A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection

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Abstract

West Nile virus (WNV) is a neurotropic flavivirus within the Japanese encephalitis antigenic complex that is responsible for causing West Nile encephalitis in humans. The surface of WNV virions is covered by a highly ordered icosahedral array of envelope proteins that is responsible for mediating attachment and fusion with target cells. These envelope proteins are also primary targets for the generation of neutralizing antibodies in vivo. In this study, we describe a novel approach for measuring antibody-mediated neutralization of WNV infection using virus-like particles that measure infection as a function of reporter gene expression. These reporter virus particles (RVPs) are produced by complementation of a subgenomic replicon with WNV structural proteins provided in trans using conventional DNA expression vectors. The precision and accuracy of this approach stem from an ability to measure the outcome of the interaction between antibody and viral antigens under conditions that satisfy the assumptions of the law of mass action as applied to virus neutralization. In addition to its quantitative strengths, this approach allows the production of WNV RVPs bearing the prM-E proteins of different WNV strains and mutants, offering considerable flexibility for the study of the humoral immune response to WNV in vitro. WNV RVPs are capable of only a single round of infection, can be used under BSL-2 conditions, and offer a rapid and quantitative approach for detecting virus entry and its inhibition by neutralizing antibody.

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Introduction

West Nile virus (WNV) is a neurotropic flavivirus that is the etiologic agent responsible for West Nile encephalitis in humans (reviewed in Brinton, 2002). Despite being endemic in many parts of Africa, Europe, Asia, and the Middle East, WNV was not introduced into the western hemisphere until the late summer of 1999 (Lanciotti et al., 1999; Marfin and Gubler, 2001). During subsequent years, WNV spread across much of North America and can now be found in a variety of avian, mosquito, and mammalian species throughout the United States, Canada, and Mexico (Granwehr et al., 2004). The rapid spread of WNV across North America during the past 5 years identifies this virus as an important emerging pathogen for the western hemisphere.

In nature, WNV exists in an enzootic cycle between birds and mosquitoes, although several incidental vertebrate species can be infected including humans (Marfin and Gubler, 2001). While the majority of individuals exposed to WNV remain asymptomatic, a spectrum of disease has been reported that ranges from a mild febrile illness to severe meningoencephalitis (Petersen et al., 2003). However, only a small fraction of individuals experience these more serious clinical complications of infection. The age and the immune status of the
affected individual are the principal factors that influence the severity of WNV associated disease (Chowers et al., 2001; Nash et al., 2001; Tsai et al., 1998). While no specific therapy for WNV infection currently exists, the development of immunotherapeutics (Oliphant et al., 2005) and vaccines (Despres et al., 2005; Karaca et al., 2005; Lustig et al., 2000; Siger et al., 2004; Tesh et al., 2002; Turell et al., 2003) is an active area of investigation. Structural and cryo-electron microscopy studies reveal that the surface of the flavivirus virion is composed of a highly ordered icosahedron of 180 envelope (E) proteins (Ferlenghi et al., 2001; Kuhn et al., 2002; Modis et al., 2003, 2005; Mukhopadhyay et al., 2003, 2005; Rey et al., 1995). The generation of antibodies capable of binding to this array of viral proteins and blocking infection is an important aspect of the immune response and a goal of vaccine development. Passive immunization studies and experimental infections of immuno-deficient mice demonstrate that antibody plays a significant role in protection from and clearance of WNV (reviewed in Diamond et al., 2003). The importance of antibodies in vivo reflects their ability to directly neutralize virus infectivity (Oliphant et al., 2005), facilitate complement-mediated lysis of virions (Mehlhop et al., 2005) and promote efficient viral clearance through Fc-dependent pathways (Oliphant et al., 2005). Characterizing the humoral immune response to WNV infection or vaccination will facilitate the development of safe and effective vaccination and immunotherapeutic strategies.

The majority of existing approaches for the study of WNV neutralization measure the inhibition of virus entry as a reduction in the number of plaques formed on monolayers of suitable cell lines (Lindsey et al., 1976; Morens et al., 1985; Russell and Nisalak, 1967; Russell et al., 1967). While a great deal has been learned about WNV and other flaviviruses using these methods, they have limitations including a requirement for an appropriate biocontainment facility and a reliance on viral strains and cell types for which plaque formation can be readily visualized and enumerated. To complement and extend these approaches, we describe a novel approach for measuring antibody-mediated neutralization of WNV infection using virus-like particles (reporter virus particles: RVPs) that encapsidate a sub-genomic WNV replicon that is packaged into an enveloped virus particle by the capsid and envelope proteins of the virus when provided in trans (Jones et al., 2005; Khromykh et al., 1998; Scholle et al., 2004). Using the work of Khromykh and colleagues as a model, we constructed a panel of replicons that express one or more reporter genes by modifying a molecular clone of a lineage II strain of WNV (Fig. 1) (Khromykh and Westaway, 1997) (reviewed in Khromykh, 2000). Each replicon was designed to facilitate the introduction of reporter genes in place of the structural genes (via a novel MluI site) or in the 3′ UTR under the translational control of an internal ribosome entry site (IRES) (via a novel NotI site). In addition, these vectors were engineered to place the sub-genomic WNV RNA under the transcriptional control of the CMV immediate early promoter/enhancer. This “DNA-launched” format allows the introduction of replicon RNA into cells using standard transfection methodologies (Pierson et al., 2005; Varnavski et al., 2000), eliminating the requirement to produce and purify the approximately 11 kb, 5mG-capped RNA that is associated with many flavivirus replicons and molecular clones.

Production of WNV RVGs was accomplished by transfection of HEK-293T cells with three plasmids that encode capsid, the envelope proteins (pre-membrane (prM) and E), and a replicon (WNIrep-GFP). Two days after transfection, RVG-containing supernatants were harvested, filtered, and used to infect target cells. RVG-infected BHK-21 cells were analyzed at 36 h post-infection for the expression of GFP by flow cytometry (Fig. 2). Using this approach, we found that infectious RVGs capable of transducing target cells were released at a titer of approximately 1.0 × 10^6 infectious particles per milliliter, as measured by GFP expression in BHK-21 cells. As expected, the production of infectious particles required expression of all three components in the producer cell (Fig. 2). To simplify this RVG production approach, without imposing limitations on the nature of structural proteins incorporated into the virus particle, we created BHK-21 cell lines that stably propagate bicistronic WNV replicons. BHK-21 cells that propagate replicons were produced by infection with RVGs that encapsidated a bicistronic replicon encoding both a reporter gene and the gene conferring resistance to blasticidin, followed by selection
neutralized WNV RNAs replicating in each of these stable cell lines were capable of being packaged into infectious RVPs when cells were transfected with vectors encoding the structural proteins. However, similar titers were obtained as compared to approaches in which the replicon was transfected along with the plasmids encoding structural proteins, indicating that these cell lines simplify but do not enhance RVP production (data not shown).

Neutralization of WNV RVPs

To evaluate the utility of RVPs for the study of antibody-mediated neutralization, we performed a series of experiments to confirm that neutralization of RVP infection could be measured as a function of reporter gene expression. These studies were performed using monoclonal antibody (mAb) 7H2, which blocks WNV infection through high affinity interactions with a neutralizing epitope on the upper lateral surface of the domain III region of the E protein (Beasley and Barrett, 2002; Oliphant et al., 2005; Sanchez et al., 2005; Volk et al., 2004). WNV RVPs were produced in WNIIrep-GFP transfected HEK-293T cells and incubated in the presence of a high concentration of mAb 7H2 prior to infection of BHK-21 cells. Incubation with mAb 7H2 blocked greater than 90% of WNV RVP infection, whereas an irrelevant control antibody had no inhibitory effect (Fig. 3A). Analysis of the level of GFP expression in cells infected with RVP in the presence of sub-neutralizing quantities of mAb confirmed that the presence of neutralizing antibody resulted in a reduction in the number of cells infected by RVPs, rather than an overall reduction in reporter gene activity in each infected cell (Fig. 3B). Although it is intuitive for experiments in which neutralization can be measured directly as a reduction in the percentage of infected cells, this control provides support for high throughput approaches in which infection is measured by catalysis of luminescent or fluorescent substrates.

Development of an assay to provide a quantitative and accurate measure of the neutralizing potency of an antibody or sera is dependent upon use of appropriate normalization conditions and validation parameters. The “percentage law” states that the proportion of virus neutralized by any given concentration of antibody will remain constant even when the concentration of the virus input is varied (Andrewes and
Elford, 1933; Burnet et al., 1937; Klasse and Sattentau, 2001 for review). The rationale behind this concept is based on the observation that antibody-mediated neutralization of virus infection typically obeys the law of mass action. In the absence of cooperative effects:

\[ [\text{virus} (V)] + [\text{mAb}] \rightleftharpoons [\text{virus-antibody complex} (\text{mAb-V})] \]

The law of mass action predicts that the fraction of virus particles neutralized by antibody \((\frac{[\text{mAb-V}]}{[\text{V}]})\) should depend on the concentration of free antibody \([\text{mAb}]\) and the affinity of the antibody for viral antigen \((K_a)\). During neutralization experiments, the concentration of free mAb is not measured directly but is assumed to be very close to the total concentration of mAb added to the neutralization reaction \(([\text{mAb}_{\text{TOT}}] = [\text{mAb}] + [\text{mAb-V}]).\) For this to occur, antibody must be present in vast excess over the virus particles at informative portions of the resulting neutralization curve, such that as the reaction approaches equilibrium, the amount of antibody in complex with virus particles will be a negligible fraction of the amount of total antibody (reviewed in Klasse and Sattentau, 2001, 2002). Conventional assays for measuring antibody-mediated neutralization are standardized using a defined number of infectious virus particles. Demonstrating that this approach satisfies the assumptions of the percentage law under all circumstances is cumbersome, particularly when studying viruses (such as flaviviruses) that secrete significant amounts of soluble antigen or defective virus particles from infected cells (Lobigs and Lee, 2004; Smith et al., 1970; Stollar, 1969), comparing different virus strains or preparations, or studying antibodies that bind viral antigens with high affinity.

Fig. 3. Neutralization of WNV RVPs. WNV RVPs were produced in HEK-293T cells by transfection with plasmids encoding WNIIrep-GFP and the structural proteins capsid and prM-E. (A) RVPs were harvested and incubated for 1 h in the presence or absence of 2.5 μg/ml of either the mAb 7H2 or an irrelevant control antibody specific for the C-type lectin DC-SIGN-R. Subsequently, RVPs were used to infect BHK-21 cells in a total volume of 250 μl. Antibody incubations and infections were performed in triplicate. At 30 h post-infection, cells were harvested, and the percentage of cells expressing GFP was determined by flow cytometry. Error bars reflect the standard deviation of the average value for each point. (B) Dot plot diagrams illustrating the intensity of replicon reporter gene expression in the presence of varying concentrations of 7H2. Geometric mean channel fluorescence of cells in the GFP positive quadrant for no RVP, no 7H2, 2500 ng 7H2, and 80 ng 7H2 conditions was 0.0 \((n = 0)\), 1183 \((n = 2342)\), 785.8 \((n = 5)\), and 1104 \((n = 436)\), respectively (with the number of positive events indicated in parenthesis).

Fig. 4. Impact of RVP concentration on neutralization potency of 7H2. WNV RVPs were produced in HEK-293T cells transfected with an equal mass of WNIIrep-GFP, WNV capsid, and WNV prM-E proteins using Lipofectamine 2000. WNV RVPs were harvested at 48 h post-transfection and diluted as indicated prior to addition of antibody. Antibodies were incubated with RVPs at the indicated concentrations for 1 h at room temperature in complete media. Pre-plated BHK-21 cells were infected in 48-well plates in a total volume of 200 μl. Infection was analyzed at 36 h post-infection by flow cytometry. Non-linear regression analysis to predict the EC50 of mAb 7H2 at each concentration of RVPs was performed using Graph Pad Prism, revealing an EC50 of 170, 32, and 11 ng/ml 7H2 for RVPs diluted 0-, 4-, or 16-fold prior to analysis. Dotted lines indicated the 95% confidence interval for the regression analysis and error bars reflect the standard deviation at each point.
To test the application of RVPs for the study of neutralization in accordance with the percentage law, neutralization curves were generated with varying concentrations of mAb 7H2 and WNV RVPs. While RVP infection was reduced in a dose-dependent fashion, the concentration of antibody required to block 50% of the WNV RVP infection events was strongly dependent upon the concentration of the virus (Fig. 4). In this regard, these initial neutralization studies violated the percentage law because relative to the affinity of mAb 7H2 for WNV antigen, the amount of viral antigen present in each neutralization reaction was too large. While this problem could be solved by a larger dilution of RVPs prior to analysis, this would have the undesirable effect of decreasing the signal to background ratio and precision of the assay. Instead, we investigated the possibility of reducing the amount of viral antigen present in RVP preparations, without affecting the infectious titer.

Manipulating the level of non-infectious virus particles present in WNV RVP preparations

An interesting aspect of flavivirus biology is the production and release of small, non-infectious subviral particles (SVPs) following expression of prM and E, which can occur in the absence of expression of the viral RNA or any of the other viral proteins (Allison et al., 1995, 2003; Schalich et al., 1996). Importantly, SVPs are structurally and antigenically similar to infectious virus particles and can be released from virus-infected cells (Brinton, 1983; Ferlenghi et al., 2001; Lobigs and Lee, 2004). Thus, factors that influence the relative amount of SVPs present in virus preparations will alter the amount of viral antigen associated with each infectious particle. Studies of several different flavivirus systems suggest that the incorporation of capsid into the virus particle is controlled at least in part by the coordinated viral cleavage of the capsid protein away from prM during translation of the polyprotein at the ER (Lobigs and Lee, 2004). Therefore, we sought to modify our complementation strategy to identify an approach that yielded infectious RVPs with the smallest amount of antigen.

Four different complementation strategies were investigated, using plasmids that expressed WNV structural genes in different configurations (Fig. 5A). Surprisingly, all four strategies produced infectious RVPs with a very similar titer (Fig. 5B). In contrast, significant differences in the concentration of antigen were observed in supernatants containing RVPs. RVPs produced using plasmids encoding a capsid-prM fusion protein (C-prM-E and C-prM + E) yielded particles with the lowest concentration of antigen per infectious unit (Fig. 5C), consistent with the importance of cleavage of the capsid-prM junction during formation of infectious virus particles (Lobigs and Lee, 2004). In contrast, RVPs secreted from cells transfected with plasmids encoding capsid and prM-E (C + prM-E) were associated with relatively high concentrations of E protein antigen. Thus, the particle to infectious particle ratio in RVP preparations, as measured by the concentration of antigen per infection event, can be manipulated using different structural protein expression systems and production conditions. Moreover, complementation strategies that employ capsid-prM and E protein expression vectors (C-prM + E) are most favorable as they allow production of RVPs associated with low concentrations of antigen and retain the additional degree of safety afforded by the use of three separate genetic elements.

Neutralization of RVPs in accordance with the percentage law

Having identified RVP production conditions that result in a more favorable ratio of infectious particles to viral antigen, we...
next developed criteria for their application in neutralization studies using an approach that satisfies the percentage law. We established the concentration of E protein antigen as the sole normalization parameter between experiments and virus particle preparations and then sought to identify a concentration of viral antigen that was sufficiently low such that the formation of RVP–antibody complexes would be negligible relative to the concentration of antibody required to neutralize half of the infectious particles (i.e., the EC50 of the mAb). Because this parameter is strongly affected by the affinity of the antibody studied, we employed high affinity antibodies for analysis, providing the most stringent validation conditions (Beasley and Barrett, 2002; Volk et al., 2004).

RVPs were produced in a BHK-21 cell line that stably replicates a WNV replicon encoding Renilla luciferase (REN) after transfection of plasmids expressing C-prM and E (C-prM + E) (Fig. 5A). RVPs were harvested, analyzed for E protein content by ELISA, aliquoted, diluted as indicated prior to incubation with varying concentrations of mAb 7H2, and then used to infect BHK-21 cells in a 96-well plate. Infection and neutralization were monitored as a function of luciferase activity at 40 h after infection. Non-linear regression analysis predicted the EC50 of 7H2 at 6.5 ng/ml (95% confidence interval (95%CI): 4.2–10 ng/ml), 3.8 ng/ml (95%CI: 2.5–5.9 ng/ml), and 6.6 ng/ml (95%CI: 3.7–11.6 ng/ml) for 10-, 20-, and 40-fold dilutions of RVPs, respectively (Fig. 6A). Thus, in contrast to data obtained with particles produced using the C + prM-E approach, the potency of mAb 7H2 did not vary significantly with increasing dilutions of particles. In addition, increasing the volume of the neutralization reaction, while maintaining the concentration of antibody, did not significantly impact the neutralization titer of 7H2 (data not shown). Both experiments directly test and validate compliance of these assay conditions with the assumptions of the law of mass action. Repeated analysis of the neutralization potency of mAb 7H2 (average EC50 of 6.8 ng/ml (±2.9 ng/ml (standard deviation): n = 9)) or 5H10 (16.0 ± 7.6 ng/ml: n = 10) performed on more than five occasions using at least three independent preparations of RVPs demonstrated a high degree of accuracy and reproducibility (Fig. 6B). Interestingly, we performed a smaller number of experiments using Vero cells and found that the potency of each antibody was somewhat greater than when BHK-21 cells were used as targets (5H10: 1.0 ± 0.6 ng/ml (n = 8) and 7H2: 1.2 ± 1.0 ng/ml (n = 5)), consistent with previous work in other viral systems that identify the impact of cellular factors on neutralization (Grady and Kinch, 1985; Kjellen, 1985; Kjellen and von Zeipel, 1984; Layne et al., 1991; Ruppach et al., 2000). This RVP system will make it possible to more readily identify cellular factors that impact WNV neutralization since they can be used to infect a variety of cell lines and primary cell types including those that do not support plaque formation. Overall, we found that RVP-based measurements of antibody-mediated neutralization could be made accurately and reproducibly while complying with the assumptions of mass action, provided that less than 1 ng of E protein antigen was used in each assay.

Fig. 6. Neutralization of WNV RVPs in accordance with the percentage law. (A) WNV RVPs were produced in BHK WNRep-REN cells transfected with plasmids encoding a WNV C-prM fusion protein and E protein (using a plasmid ratio of 3:1 by mass) using Lipofectamine 2000. RVPs were diluted as indicated and used in neutralization studies with the indicated concentrations of mAb 7H2 in quadruplicate. At these dilutions, all preparations contained less than 10 ng/ml E protein antigen. RVP–antibody complexes were added to pre-plated BHK-21 cells in 96-well plates in 200 µl total volume. Analysis of luciferase activity was performed at 40 h post-infection. Non-linear regression analysis of the resulting data set was performed to predict the EC50 of mAb 7H2 at each concentration of RVPs using Graph Pad Prism. Dotted lines indicate the 95% confidence interval for the regression analysis, and error bars reflect the standard deviation at each point. (B) The neutralization activity of mAb 5H10 was determined using three independent, antigen normalized preparations of WNV RVPs. In each case, RVPs were produced in BHK WNRep-REN using the C-prM + E production approach and frozen. The antigen concentration of RVPs was determined by ELISA and adjusted to less than 10 ng/ml for each preparation prior to incubation with mAb 5H10. Neutralization studies and analysis were performed as described above.

Antibody-dependent enhancement of WNV RVP infection

Significant experimental evidence supports a role for antibodies in protection from flavivirus infection and the establishment of immunity following vaccination (reviewed in Roehrig et al., 2001). However, under some circumstances, antibodies may have pathogenic consequences as they can enhance infection of FcγRII-expressing cells (reviewed in Halstead, 2003). To evaluate the feasibility of investigating antibody-dependent enhancement of infection (ADE) using our assay, WNV RVPs were incubated in the presence of decreasing concentrations of mAb 7H2 for 1 h, and then incubated with K562 cells, an erythroleukemic human cell line that expresses FcγRII (CD32) and has been
used previously in the study of ADE of dengue virus (Guy et al., 2004; Littaua et al., 1990). Under typical infection conditions, these cells are poorly permissive for WNV infection (less than 0.5% GFP positive cells in the absence of antibody). We observed a large (>50-fold) degree of enhancement of RVP infection in the presence of sub-neutralizing concentrations of mAb 7H2 (Fig. 7). Similar results were obtained if infections were performed with RVPs encapsidating a replica encoding luciferase and with a replication competent molecular clone of WNV (Pierson et al., 2005).

Discussion

Genetically modified virus particles that encode reporter genes provide a rapid and quantitative method for the study of virus entry in vitro. In this study, we describe the production and application of virus-like particles that encapsidate a WNV replicon for the study of antibody-mediated neutralization of WNV infection. This approach is well suited for neutralization studies because RVPs are capable of only a single round of infection and share many of the structural, biochemical, and functional properties of fully infectious virus particles (Khromykh et al., 1998; Scholle et al., 2004). Methods for the production of RVPs were adapted from an elegant series of studies by Khromykh and colleagues that describe both Kunjin virus replicons (Varnavski and Khromykh, 1999; Varnavski et al., 2000) and the complementation of these RNAs with structural proteins provided in trans by alphavirus vectors (Jones et al., 2005; Khromykh et al., 1998; Scholle et al., 2004) or stable packaging cell lines (Harvey et al., 2004). The criteria used to develop our RVP production approach were to perform RVP-based measurements of neutralization under conditions that satisfy the assumptions of the law of mass action. To achieve this, three aspects of RVP production were considered.

First, we sought to develop a flexible strategy to study antibody-mediated neutralization of envelope proteins from a variety of different WNV isolates. We optimized methods for the production of WNV RVPs by complementation of a DNA-launched WNV replicon with standard eukaryotic expression plasmids that encode the capsid and prM-E proteins of WNV. A strength of this complementation approach is that it allows the production of RVPs that incorporate the structural proteins of WNV using expression vectors that are readily transfected into cells, in widespread use in the field, and amenable to straightforward and high-throughput cloning methods such as site-directed mutagenesis. By comparison, complementation strategies employing alphavirus vectors (Jones et al., 2005; Khromykh et al., 1998; Scholle et al., 2004) or stable packaging cell lines (Gehrke et al., 2003; Harvey et al., 2004) are less versatile but may be capable of production at higher titers. Our complementation approach confers a considerable degree of flexibility by permitting the production of RVPs that incorporate the structural components of different virus strains simply by changing the combination of plasmids transfected into producer cells. To date, we have produced RVPs incorporating the prM-E proteins of several different strains and mutants of WNV (Hanna et al., 2005; T. Pierson, unpublished data), as well as the distantly related dengue and Langat viruses (Whitby et al., 2005; Davis et al., in press).

Second, RVP production approaches were optimized for the study of antibody-mediated neutralization under conditions that satisfy the assumptions of the law of mass action. In this regard, minimizing the concentration of non-infectious E protein antigen present in RVP preparations was essential. Although infectious RVPs were produced at similar titers after complementation with plasmids encoding the WNV structural genes in four different configurations, the ratio of infectious to non-infectious particles was markedly different. The two production strategies that utilized a capsid-prM fusion protein resulted in RVP stocks with a relatively low concentration of non-infectious E protein. When considered as a function of the infectious titer, the concentration of antigen present in stocks of RVPs produced using C-prM-E and C-prM + E strategies is quite similar to that of stocks of infectious WNV harvested from mosquito cell cultures three days after infection (approximately 3800–10,000 PFU of WNV/ng E protein vs. +1000 infectious RVP/ng E protein; data not shown). In contrast, RVPs prepared by expression of capsid and prM-E proteins on two separate plasmids yielded significantly higher concentration of antigen (>50-fold), despite an equivalent infectious titer. This in turn decreases the utility of particles produced using this strategy for neutralization studies.

We suggest that the discrepancy in the relative proportions of infectious and non-infectious particles may reflect differences between the requirements for the release of non-infectious subviral particles (composed solely of envelope prM and E proteins) (Konishi et al., 1992), from those directing...
secretion of infectious virus particles (incorporating capsid and RNA) (Lee et al., 2000). In our system, RVPs were produced by transfection of three or four different plasmids into cells. Although a significant percentage of cells will take up all of the plasmids present in the transfection mixture, a fraction of cells will receive only a single plasmid. When producing RVPs with capsid and prM-E expression vectors (C + prM-E) the fraction of cells transfected with only the prM-E expression plasmid will have the ability to release large amounts of non-infectious SVPs into the supernatant. In support of this, reducing the mass of the prM-E expression plasmid in the transfection mix relative to the other plasmid components allowed RVP production associated with significantly reduced concentration of antigen (data not shown). In contrast, for C-prM + E and C-prM-E production strategies, a requirement for cleavage at the C-prM junction coupled with the required role of prM in folding and trafficking of E protein (Allison et al., 1995) imposes a requirement for the simultaneous expression of all four components (replicon, capsid, prM, and E) on secretion of E protein antigen. Our results provide direct support for the importance of the viral protease-mediated cleavage event between capsid and prM in regulating the kinetic and temporal relationships that balance prM-E mediated formation of a virus particle and the requirements for incorporation of RNA and capsid protein (Lobigs and Lee, 2004). While an important caveat to consider for structure–function studies, the ability to manipulate the relative proportion of infectious SVPs, and thus the concentration of E protein antigen, present in RVP preparations proved to be a critical aspect of the development of RVP-based methods for investigating antibody-mediated neutralization.

A third consideration when selecting methods for the production of WNV RVPs is the potential for the formation of infectious WNV genome by recombination. While recombination during trans-encapsulation of flavivirus replicons to yield infectious viral genomes has not yet been reported, it has been proven to be of significant concern in related alphavirus replicon systems (Vasilakis et al., 2003 and references within). The use of DNA plasmids to launch both replicon and structural gene RNAs confers a significant degree of flexibility to our approach but may also increase the potential for recombination during RVP production. Recombination between DNA plasmids following transfection is well documented and is a property often exploited for the production of infectious virus using molecular clone technology of other viral systems including HIV-1, adenovirus, and vaccinia virus (Skrabal et al., 2003). Recombination is particularly efficient when significant regions of homology exist between exogenously added plasmids. Unfortunately, the overlap between the amino-terminus of WNV capsid and a required RNA cis element in the replicon (C20) imposes a requirement for sequence homology between the replicon and capsid expression vector. Likewise, homology exists between the carboxy-terminus of E protein and the ER translocation signal of the remaining polyprotein encoded by the replicon. To reduce the possibility for reversion to a fully infectious virus, our production strategies used at least three components imposing a requirement for at least two independent recombination events for the restoration of a wild type genome. Since RVPs produced using C-prM-E and C-prM + E complementation strategies are equivalent with respect to both infectious titer, concentration of E protein antigen present, and recognition by neutralizing antibodies (data not shown), the C-prM + E strategy was used for neutralization studies.

The standard method for detecting neutralizing antibodies against WNV is the plaque reduction neutralization test (PRNT) (Lindsey et al., 1976; Morens et al., 1985; Russell and Nisalak, 1967; Russell et al., 1967). Using this approach, the ability of antibody to bind virus and prevent infection is measured as a reduction in the number of plaques formed following infection of monolayers of permissive cells. While the use of this methodology has allowed for great insight into flavivirus biology and pathogenesis, it has several limitations. First, the PRNT assay is relatively slow, requiring 3–4 days for plaque formation and analysis, and is logistically difficult to perform in large scale. Second, only cell types upon which the virus readily forms plaques may be used to investigate neutralization of WNV. Thus, the PRNT approach does not allow the neutralization capacity of antibodies to be detected using virus strains that plaque poorly or on permissive cell types that do not form monolayers. Because cellular factors may impact the outcome of antibody–virus interactions, a capacity to measure neutralization in several cellular contexts, including those that reflect targets of WNV infection in vivo, is strongly desirable. Third, the quantitative power of the PRNT is subject to the number of wells examined and number of plaques counted manually by the investigator. The latter process is somewhat subjective when plaque size and morphology is variable. Finally, the PRNT approach involves the use of live infectious virus, which must be handled by a skilled investigator in an appropriate biocontainment facility.

The application of RVPs for the study of antibody-mediated neutralization has several desirable features that complement the PRNT. Infection of cells by RVPs is measured directly as a function of reporter gene activity, allowing the study of virus entry and its inhibition on a variety of cells types, including those that do not support the formation of plaques. This is particularly useful when studying ADE on non-adherent Fc-receptor bearing cell lines. RVP-based measurements of neutralization studies can be performed rapidly (2 days) and on large scale using a 96-well plate format. Analysis of a large number of independent experiments assayed on different days with different preparations of RVPs revealed that this approach allows a significant degree of precision and reproducibility. Significantly, neutralization studies using RVPs allow the analysis of a panel of different WNV strains or lineages without a requirement to isolate infectious clones of each virus strain of interest nor manipulate them in a BSL-3 facility.

A critical and innovative aspect of an RVP approach for measuring neutralization is the normalization criteria employed for each experiment. In contrast to the PRNT assay, the amount of RVPs used in each experiment was determined as a function
of the antigen content of the preparation, rather than its infectious titer. Importantly, no assumptions are made regarding the nature of the antigen in RVP preparations, reducing the impact of variations in the particle to infectious particle ratios of RVP (or virus) preparations. We arrived at these criteria by determining experimentally that it is not appropriate to assume the antigen content of virus preparations is sufficiently low to allow study under conditions of “antibody excess”, particularly when the affinity of the antibody studied is high. This criterion, however, is not uniquely suited to the use of RVPs. We have also observed the concentration of antigen present in stocks of infectious WNV can vary significantly (100-fold) depending upon the cell line and conditions of production, and that the infectious to non-infectious particle ratio varies greatly among different strains of flaviviruses (M. Diamond, E. Harris, and T. Pierson, unpublished data). The ability of flavivirus infected cells to shed viral antigen in the form of defective mature virus particles, or SVPs composed solely of pm-E, introduces additional challenges for the study of antibody-mediated neutralization or ADE. For both infectious virus and RVPs produced by complementation, the use of antigen as a critical normalization parameter provides a direct method to ensure each experiment is performed in accordance with the percentage law.

Understanding the biochemical and cellular factors that determine the fate of a virus particle bound by antibody is an important aspect of understanding the requirements for an effective and safe vaccine. For flaviviruses, this is complicated by an ability of both neutralizing and non-neutralizing antibodies to facilitate infection under some circumstances and possibly, as in secondary DEN infection, contribute to pathogenesis (Halstead, 2003). The application of RVPs to study the mechanism of neutralization and enhancement allows a direct and quantitative measurement of antibody–virus particle interactions in variety of cellular contexts and will allow novel approaches to determine the mechanisms of antibody-mediated neutralization and enhancement.

Materials and methods

Cell lines

Human embryonic kidney cells (HEK)-293T, HeLa cells, Vero, and Baby hamster kidney (BHK-21) clone 15 cells were maintained in Dulbecco’s modified essential media (DMEM) (Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum (FCS), and 1% penicillin/streptomycin (P/S) (Invitrogen, Carlsbad, CA). BHK-21 cell lines that stably propagate WNV replicons were maintained in DMEM/10% FCS/1% P/S supplemented with 10 μg/ml of blasticidin S (Invitrogen, Carlsbad, CA). K562 cells were grown in RPMI supplemented with 10% FCS and 1%P/S. All cell lines were maintained at 37 °C in 5% CO2.

Construction of a sub-genomic WNV replicon

Construction of a panel of DNA-launched sub-genomic WNV replicons was accomplished by modification of a previously described infectious molecular clone of a lineage II strain of WNV (strain: WN 956 D117 3B). The objective was to build a replicon in a configuration that simplified the introduction of one or more reporter genes and allowed expression in eukaryotic cells following transfection of the plasmid, eliminating a requirement for production and purification of RNA in vitro. The starting material for these studies was plasmid (pSP6WNV/Xba) encoding WNV under the transcriptional control of the SP6 promoter (kindly provided by Dr. Vladimir Yamschikov (University of Kansas))(Wengler and Gross, 1978; Yamschikov et al., 2001). Replicons were constructed in several steps employing standard methods of molecular cloning.

The first step in construction involved the introduction of a unique NotI restriction endonuclease recognition site into the 3′ UTR, positioned 24 nucleotides downstream of the stop codon of the WNV ORF. The purpose of this site was to allow the introduction of reporter genes or drug resistance markers into the replicon under the translational control of an internal ribosome entry site (IRES). This was accomplished using an overlap extension PCR approach to synthesize a DNA fragment containing the NotI site into the 3′ UTR, followed by insertion back into the parental vector using unique AvrII and XbaI sites in SP6WN/Xba (to create vector pSP6WNV-Not). To minimize the potential for recombination during this and subsequent cloning steps, plasmid propagation was performed in Stbl2 rec bacteria (Invitrogen, Carlsbad, CA) grown for 22–24 h at 30 °C in LB broth. Next, a reporter gene cassette composed of the green fluorescent protein (GFP) and hygromycin B phosphotransferase (Hph) positioned downstream of the EMCV IRES was cloned into the NotI site (to generate vector pSP6WNV-IHF).

A second major modification involved replacement of the majority of sequence encoding the viral structural proteins with the 2A autoprotease of foot and mouth disease virus (FMDV) and a second unique MluI restriction site. This step was accomplished using overlap extension PCR method in which the novel MluI site and the 19 amino acids of the FMDV 2a protease was engineered into the overlapping primer set. The resulting PCR fragment was cloned into unique ClaI and NsiI sites in pSP6WNV-IHF (to create the replicon vector pWrep2a-IHF).

To eliminate a requirement for the synthesis and purification of replicon RNA in vitro, two further modifications of pWrep2a-IHF were made. First, a ribozyme from the hepatitis delta virus (HDV) and a SV40 polyadenylation signal were cloned adjacent to the 3′ terminus of the WNV cDNA (Fodor et al., 1999) such that ribozyme-mediated cleavage of the replicon RNA occurs to define the end of the RNA as the terminal nucleotides of the WNV genome. A fragment containing these two components was assembled using overlap extension PCR and cloned into a unique XbaI site (blunted with mung bean nuclelease) of pWrep2a-IHF. Next, a CMV promoter/enhancer was engineered upstream of the first nucleotide of the WNV genome using an overlap extension PCR strategy and unique Clal and NsiI sites in pWrep2a-IHF, creating a “DNA-launched” replicon (pWIIrep-IHF). Care was taken to ensure
the eukaryotic promoter was positioned to allow transcription to initiate at the 5' terminal nucleotide of the WNV genome. Correct placement of both the CMV promoter and HDV ribozyme were required to ensure that the RNA pol II messages produced following plasmid transfection would result in the production of WNV RNAs with the authentic terminal residues required for RNA replication.

Subsequently, a large number of additional replicons were constructed by simply substituting different gene cassettes into the unique MluI and NotI sites present at the 5' and 3' ends of the replicon (no reporter genes: WNIIrep, GFP: WNIIrep-GFP, Firefly luciferase: WNIIrep-FLUC, Renilla luciferase: WNIIrep-REN, GFP + blasticidin resistance: WNIIrep-GFP-IB, Renilla luciferase + blasticidin resistance: WNIIrep-REN-IB). Replicons were characterized by transfection of HEK-293T cells with 4µg of plasmid using Lipofectamine 2000 (according to the manufacturer's protocol) and quantifying reporter gene expression 48 h later. In addition, replicon RNAs were evaluated for their ability to replicate and be packaged into pseudo-infectious reporter virus particles as described below.

WNV structural protein expression vectors

The structural genes of WNV were cloned into several different expression vectors for use in the production of RVPs. A plasmid encoding the prM-E genes of the NY99 strain of WNV (pCBWN) was provided by Dr. Gwong Jen J. Chang (CDC) (Davis et al., 2001). pCBWN is a pcDNA3-based plasmid encoding a modified form of the WNV prM-E protein in which the signal sequence of WNV prM has been replaced with that of Japanese encephalitis virus in order to improve expression. The prM and E genes were amplified individually and as a prM-E fusion protein from this vector using PCR and cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) using topoisomerase I-mediated cloning methods. Subsequently, these genes were transferred by Gateway LR recombination into pcdNA6.2/V5-DEST (Invitrogen) to generate WNI prM 6.2, WNI E 6.2, and WNI prM-E 6.2. Plasmids encoding capsid-prM (C-prM) or capsid-prM-E (C-prM-E) fusion proteins created by cloning PCR fragments into unique NheI and BamHI sites of pIREShygro2 (Clonetech). WNV capsid was cloned from SP6WN/Xba into the Gateway system and subsequently into pcdNA 6.2 as described above (WNII Cap).

Production of pseudo-infectious WNV reporter virus particles (RVPs)

WNV RVPs were produced in HEK-293T cells by transfection with an equal mass (1.3 µg) of three DNA plasmids encoding: a replicon, capsid, and prM-E using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Preparation of RVPs using this system was accomplished by transfection of pre-plated cells with plasmids encoding capsid and prM-E. RVPs were produced by transfection of this replicon stable cell line with WNV structural protein expression vectors in several configurations and with varying ratios of DNA plasmids. Optimized RVP production conditions were determined and involved transfection of replicon stable cells in a T75 flask with 30 µg of C-prM vector and 10 µg of WNV E expression plasmid.

Cryo-preservation of RVPs was performed using two methods. Our initial studies were performed by mixing RVPs with an equal volume of heat-inactivated FCS containing 25 mM HEPES buffer, pH 8.0, followed by storage at −80 °C. In subsequent studies, we found that the addition of serum was not as important for virus stability as the presence of HEPES buffer. Therefore, RVP production conditions were modified slightly by producing virus particles into a commercially available low glucose formulation of media that already contains 25 mM HEPES (Invitrogen). RVP containing supernatants were then frozen directly. The stability of RVPs stored using this approach was evaluated and confirmed by periodically (n = 6) measuring infectious titer over the course of 2 months. We found that infectivity did not vary significantly over this period of time.

Infection of target cells with RVPs

Conditions for the infection of target cells were selected to maximize the linear portion of the curve defining the relationship between virus/RVP input and the number of infected cells. Target cells for WNV RVP infection were plated for infection experiments to achieve approximately 25–50% confluence at the time of infection. While infection of cells at higher density was possible, we found the relationship between virus input and infection saturated at lower levels of infection. Infections were harvested at 36–48 h post-infection, depending on the kinetics and quantity of reporter gene expression in the cell type studied.

Antigen capture ELISA for WNV E protein

To quantify the amount of E protein antigen in virus and reporter virus preparations, an antigen capture ELISA was developed based on methodologies developed in our laboratory for the characterization of WNV monoclonal antibodies (Sanchez et al., 2005). ELISA plates were prepared by coating 96-well plates for 8–12 h at 4 °C with the E-specific group reactive mAb 4G2 (100 ng per well) in a buffer composed of 15 mM sodium carbonate and 35 mM sodium bicarbonate in PBS (pH 9.6). 4G2 coated plates were washed three times prior to the addition of WNV antigens (wash buffer: 1X PBS, 0.05%
Tween) and blocked for 1 h in 1× phosphate-buffered saline (PBS), 0.05% Tween-20, 1% bovine serum albumin (BSA) (blocking buffer). Prior to analysis, E protein-containing supernatants were lysed in blocking buffer containing 1% Triton X-100 for at least 5 min, followed by serial two-fold dilutions in blocking buffer containing 0.1% Triton X-100. The standard used in this assay was a purified soluble form of the E protein (NY1999 strain) produced in HEK-293T cells using a recombinant vaccinia virus expression system as described in detail (Sanchez et al., 2005). These standards, initially at 125 ng/ml, were lysed and diluted as described above. Each sample was incubated in 4G2-coated wells for 2 h with gentle shaking to promote antigen capture, followed by six washes. Wells were then incubated with 2 μg/ml of a biotin conjugate of the E protein-specific monoclonal antibody E1 (Oliphant et al., 2005) for 1 h, followed by six washes. A horseradish peroxidase conjugate of streptavidin (400 ng/ml) was used to detect bound E1 (1-h incubation) and was developed using TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD). The reaction was stopped with 2N H₂SO₄ and read on a MRX Revelation plate reader at a wavelength of 450 nm.

Neutralization of WNV reporter virus particles

RVPs were produced as described above and frozen. The concentration of E protein present in each RVP preparation was measured using an antigen-capture ELISA and diluted in media to a concentration of less than 10 ng/ml. One hundred microliters of RVPs was incubated with an equal volume of media containing monoclonal antibody at varying concentrations at room temperature for 1 h. Targets for RVP infection were prepared by plating 6–8 × 10³ cells per well of a 96-well plate 5–6 h prior to infection. Cells were infected in quadruplicate by replacing the media of the target cells with RVP–antibody complexes and returned to the incubator for 40 h. For experiments with RVPs encapsidating a replicon encoding GFP, target cells were harvested by trypsinization, fixed in 2% paraformaldehyde in PBS, and analyzed by flow cytometry. For experiments with RVPs encapsidating a replicon encoding luciferase, infected cells were lysed in 100 μl of commercial lysis reagent (Promega) and assayed using luciferin-containing substrate as described. Luminescence was measured using a Wallac luminometer. Data obtained from neutralization assays were analyzed using non-linear regression analysis (GraphPad Prism 4, GraphPad Software, Inc). Raw data were fit to a sigmoidal dose-response (one site competition) curve constrained with the value of non-infected cells. The $EC_{50}$ of an antibody was determined for curves with an $R^2$ value of greater than 0.8.

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