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Baboon model for West Nile Virus infection and vaccine evaluation

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Abstract

Animal models that closely mimic the human condition are of paramount significance to study pathogenic mechanisms, vaccine and therapy scenarios. This is particularly true for investigations that involve emerging infectious diseases. Nonhuman primate species represent an alternative to the more intensively investigated rodent animal models and in a number of instances have been shown to represent a more reliable predictor of the human response to infection. West Nile virus (WNV) has emerged as a new pathogen in the Americas. It has a 5% fatality rate, predominantly in the elderly and immune compromised. Typically, infections are cleared by neutralizing antibodies, which suggests that a vaccine would be efficacious. Previously, only macaques had been evaluated as a primate model for WNV vaccine design. The macaques did not develop WNV disease nor express the full complement of IgG subclasses that is found in humans. We therefore explored baboons, which exhibit the similar four IgG subclasses observed in humans as a new model for WNV infection and vaccine evaluation. In this present report, we describe the experimental infection of baboons with WNV and test the efficacy of an inactivated WNV vaccination strategy. All experimentally infected animals developed transient viremia and subsequent neutralizing antibodies. Anti-WNV IgM antibodies peaked at 20 days post-infection. Anti-WNV IgG antibodies appeared later and persisted past 60 days. Prior vaccination with chemically inactivated virus induced neutralizing titers and a fast, high titer IgG recall response, which resulted in lower viremia upon challenge. This report is the first to describe the development of the baboon model for WNV experimental infection and the utility of this model to characterize the immunologic response against WNV and a candidate WNV vaccine.

Keywords: West Nile virus; Vaccine; Baboon

Introduction

Fever, peripheral viremia and an ensuing IgM, IgG and neutralizing antibody response characterize WNV infection in humans (Hayes et al., 2005a, 2005b; Lanciotti et al., 1999). Since no WNV-specific anti-viral drugs are available, patients can only be treated with palliative therapy (e.g. intravenous fluids). Still, most hospitalized patients recover and ultimately clear viral infection due to the inherent ability in even an immunologically naive host to mount a timely immune response and to control WNV infection. Upon natural exposure of humans, WNV clearance is found most often, WNV-associated clinical disease is seen as an exception. If the immune system fails, however, uncontrolled WNV spreads to the central nervous system and causes death by flaccid paralysis. The most severe disease phenotypes are observed after WNV infection of immune compromised individuals, such as after blood transfusion or organ transplantation (Iwamoto et al., 2003; Pealer et al., 2003). During WNV epidemics, as many as 1 in 150 blood donations can have detectable levels of viremia. During the 2002–2003 WNV epidemic in the United States, all cases of transfusion–transmission resulted from WNV IgM-negative donations (Busch et al., 2005; Petersen and Epstein, 2005; Stramer et al., 2005), which is consistent with the

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ability of IgM and/or IgG to prevent transmission. Hence, there exists a continued need to develop WNV vaccines and the hope, based on natural transmission data, that they would be efficacious. The murine model of WNV infection has been the most intensively investigated and is presently the preferred animal model for WNV research. The mouse model has allowed investigators to gain valuable information with regard to the immune response to WNV infection. Mice are susceptible to WNV infection, with LD50s as low as 1 pfu after peripheral injection (i.p., i.v. or foodpad) (Beasley et al., 2002; Papin et al., 2005). The small size of mice and their high susceptibility offer enormous practical benefits but may yield overly pessimistic results in vaccine studies compared to the much less susceptible primates (including humans). Susceptibility to infection in mice has been mapped to 2′–5′-oligoadenylate synthetase (OAS) (Mashimo et al., 2002; Perelygin et al., 2002). OAS limits WNV replication cell autonomously, even after intracranial (i.c.) injection, which bypasses the normal innate and adaptive immune responses. Studies in B-cell-deficient mice showed that neutralizing antibodies limit systemic spread and mortality (Diamond et al., 2003a, 2003b). Much has been learned about the antibody response to WNV using murine challenge models, in which prior immunization and post-exposure IgG treatment protects against disease. This body of work implicates the humoral immune response and neutralizing antibodies as correlates of protective immunity in WNV infection (Engle and Diamond, 2003; Oliphant et al., 2005; Tesh et al., 2002; Gould et al., 2005).

There are drawbacks to the mouse model of WNV infection and its use to assess the induction of immune responses and vaccine efficacy when testing new approaches. An example is that the mouse immune system and how it responds to WNV infection differs from the human immune response to this organism and other viral infections (Mestas and Hughes, 2004). Additionally, not all disease manifestations observed in humans were also observed in mice following WNV infection. As an alternative to mice, macaque species have been the nonhuman primate model of choice to study viral infections and provide the closest animal model to study the immune response to infection and vaccination. However, this model also has its limitations for investigations involving WNV. Macaques often carry the herpes B virus, which can be fatal to animal handlers that become inadvertently exposed to infected animals or tissues. More important to vaccine design, macaques lack the opsonizing antibody subclass IgG3, which in general reacts against protein antigens and readily activates complement (Kennedy et al., 1997). Recently, macaques, particularly rhesus monkeys, have been difficult to obtain and have become expensive due to their limited supply and use as the preferred nonhuman primate model for AIDS research. In contrast, the baboon (*Papio* species), another nonhuman primate model, are widely available and cost efficient mainly because they breed well in captivity. Unlike macaques, baboons do not harbor herpes B virus and have four IgG subclasses analogous to humans, which allows for more thorough investigation of vaccine aimed at inducing a protective antibody response. Hence, baboons have been utilized as animal models in the infectious disease arena and to test the safety and immunogenicity of human candidate vaccines. This includes bacterial, protozoal and viral infections, such as group A and B *Streptococcus*, group B meningococcus, *Haemophilus influenzae* type b, *Schistosoma mansoni*, *Trypanosoma cruzi*, human immunodeficiency virus, human T cell leukemia virus, hepatitis B virus, Epstein–Barr virus and rotavirus, among others (for example, Bot et al., 2001; Jenson et al., 2000; Kalina et al., 2005; Kariuki et al., 2004; Kennedy et al., 1997; Payton et al., 2004; Siddiqui et al., 2005; Watts et al., 1999; Wolf et al., 2006). Thus, the aims of this study were to determine (a) whether we could develop an experimental infection with WNV in baboons and (b) whether this experimental infection model could be used to assess the immune response and efficacy of a prototypic WNV vaccine.

Results

WNV infection in baboons

We infected three baboons (*Papio hamardryas anubis*) with 10^5 pfu of WNV OK02 in 0.1 ml PBS by intradermal injection. OK02, like NY99 (Beasley et al., 2002), is neuroinvasive and causes 100% mortality in mice (Papin et al., 2005). The animals were monitored daily and blood drawn prior to infection and at daily intervals up to day 14 and thereafter at days 21, 28, 42 and 56. Viral RNA was isolated from serum using QiAmp Viral RNA (Quiagen Inc.) according to manufacturer's recommendations. We used our previously developed quantitative real-time PCR assay to determine viral load (Papin et al., 2004b). WNV replicated in each animal with peak serum titers of 10^5–10^6 copies/ml at day 4 post-infection (Fig. 1). By day 10, virus was no longer detectable in serum. This kinetic pattern is similar to human infection (Hayes et al., 2005a). One animal developed a transient macular rash but showed no other clinical signs that might typify more severe disease such as weakness, disorientation or flaccid paralysis. This is not surprising since, in human infection as well, the ratio of clinically unapparent to apparent infections is estimated at 140–320:1 (Richman et al., 2002). Hence, the baboon infection model mimics the clinical course of primary human infection by WNV.

The antibody response to WNV in the three baboons followed the prototypical pattern of WNV infections in healthy human adults (Fig. 2). We were able to use a commercial WNV ELISA (Focus Diagnostics, Inc., Herndon, VI) since anti-IgG and anti-IgM antisera recognized baboon heavy chains as well as human heavy chains. This test is used to detect WNV in patients, and WNV reactivity is expressed as index value relative to positive and negative control. An index value >1 is considered positive. The response across the three experimental animals was remarkably homogenous. Anti-WNV-specific IgM antibodies peaked at approximately 20 days post-infection and then decreased to within two-fold above normal at day 57. Anti-WNV-specific IgG antibody levels increased up to day 25 and then more slowly until day 57, the end of the observation period. Next, we determined

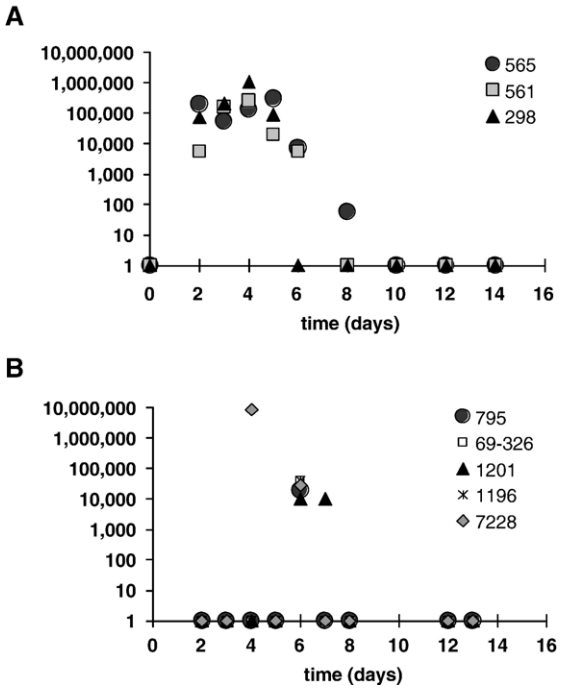

Fig. 1. Peripheral viremia in WNV-infected baboons (A) without immunization and (B) after immunization with killed virus vaccine.

neutralizing antibody titers by plaque reduction assay (Table 1). At day 21 p.i., the geometric mean neutralizing titers were 1:1250, 1:731 and 1:146. This establishes that the immune response to WNV in the baboon is similar to the human response to WNV exposure.

IgG, IgM and neutralizing antibodies are induced by a chemically inactivated WNV vaccine

To evaluate a killed WNV vaccine (West Nile-Innovator, Ft. Dodge Animal Health Inc., Ft. Dodge, IO) in baboons, we vaccinated five animals i.m. and boosted twice at 2-week intervals using the manufacturer's recommended dose (approximately 10^7 TCID50). Three weeks after the last boost, the animals were infected with 10^5 pfu WNV-OK02 i.d. WNV-specific IgG and IgM titers were determined by ELISA. With the exception of animal 795, there were no pre-immunization (PI) IgM or IgG antibodies to WNV (Fig. 3B). After immunization and prior to challenge (PCS), each animal developed significant WNV-specific IgG antibodies (Fig. 3B, compare group PCS to PI, $p \leq 0.001$ by Student's t test, $n=5$). The IgG levels induced by the killed virus vaccine after two boosts were as high as IgG levels at 21 days after challenge of the same animals with live virus (Fig. 3B, compare group d21 to PCS) and significantly higher than the positive control (PC). This suggests that chemically inactivated WNV was as efficient as natural infection in inducing IgG antibodies.

We did not detect IgM antibodies prior to immunization (PI) except for animal #795 (Fig. 3A). The IgM levels for all animals including #795 were barely above the cut-off value, but not significantly so at 3 weeks after the last boost (PCS) (Fig. 3A

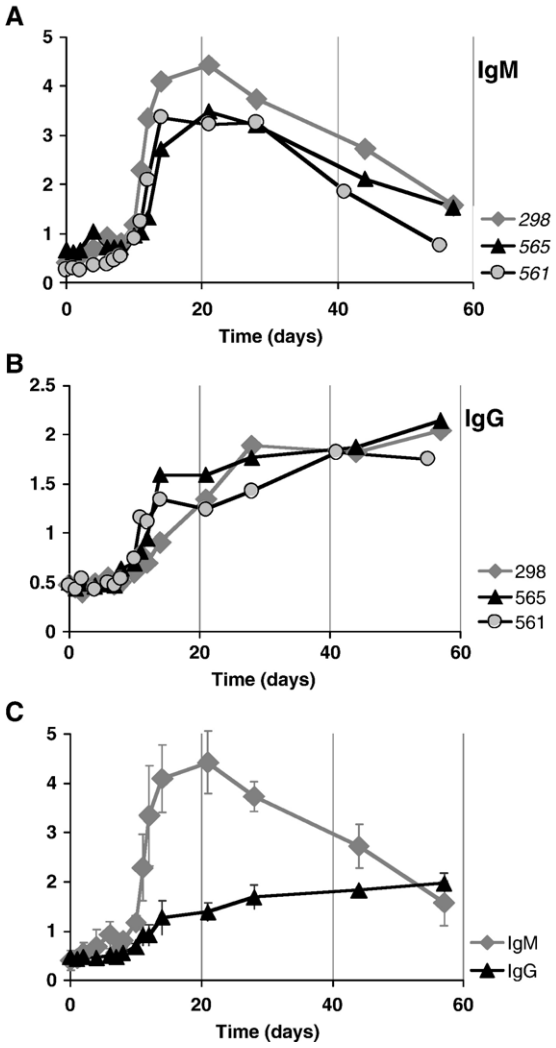

Fig. 2. Individual, (A) IgM and (B) IgG response to WNV in $n=3$ baboons. (C) Mean and SD of IgM and IgG responses. Index >1 indicates the presence of WNV antibodies.

compare group PI to PCS). This was expected since we wanted the IgM levels, i.e. the primary response, to decline prior to challenge such that we could measure the vaccine-primed secondary response. In contrast to naive animals (298, 561,

Table 1
Geometric mean neutralizing titer

Animal	Sex	Age/Years	p.i.[a]	Day 11	21 days
#795	f	9	1855	3363	nd[b]
#1201	m	3	736	5750[c]	nd
#69–362	f	5.5[d]	464	5750	nd
#7228	f	16.5	292	5750	nd
#1196	f	7.5	736	5750	nd
#298	m	7	na[e]	nd	1250
#561	m	7.5	na	nd	731
#565	m	8[d]	na	nd	146

[a] Post-immunization (three weeks after last boost).
[b] Not determined.
[c] Maximal dilution.
[d] Estimated; wild-caught animal.
[e] Not applicable.

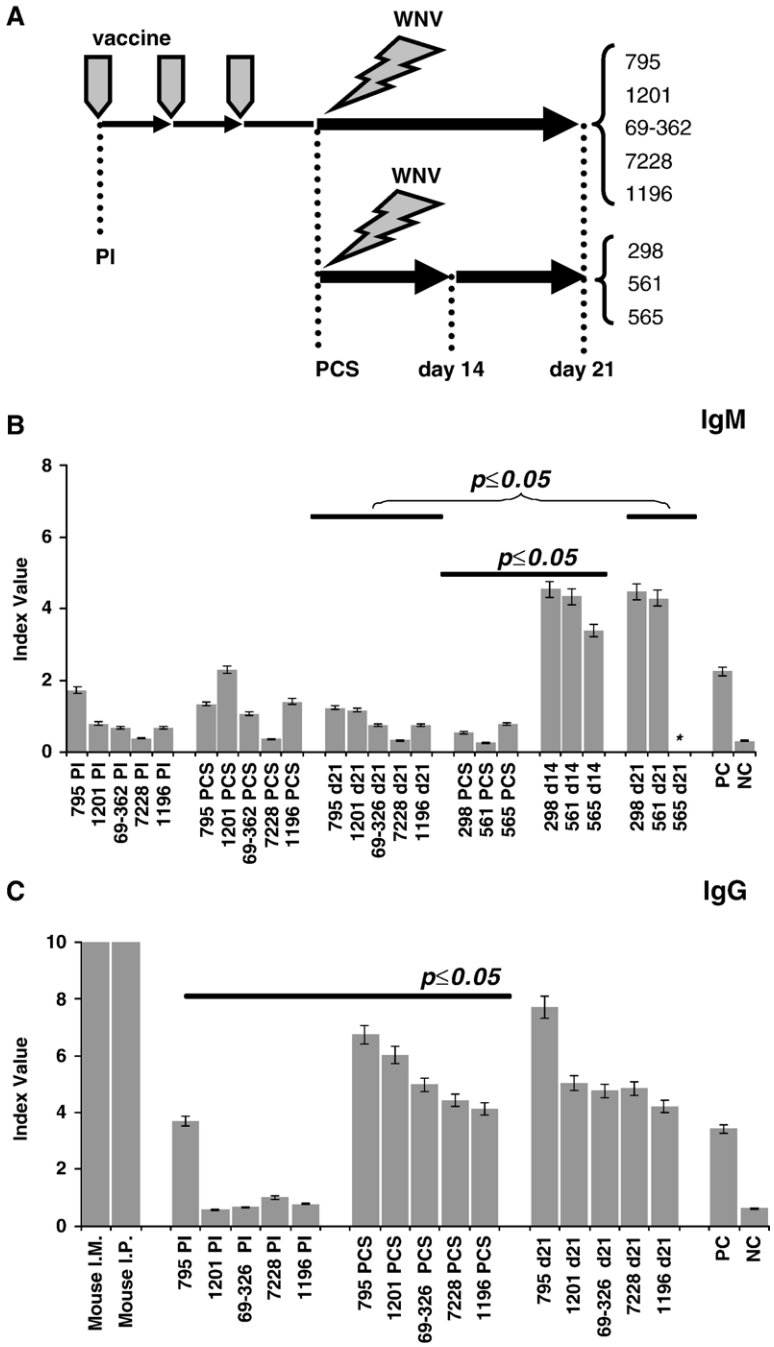

Fig. 3. (A) Outline of the vaccination regimen. (B) IgM response to WNV immunization. (C) IgM response to WNV immunization. PC, NC positive, negative control from Focus Inc. ELISA. Mouse control serum from IM or IP challenge. PI, pre-immunization with fixed WNV vaccine, PCS, pre-challenge, d21 and d14, days 14 and 21 post-challenge with WNV. Index >1 indicates the presence of WNV antibodies. Numbers indicate individual animals.

565), which developed a rigorous IgM response at day 14 and day 21 after WNV infection (Fig. 3A group naive compare PI to d14 and d21, $p \leq 0.05$ by Student's t test), the immunized animals (795, 1201, 69–326, 1196, 7228) did not develop IgM antibodies after WNV infection. Rather, they developed IgG antibodies. Class switching from IgM to IgG due to vaccination at day 21 was significant to $p \leq 0.008$ when comparing vaccinated ($n=5$) to naive animals ($n=3$) (Fig. 3A compare d21 for animals 795, 1201, 69–326, 1196 and 7228 to naive animals 298, 561 and 565). This demonstrated that our prime/boost regimen induced a WNV-specific, IgG-dominated B cell memory response.

Next, we determined WNV plaque reduction neutralizing titers (PRNT). For PRNT, virus was mixed with antiserum at 5-fold dilutions and incubated at room temperature for 60 min prior to plaque assay on Vero cells (Beasley and Barrett, 2002). PRNT were induced by killed WNV vaccine prior to challenge (1:816, $n=5$) similar to live virus infection (1:709, $n=3$), but challenge of immunized animals induced even higher titers (1:5272, $n=5$) at day 11 after WNV infection (Table 1). This is

consistent with B cell memory. By comparison to the lower PRNT titers observed in infected, naive animals, it suggests that even if PRNT titers are not dramatic after vaccination, the host can mount an accelerated recall response to subsequent exposure. We also evaluated peripheral viremia (Fig. 1B) and found it reduced in peak level (5×10^4 at day 6) in 4/5 animals and in duration in 5/5 animals relative to naive WNV-infected baboons. This establishes that killed WNV vaccine induced a protective immune response in baboons.

Discussion

We developed baboons as a novel nonhuman primate model for WNV. Studying WNV infection in nonhuman primates offers unique insights into flavivirus biology and is a prerequisite step for vaccine evaluation in clinical trials (Arroyo et al., 2004; Olberg et al., 2004; Ratterree et al., 2004). Mice represent a wonderful model to uncover the principal components of host–pathogen interactions, but exclusive use of murine models runs the risk of overlooking key aspects of human (primate) immunology (Mestas and Hughes, 2004). For instance, mice lack the IgA-specific FcalphaR1 receptor and presumably use CD71, which binds IgM as well as IgA. Mice also lack homologs to the human FcGammaRIIA and IIC. Even though we do not know how these differences may affect the validity of WNV studies in mice, it is prudent to assume that species differences in immune modulation can lead to susceptibility differences to human pathogens and to vaccine efficacy. Furthermore, primate blood is neutrophil-rich whereas mouse blood is not (50–70% compared to 10–25% of total white cell count, respectively). This may provide a much greater reservoir of innate effector cells, which in primates may contain WNV until the humoral response develops. Hence, limited systemic viremia and high-grade fever rather than death (as in mice) are the normal outcomes of flavivirus infection in macaques, baboons and humans.

Our studies are significant because they are the first to describe the controlled experimental infection of baboons with WNV. WNV-antibody-mediated responses are key to limiting flavivirus infection (Ben-Nathan et al., 2003; Diamond et al., 2003a, 2003b; Engle and Diamond, 2003), and primate-specific properties, such as isotype and repertoire preferences of the antibody response, need to be considered for WNV vaccine design and evaluation. The establishment and maintenance of WNV infection in baboons then allowed us to evaluate the immune response and efficacy of a prototypic human vaccine candidate. Other primate species, such as macaques, can be used to study WNV infection and yield a similar phenotype upon infection (Arroyo et al., 2004; Goverdhan et al., 1992; Olberg et al., 2004; Pletnev et al., 2003; Pogodina et al., 1983; Ratterree et al., 2003, 2004). However, the biomedical community is experiencing an acute shortage of macaques (Health, 2003), as wild macaques can no longer be imported or used, macaques breed in captivity only seasonally, and most *Rhesus* macaques born in captivity are slated for HIV vaccine research. This leaves a significant gap as studies on flavivirus vaccines have progressed rapidly and need to move from the murine to primate models. The baboon is underutilized for infectious disease research and offers an alternative to macaque studies. Baboons have been used since the 1950 for the purpose of transplantation research including baboon-to-human and pig-to-baboon xeno-transplants (Baker, 1995; Kalina et al., 2005; Michaels et al., 2004; Platt, 2001; Starzl et al., 1993), breed easily in captivity year round and have a cost advantage over macaques. With regard to vaccine evaluation against flaviviruses, baboons provide a unique advantage, namely the presence of multiple IgG isotypes, which are missing in macaques (Shearer et al., 1999). Here, we built upon our prior work on baboon models of viral infections (Kalina et al., 2005; Whitby et al., 2003) and the accumulated knowledge about baboon physiology, virology and immunology (Hainsey et al., 1993; Kennedy et al., 1997; Whitby et al., 2003) to establish a novel nonhuman primate model for WNV infection and flavivirus vaccine evaluation.

WNV infection of three baboons yielded uniform peripheral viremia and humoral immune responses that were virtually identical to those found in healthy humans exposed to WNV. During WNV infection of humans, WNV RNA can be detected between day 2 and 12 post-mosquito bite, IgM and IgG develop at days 11 and 12, respectively. During WNV infection of baboons, we detected WNV RNA between day 2 and 6 post-inoculation and IgM and IgG were first detectable at days 10 and 12, respectively. A chemically inactivated WNV vaccine reduced peripheral viremia and induced high titer neutralizing antibodies. Immunized animals developed a faster, IgG-dominated antibody response than naive animals. This suggests that vaccination with chemically inactivated virus accelerates the WNV-specific adaptive immune response and will offer clinical benefits.

Materials and methods

Virus strains

We used two plaque-purified low passage (≤ 5 passages on Vero cells) strains (OK02 and OK03) isolated from blue jay brains during the 2002 and 2003 epidemics (Papin et al., 2004b). Both strains have been sequenced to confirm their identity (Papin and Dittmer, unpublished) and belong to US lineage I (Davis et al., 2005). OK02 and OK03, like NY99 (Beasley et al., 2002), are neuroinvasive and cause 100% mortality in mice after peripheral administration of $\leq 10^3$ pfu per animal (Floyd et al., 2004; Papin et al., 2005).

Plaque assay and plaque reduction neutralization titer (PRNT)

WNV was titered on Vero cells by plaque assay according to standard procedures (Beasley and Barrett, 2002). For PRNT, virus was mixed with antiserum at 5-fold dilutions and incubated at room temperature for 60 min prior to plaque assay.

Viral load assay

We used our previously developed quantitative real-time PCR assay for WNV (Papin et al., 2004a). This assay uses SYBR green as the method of detection and is as sensitive and as specific as TaqMan-based real-time QPCR assays. Viral RNA was isolated from serum using QiAmp Viral RNA (Quiagen Inc.) according to manufacturer's recommendations, reverse-transcribed and real-time QPCR conducted as described (Hilscher et al., 20005; Papin et al., 2004a, 2004b).

ELISA

We used a commercial WNV ELISA (Focus Technologies Inc.) following the manufacturer's protocol. The Focus Inc. IgM ELISA is a sandwich ELISA using rabbit anti-human IgM-coated wells to capture all IgM in serum and WNV antigen and a specific anti-WNV-horseradish-peroxidase (HRP)-conjugated secondary mouse antibody to recognize the complex. We were able to utilize this ELISA since the anti-IgM antiserum recognizes baboon heavy chains as well as human heavy chains. The Focus Inc. IgG ELISA uses WNV-antigen-coated wells and anti-IgG/HRP-conjugated detection. We were able to utilize this ELISA since the anti-IgG antiserum recognizes baboon as well as human IgG. WNV reactivity is expressed as index value relative to a positive and negative control. For IgG, baboon serum, Cut-off Calibrator or controls were added to antigen-coated polystyrene microwells. After incubation, peroxidase-conjugated goat antihuman IgG was added followed by tetramethylbenzidine and hydrogen peroxide substrate reagent and then stop solution. For IgM, baboon serum, Cut-off Calibrator or controls were added to rabbit anti-human IgM-coated polystyrene wells. Following incubation, recombinant West Nile virus antigen was added followed by peroxidase-conjugated mouse monoclonal anti-flavivirus, tetramethylbenzidine and hydrogen peroxide substrate reagent and then stop solution. Wells were read at a wavelength of 450 nM. Results are reported as index values relative to the Cut-off Calibrator. An index value >1 is considered positive.

Animals

Eight baboons (*P. hamadryas anubis*) of both sexes and various ages (Table 1) were single-housed in aluminum cages at the University of Oklahoma Health Sciences Center (OUHSC) Department of Comparative Medicine's animal facility which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures were approved by the OUHSC Institutional Animal Care and Use Committee and are in accordance with the *Guide for the Care and Use of Laboratory Animals* and National Research Council guidelines (Council, 1996). Baboons were fed monkey chow twice daily and given fruit, popcorn, peanuts and other treats once a day. For infection, baboons were sedated with an intramuscular injection of ketamine hydrochloride (approximately 10 mg/kg). Blood was collected from sedated animals using a 20-gauge needle and a 10 ml syringe. Samples were collected prior to each vaccination, prior to inoculation (day 0) and on days 1–14, 21, 28, 42 and 56. Blood was divided into a serum separator tube and a heparin tube and then placed on ice until it was centrifuged. After clotting for about 2 h, the samples were centrifuged for 15 min at high speed. The serum or plasma was decanted and stored at −80 °C. For vaccination, 5 baboons were injected intramuscularly with killed West Nile Virus Vaccine (West Nile—Innovator, Fort Dodge Animal Health, Fort Dodge, Iowa) labeled for horses. Three vaccinations were given at 2-week intervals. For virus inoculation, a small spot was shaved in the middle of the back. The area was cleaned with betadine scrub and then sprayed with alcohol. West Nile virus (OK03) 10^5 pfu in a volume of 0.1 ml was injected intradermally.

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