koutango viruses, none of which have a serine at position 156, suggested that this is an important residue for recognition by the mAb. Increased reactivity of mAb17D7 to KUNV Hu6774, which differs from KUNV MRM61C only by having a serine at position 156, was consistent with this conclusion. This was further supported by the inability of mAb17D7 to bind KUNV MRM16, or recombinant KUNV MRM61C E protein, both of which have a phenylalanine at position 156. However, it is important to note that the strong reactivity of mAb17D7 to the non-glycosylated Alfuy virus (May et al., 2006) indicated that the requirement of 156Ser for reactivity was not associated with the presence of N-linked glycosylation at this site.

Based on the sequence alignments in Table 1, two synthetic peptides were prepared, a 20 mer (sWN19) comprising E147–165 plus an N-terminal cysteine to allow for post-synthesis modifications, and a 10 mer (sWN10) comprising E151–160. In ELISA, mAb17D7 bound only to sWN19 (Fig. 2). When assessed in a microsphere assay, mAb17D7 bound both sWN10 and sWN19-coated microspheres (Fig. 3). This result may be due to the increased sensitivity of microsphere assays as compared with ELISA (Bossart et al., 2008), or to differences in the conformation and presentation of the peptide on the beads. These data confirmed that mAb17D7 recognized a 10 aa region of the WNV E protein.

**Peptide WN19 expressed as a fusion protein in mammalian cells is bound by WNV-specific antibodies in horse serum**

WNV-specific antibodies in the serum of mice and horses failed to bind the synthetic WNV peptides in ELISA (data not shown) and microsphere-based assays (Fig. 3). These data were inconsistent with previous results, which had demonstrated that antibodies in WNV-positive horse sera were capable of blocking the binding of mAb17D7 to WNV (Sanchez et al., 2007). The inability of the horse antibodies to bind the synthetic peptides indicated that there was a requirement for either additional contact residues or an N-linked glycan (which is absent in synthetic peptides) for binding. To further investigate these possibilities, recombinant proteins incorporating peptide WN19 or a larger variant (WN67) encoding 67 aa, predominantly of DI (E127–193), were expressed in mammalian cells. Both recombinant molecules were expressed as fusion proteins with an extended scFv carrier protein (Coia et al., 1996) specific for human glycophorin. Analysis of the recombinant proteins with a goat anti-mouse polyclonal antibody, which bound the extended scFv carrier protein, showed similar quantities of scFv and scFv–WN19 fusion proteins (Fig. 4a), whereas less scFv–WN67 fusion protein was produced by the COS-7L cells. The presence of peptide WN19 on the scFv–WN19 fusion protein was confirmed by the specific binding of mAb17D7 (Fig. 4b). Serum from a WNV-seropositive horse (sample 3, Table 2) specifically bound the scFv–WN19 and scFv–WN67 fusion proteins (Fig. 4d). These data demonstrated that additional amino acid residues were not needed for binding of WNV-infected horse serum to peptide WN19 and suggested that glycosylation was associated with the immunogenic properties of this epitope in a WNV-infected horse.

A weakly reactive protein was also detected by some horse sera in COS cell antigen controls (Fig. 4d), suggesting non-specific reactivity with a COS cell protein.

**The N-linked glycan is important for binding of peptide WN19 by WNV-positive horse sera**

When the scFv–WN19 and scFv–WN67 fusion proteins were probed with anti-mouse polyclonal antibodies or mAb17D7, a distinct doublet was detected (Fig. 4a and b). This was most likely due to a mixture of glycosylated and non-glycosylated fusion proteins in the cell lysate. The observation that the WNV-positive horse serum recognized only the larger, and presumably glycosylated form, of these proteins (Fig. 4d) strengthened the hypothesis that N-linked glycosylation was required for immunogenicity. To confirm this, the scFv–WN19 fusion protein was digested with PNGase F to remove the N-linked glycan. As expected, removal of the glycan was characterized by an increase in mobility of the protein and the detection of a single band consistent with the smaller doublet fragment previously observed (Fig. 5a and b). Fig. 5(c) shows the results
obtained with WNV-infected horse sera (sample 3, post-infection), in which no detectable binding was observed with the PNGase F-treated protein. Additional results with three other WNV-positive horse sera showed that two of the sera (samples 11 and 13) recognized the glycosylated protein only and one serum (sample 12) bound to both forms (Fig. 5d). We concluded that glycosylation of WN19 is generally required for recognition by antibody from WNV-infected horses.

**Peptide WN19 is recognized by serum antibodies from horses infected experimentally and naturally with WNV**

A larger panel of sera from experimentally infected horses was assessed using the recombinant fusion protein (secreted by NS0 cells) in Western blot (Table 2). Antibodies in the post-infection samples from horses 1 and 2 bound the scFv–WN19 fusion peptide, with no detectable reactivity with the pre-infection samples. This suggests that reactivity to the peptide is specifically induced during infection with WNV. Serum from horse 3, post-infection with WNV, also specifically reacted with the scFv–WN19 fusion protein; however, there was no pre-infection serum available for this horse. Cross-reactivity with the scFv carrier protein was observed for the serum from two horses (4 and 5).

Further testing was performed using sera from eight horses shown to be positive for natural, subclinical WNV infections by VNT (horses 7–14). Antibodies in six of these samples specifically bound the scFv–WN19 fusion protein. Reactivity of the horse sera to recombinant WNV NY99 E protein was also assessed as a control. Two WN19-positive sera (1 post and 10) did not detect E protein (possibly due to an inadequate amount of E protein on the blot), while one WN19-negative serum (sample 14) reacted strongly with E protein and in VNT. Nevertheless, the recognition of recombinant peptide WN19 by most sera collected from horses exposed to experimental or natural infection indicated the potential of this peptide for equine WNV infection diagnosis.

**Peptide WN19 is also recognized by MVEV-immune horse sera**

To assess the specificity of peptide WN19 and to explore the association of reactivity with this peptide and infection with a glycosylated WNV strain, we tested sera from horses with virus-neutralizing antibodies to KUNV and or MVEV (Table 2, samples 15–22). MVEV and KUNV are endemic...
Australian flaviviruses which generally cause subclinical to mild disease in horses (Kay et al., 1987; Hall et al., 2002). The E protein is glycosylated in strains of MVEV, but not in many strains of KUNV (Wright, 1982; Lobigs et al., 1988; Adams et al., 1995). Four sera (samples 15–18) that neutralized the infectivity of KUNV, but not MVEV, reacted weakly or not at all with WN19, despite clearly recognizing the WNV NY99 E protein. The weak reactivity to the scFv–WN19 fusion protein for three of these sera (samples 16–18) was attributed to cross-reactive binding to the scFv carrier protein. In contrast, all four sera containing MVEV-neutralizing antibodies (samples 19–22) specifically reacted with the scFv–WN19 fusion protein. These data suggest that recognition of the scFv–WN19 fusion peptide is generally restricted to sera from horses infected with WNV strains and closely related flaviviruses that exhibit E protein glycosylation. However, it should be noted that the four MVEV-neutralizing sera also neutralized KUNV to an equivalent or lower titre. While a similar pattern of cross-neutralization of KUNV by MVEV-immune horse sera has

### Table 2. Reactivity of horse sera to recombinant proteins in Western blot

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<tr>
<td>mAb2B2/3.91D</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Negative horse pool‡</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Results were scored based on band intensity. Clearly visible bands were graded 1+ (weakest) → 3+ (strongest). Bands which were only just visible were graded +/− and no band −.
†A VNT titre <40 was considered negative.
‡Samples 12 and 22 were assessed independently using recombinant cell lysates.
§Control blot results were graded on the presence (+) or absence (−) of a visible band.
||Pooled sera from a flavivirus-naïve herd. WNV NY99 VNT <40.
ND, Not done.
been reported previously (Gard et al., 1977), we cannot be certain of the aetiological agent of these infections. Sera from two MVEV-immune horses (20 and 22) were further assessed for reactivity to PNGase F-treated scFv–WN19 fusion protein and did not recognize the unglycosylated peptide (Fig. 5d). These data provide further evidence that reactivity to peptide WN19 is associated with infection of horses with glycosylated flaviviruses.

DISCUSSION

We have identified an immunogenic, 19 aa WNV peptide (WN19) that may be a useful diagnostic marker of WNV infection in horses. The peptide was identified using mAb17D7, which had previously been shown to bind an immunogenic epitope in DI of WNV NY99 E protein (Sanchez et al., 2005, 2007). In this investigation, the ability of this mAb to bind reduced and carboxymethylated antigen confirmed its recognition of a linear epitope. The unique reactivity profile of this mAb to the various strains of WNV and other related flaviviruses made it possible to predict and map its contact residues to 151–160 of WNV NY99 E protein using synthetic peptides. The binding pattern of mAb17D7 also indicated that residue 156Ser was required for recognition of the epitope. This was demonstrated by the lack of reaction to the E protein of KUNV strain MRM16 and recombinant KUNV E protein, both of which contained phenylalanine at this position. The
A fourth WNV-positive horse serum could recognize both glycosylated and unglycosylated forms of the fusion peptide, which suggests that glycosylation of the recombinant fusion protein may not be required for the binding of sera from all WNV-infected horses. Previous studies have shown that the analogous region of DI of the E protein in yellow fever virus was bound by murine and human mAbs, and that the glycosylation status contributed to the nature of mAb binding (Ryman et al., 1997; Daffis et al., 2005). Furthermore, tick-borne encephalitis virus E protein glycosylation has been shown to stabilize DI epitopes (Guirakhoo et al., 1989).

Analysis of sera from 13 horses, experimentally or naturally infected with WNV, revealed that nine (69%) specifically bound the scFv–WN19 fusion protein. There was no reactivity to the peptide of two sera and cross-reactivity to the extended scFv carrier protein for another two. Analysis of paired horse sera from a WNV experimental infection trial confirmed that peptide WN19-reactive antibodies are associated with WNV infection and that the recombinant fusion peptide is recognized by 21–26 days post-infection with WNV, albeit weakly in most cases.

The reactivity of MVEV-neutralizing horse sera to the scFv–WN19 fusion protein, in only its glycosylated form, highlighted the immunogenicity of the E protein N-linked glycan during flaviviral infection of horses. This is supported by data from a limited number of sera with KUNV-neutralizing antibodies, but no MVEV-neutralizing antibodies, which indicated that infection with a flavivirus that is generally considered to be unglycosylated (Wright, 1982) did not induce a clear response to the peptide. Although a number of glycosylated KUNV strains have been reported (Adams et al., 1995), acquisition of E protein glycosylation of WNV strains can occur during limited passage in cell culture (Scherret et al., 2001; Beasley et al., 2004a). Thus, further sequencing of low-passage KUNV isolates is required to confirm that a lack of glycosylation is the predominant phenotype in the field. Considering that MVEV displays a glycosylated E protein and high amino acid identity in the region E151–160 to WNV NY99, it was not surprising that sera containing MVEV-neutralizing antibodies reacted with the scFv–WN19 fusion protein. We hypothesize that it is therefore unlikely that this peptide will enable differentiation between other similarly glycosylated flaviviruses of high sequence identity in this region, such as WNV Sarafend and glycosylated KUNV strains. Furthermore, the non-specific reactivity of most of the KUNV-neutralizing sera (which contained no MVEV-neutralizing antibodies) to the extended scFv carrier protein made it difficult to make any firm conclusions as to whether this peptide confers any diagnostic advantage over other described WNV antigens.

Several groups have recently attempted to improve the specificity of WNV E antigens by focussing on subunits of the E protein. By eliminating the cross-reactive epitopes in the E protein domain II, Roberson et al. (2007) reported a...
sensitive WNV diagnostic antigen that was considerably more specific than its wild-type counterpart. In a different approach, Beasley et al. (2004b) developed a recombinant protein based on E protein domain III. However, only limited information on the specificity of this antigen was reported for sera from natural infections. In a more defined analysis of domain III antigens, a linear, 15 aa fragment of this domain has been successfully used in ELISA (Herrmann et al., 2007) for human WNV infection diagnosis, and has also been assessed as an antigen on an amperometric immunosensor (Ionescu et al., 2007). The comparative specificity of these E-protein-subunit antigens has not been fully investigated.

The downside of compartmentalizing any target antigen is that there is often a reduction in sensitivity. This is evidenced by our data where a number of samples with VNT titres ≥ 160 reacted only weakly with the scFv–WN19 fusion protein, or not at all. Microsphere immunoassays have superior sensitivity due to the fluorescent reporters used, the availability of more epitopes and the greater surface area (Shi & Wong, 2003). This platform has been investigated for WNV diagnosis (Wong et al., 2003, 2004; Johnson et al., 2005; Balasuriya et al., 2006), and we believe that the scFv–WN19 fusion protein could be a valuable inclusion in a similar assay.

The results reported here highlight the importance of considering glycans when searching for potential diagnostic antigens. The glycan-dependent binding of infected sera to pathogen antigens has been reported previously. One example is the mannose-dependent binding of an HIV-specific neutralizing antibody (Sanders et al., 2002). Furthermore, a species-specific Ehrlichia canis peptide of high diagnostic potential has been shown in its glycosylated form to be much more immunoreactive than its nonglycosylated counterpart (McBride et al., 2007).

Our results suggest that induction of antibodies to the WN19 epitope during WNV infection of horses is generally associated with E protein glycosylation of the infecting viral strain. Our future studies will examine the specificity and sensitivity of this peptide as a diagnostic antigen by analysing a wider range of sera from animals known to be infected with glycosylated and nonglycosylated WNV strains and related flaviviruses.

ACKNOWLEDGEMENTS

We thank Gwong-Jen J. Chang, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA, for the pCBWN plasmid; David Chang, Alexander Khromykh and Yin Xiang Setoh, The University of Queensland, Australia, for KUNV prME plasmid and preparation of recombinant KUNV antigen; Gregory Coia, CSIRO, Victoria, Australia, for the pGCO38C, plasmid; Richard Bowen, Colorado State University, Fort Collins, CO, USA, Lorna Melville, Department of Primary Industry Fisheries and Mines, Northern Territory, Australia, Carolina Arevalo, Alexis Sandis, Luis Nazario Araya, Jose Luis Hernandez, Universidad Nacional, Heredia, Costa Rica, and Aaron Brault, University of California, Davis, CA, USA, for generous supply of horse serum; Kim Pham, The University of Queensland, Australia, for excellent technical assistance in VNT and antigen preparation; Kym Hoger and Kirstie Breslin, AGEN Biomedical Ltd, Queensland, Australia, for invaluable advice and assistance with cell culture; and Robert Doms, University of Pennsylvania, Philadelphia, PA, USA for useful discussions and supply of mAb17D7. This research was supported by the Australian Biosecurity CRC for Emerging Infectious Disease and AGEN Biomedical Ltd.

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