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Recommended Citation
Humanized Monoclonal Antibody against West Nile Virus Envelope Protein Administered after Neuronal Infection Protects against Lethal Encephalitis in Hamsters

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Humans infected with West Nile virus (WNV) may clinically present with symptoms that are suggestive of neurological infection. Nearly all treatments of WNV disease have been effective in animal models only if administered before or soon after viral challenge. Here, we evaluated whether a potent neutralizing anti-WNV humanized monoclonal antibody (MAb), hE16, could improve the course of disease in a hamster model when administered after the virus had infected neurons in the brain. Five days after viral injection, WNV was detected in the brains of hamsters by cytopathic assay, quantitative reverse-transcription polymerase chain reaction, and immunohistochemical staining of WNV envelope in neurons. Notably, 80%–90% of the hamsters treated 5 days after viral injection by intraperitoneal injection with hE16 survived the disease, compared with 37% of the placebo-treated hamsters (P < .001). The hamsters that received hE16 directly in the brain also exhibited markedly improved survival rates, compared with those in the placebo-treated hamsters. In prospective experiments, hamsters with high levels of infectious WNV in their cerebrospinal fluid were also protected by hE16 when administered 5 days after viral injection. These experiments suggest that humanized MAbs with potent neutralizing activity are a possible treatment for human patients after WNV has infected neurons in the central nervous system.

Many preclinical studies [1–4] of treatment of West Nile virus (WNV) infection have involved intervention before or soon after viral challenge of rodents. Because patients often present with symptoms that suggest possible brain infection [5], the development of treatment for WNV disease, therefore, should include intervention after the virus has entered the brain. Currently, a controlled clinical trial is under way to assess the safety and efficacy of immune human IgG in patients with known or suspected WNV infection (National Institutes of Health identifier NCT00068055). This product, Omr-Ig-Gam, was generated from pools of nonimmune and immune plasma and has relatively low neutralizing activity against the strains of WNV that currently circulate in North America [3, 6]. Because WNV encephalitis alters the permeability of the blood-brain barrier [7, 8], serum immunoglobulins may penetrate the central nervous system (CNS) of infected patients.

The role played by WNV-specific antibody (Ab) in protection against severe disease has been studied in
several rodent models [3, 6, 9–13]. Natural IgM Abs [10] or passively administered WNV-specific Abs [12] prevent WNV disease when administered early during the course of infection. Studies [3, 6, 11] in which WNV-reactive Abs were administered at later times, when WNV could be isolated from homogenized brain tissue, suggests that Ab therapy may be efficacious even after the virus has infected the brain. Humanized monoclonal Abs (MAbs) against WNV have been developed and show therapeutic efficacy in mice even when administered as a single dose several days after viral challenge [9, 14]. Here, we evaluated the efficacy of the humanized MAb hE16 in a second animal species, hamsters, and tested whether therapy improves survival even when administered after the virus has infected neurons in the brain.

MATERIALS AND METHODS

Hamsters and virus. Adult female Syrian golden hamsters >7 weeks of age were obtained from Charles River Laboratories. Animal use was in compliance with the guidelines of the Utah State University Institutional Animal Care and Use Committee was done in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility. Prototype NY99 WNV crow-brain stock (gift of R. Lanciotti, Centers for Disease Control and Prevention) was grown on Prototype NY99 WNV crow-brain stock (gift of R. Lanciotti, of Laboratory Animal Care International–accredited facility. was done in an Association for Assessment and Accreditation State University Institutional Animal Care and Use Committee Animal use was in compliance with the guidelines of the Utah were assayed using a Vero cell cytopathic assay [15], to identify the end point of infection [16]. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to determine the number of WNV-specific RNAs [17, 18]. Fresh tissue, serum, or cerebrospinal fluid (CSF) samples were homogenized in Trizol RNA purification reagent (Sigma-Aldrich Chemical). Linear acrylamide (Ambion) and total normal mouse RNA was added to the homogenates containing serum or CSF before RNA purification. Primer pairs and qRT-PCR algorithms for measurement of WNV RNA and mouse glyceraldehyde phosphate dehydrogenase (mGAPDH) in serum and/or tissues have been described elsewhere [19]. Data are reported as WNV genome equivalents (ge) per milliliter for serum and CSF samples and as WNV transcript equivalents per gram for all other tissues.

Stereotaxic implantation of cannula into the brain ventricle. The procedure used for stereotaxic implantation of cannula into brain ventricles was modified from a published stereotaxic cannulation procedure in rats [20, 21]. With a stereotaxic device (David Kopf Instruments), all measurements for placement of the cannula used the bregma as a reference point. A hole that was 2 mm lateral of midline and even with the bregma in the rostral-caudal plane was drilled for placement of a 4.5-mm cannula into the parenchyma of the cerebrum [22]. An Alzet osmotic pump (Durect) was attached to the cannula and placed sc over the shoulder region of the hamster. Artificial CSF (Alzet; Durect) was used as a vehicle control. An Alzet osmotic pump was loaded with 200 μL of hE6 at a concentration of 25 mg/mL, which delivers 8 μL/h or 5 mg/hamster over a course of 24 h. The hamsters weighed an average of 116 g, so they received an average dose of 43 mg/kg of whole body weight.

Collection of CSF from hamsters. CSF was collected from the cisterna magna of live hamsters in a manner similar to that described elsewhere for rats [23]. Thirty to seventy microliters of CSF was collected per hamster. Red blood cells (RBCs) were counted on a hemocytometer to determine the extent of blood contamination.

Immunohistochemical analysis. Infected and uninfected hamsters were administered ketamine and xylazine and then perfused directly with PBS and 4% paraformaldehyde after cardiac puncture. After fixation overnight, the sections were deparafinized and rehydrated by standard histological procedures with xylene-ethanol, 95% ethanol, 70% ethanol, and distilled water. The sections were filled with DakoCytomation Target Retrieval Solution (DakoCytomation) diluted 1:10 in distilled water and were boiled in a microwave for 4 cycles of 1 min in a Coplin jar or boiled at 125°C for 4 min in a decloaking chamber (Biocare Medical). Sections were permeabilized with 0.5% Triton X-100 in PBS for 5 min and were blocked using 10% normal goat serum in 0.2% Triton X-100 in PBS blocking solution. Slides were incubated with primary MAb (7H2 or 5H10; 1:200) against WNV (BioReliance; Invitrogen Bioservices), polyclonal anti-calbindin D28K (1:500; Sigma-Aldrich), and polyclonal anti-neuron specific enolase (NSE; 1:20; Chemicon) in dilution fluid containing 5% normal goat serum, 0.2% Triton X-100 in PBS for 2 h. After washing with PBS, Alexa Fluor 568 goat anti–mouse IgG secondary Ab, Alexa Fluor 488 goat anti–mouse IgG secondary Ab, and Alexa Fluor 488 goat anti–rabbit IgG secondary Ab (1:200; Molecular Probes) were diluted and incubated for 2 h. The slides were washed and mounted with VECTASHEILD mounting medium (Vector Laboratories). Stained slides were visualized using a Nikon Eclipse TE300 microscope (Nikon) with an attached Lambda DG4 (Sutter Instrument Company) and a Bio–Rad MRC 1024 confocal microscope (Bio–Rad). Captured images were processed using Confocal Assistant software (version 4.02; Bio–Rad), and
Figure 1. Therapeutic effect of hE16 monoclonal antibody (MAb) in West Nile virus (WNV)–infected hamsters. A, Intraperitoneal (ip) administration of 60 mg/kg hE16 (n = 20), 60 mg/kg palivizumab (negative control Ab; n = 20), or sterile saline (n = 20) 2 days after viral injection. B, ip administration of 100 mg/kg hE16 (n = 30) or sterile saline (n = 30) 5 days after viral injection. C, Brain cannulation of 48 mg/kg hE16 (n = 8) or sterile saline (n = 9) 5 days after viral injection.
the plates were made using Adobe Photoshop (version 6.0). The control and experimental images were collected and processed using the same instrument settings.

For immunoperoxidase localization of anti–human IgG, tissue sections were processed in a fashion similar to the immunofluorescence protocol up to the antigen-retrieval step. Subsequently, by use of the Ventana NexES IHC–Full System (Ventana Medical Systems), the sections were stained for polyclonal anti–human IgG Fc-specific alkaline phosphatase–conjugated Ab (Sigma-Aldrich), and detection was done using the Ventana Basic AEC Detection Kit (Ventana Medical Systems), the sections were stained for poly

Statistical analysis. Survival data were analyzed using the Wilcoxon log rank survival analysis, and other data were evaluated by 1-way analysis of variance (JMP Statistical Discovery software; version 6.0; SAS Institute).

RESULTS

Efficacy of hE16 2 days after viral injection. Previous experiments have demonstrated that the mouse MAb E16 protects against lethal WNV infection in mice even when administered as a single dose (~100 mg/kg) 5 days after infection [9]. To evaluate whether the humanized version of this MAb, hE16, has therapeutic activity in a second animal species, we tested its efficacy in hamsters. Administration of a single dose of hE16 (60 mg/kg) 2 days after viral injection protected 95% of hamsters (19/20) up to 21 days after the injection (figure 1A), whereas only 20% and 35% of hamsters survived after treatment with a control MAb and saline, respectively (P < .001). Hamsters treated with hE16 gained weight at essentially the same rate as did the uninfected control hamsters, whereas only 20% and 35% of hamsters survived after treatment with a control MAb and saline, respectively (P < .001).

The effect of hE16 on viral burden was also evaluated (table 2). Administration of hE16 2 days after viral infection effectively reduced titers in brains and spleens to below the limits of detection as measured 7 days after viral infection. No infectious virus was detected in the kidneys or brains of hE16-treated hamsters, although qRT-PCR did detect low levels of viral RNA in the spleens of 2 of 5 hamsters.

Detection of WNV in CNS tissues 5 days after viral injection. To evaluate when WNV disseminated into CNS tissues in hamsters, we harvested the cerebellum, cerebrum, midbrain with brain stem, cervical spinal cord, and thoracic and lumbosacral spinal cord 2, 5, and 6 days after viral infection and assayed for viral RNA using qRT-PCR. Two days after viral injection, viral RNA in neurological tissues were at or below the levels of detection (figure 2A). However, days 5 and 6 after infection, viral RNA was present in CNS tissues at levels that were at least 3–4 logs higher than those 2 days after viral infection. The virus identified 5 and 6 days after viral injection in CNS tissues was
likely not of intravascular origin, because serum samples from the same hamsters had levels of viral RNA that were >2 logs lower than those observed in neuronal tissue (figure 2B).

Because detection of infectious WNV in homogenized brain could be the result of infection of nonneuronal cells (i.e., infiltrating macrophages, microglia, or glia), immunohistochemical analysis was performed on neurological tissues. Thin sections were immunostained for WNV envelope antigen, neuron-specific enolase (CNS neuron specific), and calbindin (Purkinje neuron specific) [24, 25]. As expected, staining for WNV antigen in CNS tissues from uninfected hamsters was near background levels (figure 3A, 3D, 3G, and 3J). In contrast, WNV antigen was readily detectable in kidney tissues from infected hamsters 3 and 5 days after viral injection (figure 3N–3O); these results are consistent with the findings of previously published studies [4, 26]. WNV-specific antigen staining was identified in several different CNS tissues 5 days after viral injection. Many of the WNV antigen–positive cells were clearly stained with NSE (figure 3A–3C, arrows). Similarly, neurons in the cervical spinal cord (figure 3G–3I) and midbrain/brain stem (figure 3J–3L) also were infected with WNV. In the cerebellum, WNV colocalized with calbindin expression, a marker of Purkinje neurons (figure 3D–3F); these results also are consistent with the findings of previous experiments in mice [27]. WNV antigen expression did not colocalize with glial fibrillary acidic protein staining of astroglial cells 5 days after viral injection (data not shown), suggesting that the tropism of WNV in the CNS was restricted primarily to neurons.

**Efficacy of hE16 5 days after viral injection.** Given that hamsters had pathologic evidence of an active CNS infection 5 days after infection, we investigated whether hE16 could ameliorate WNV disease if the Ab was administered to hamsters at this time point. Administration of hE16 (100 mg/kg) 5 days after infection as a single intraperitoneal (ip) dose markedly improved the survival of hamsters ($P \leq .001$) (figure 1B). Eighty percent of the hamsters treated with hE16 survived up to 21 days after viral injection, compared with 37% survival in the saline-treated group. Correspondingly, hamsters receiving hE16 also showed improved weight gain ($P \leq .05$) (table 1, experiment B).

To assess the effect of therapeutic Ab intervention on viral burden in tissues, hamsters were infected with WNV and treated 5 days after viral injection with either hE16 or saline, and WNV RNA was measured by qRT-PCR on day 8 in the cerebellum, cerebrum, brain stem, spinal cord, and kidney (table 2). WNV RNA was detected in all tissues from hE16-treated hamsters by qRT-PCR, but the tissue RNA levels in the saline-treated hamsters were >1 log higher. Correspondingly, hE16 treatment also reduced WNV antigen detection by immunohistochemical analysis in the brain. When the cerebral cortex

<table>
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<tr>
<th>Assay, tissue</th>
<th>Treated at 2 and assayed at 7 days after viral injection</th>
<th>Treated at 5 and assayed at 8 days after viral injection</th>
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<tbody>
<tr>
<td>qRT-PCR</td>
<td>hE16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>hE16&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Whole brain&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.9&lt;sup&gt;d&lt;/sup&gt; (0/5)</td>
<td>7.0 ± 0.5 (5/5)</td>
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<td>Cerebellum</td>
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<td>Midbrain, stem</td>
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<td>Spleen</td>
<td>5.9&lt;sup&gt;d&lt;/sup&gt; (2/5)</td>
<td>9.3 ± 0.8 (4/4)</td>
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<tr>
<td>Kidney</td>
<td>5.9&lt;sup&gt;d&lt;/sup&gt; (0/5)</td>
<td>6.5 ± 0.3 (5/5)</td>
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**Cell culture**

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<th>Assay, tissue</th>
<th>Treated at 2 and assayed at 7 days after viral injection</th>
<th>Treated at 5 and assayed at 8 days after viral injection</th>
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<tr>
<td>qRT-PCR</td>
<td>hE16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>hE16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain</td>
<td>&lt;3&lt;sup&gt;f&lt;/sup&gt; (0/5)</td>
<td>4.1 ± 1.0 (4/5)</td>
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<tr>
<td>Kidney</td>
<td>&lt;3&lt;sup&gt;f&lt;/sup&gt; (0/5)</td>
<td>5.7 ± 0.5 (5/5)</td>
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**NOTE.** For quantitative reverse-transcription polymerase chain reaction (qRT-PCR), data are mean ± SD log WNV transcript equivalents per gram of tissue (no. positive/total no.); for cell culture, data are mean ± SD log TCID<sub>50</sub> per gram of tissue (no. positive/total no.). Limits of detection were determined by calculating 2 SDs above the mean for all uninfected samples for all tissues from hamsters treated either on day 2 or on day 5; the limit of detection was <log 5.9 for the qRT-PCR assay and <log 3 for cell culture. P values are for comparisons with the saline-treated group by the chi<sup>2</sup> test, Wilcoxon rank sum analyses, or Student’s t test.

<sup>a</sup> Single ip injection at 60 mg/kg.
<sup>b</sup> Single ip injection at 100 mg/kg.
<sup>c</sup> Whole brain consisted of homogenized whole brain and brain stem.
<sup>d</sup> P < .01.
<sup>e</sup> P < .05.
<sup>f</sup> P < .001.
was stained for WNV antigen in hamsters treated 5 days after viral injection and assayed 7 days after viral injection, markedly reduced staining was evident in the hE16-treated hamsters (figure 3P and 3Q).

**Efficacy of hE16 administered directed into the brain 5 days after viral injection.** The experiments above establish that peripheral injection of hE16 effectively controls WNV infection and prevents mortality even after virus has disseminated to neurons in the CNS. To test the hypothesis that hE16 in the brain correlated with its ability to control WNV mortality, we administered Ab directly into the cerebrum of WNV-infected hamsters 5 days after viral injection by stereotaxic surgery with an Alzet pump. hE16 delivered directly into the brain was detected by immunohistochemical analysis in 2 uninfected hamsters 3 days after surgery. Intense staining of hE16 was observed in the cerebral cortex at the cannulation site, and lower levels were seen in the cerebellum and brain stem (data not shown). Thus, this delivery system facilitated broad delivery of Ab into the brain, with the highest levels near the site of cannulation. Importantly, delivery of hE16 directly into the brain 5 days after viral injection \( (P \leq 0.01) \) improved the survival of hamsters (figure 1C), with 88% percent of the hamsters treated with hE16 surviving, compared with 22% survival in the saline-treated group (table 1, experiment C).

**Efficacy of hE16 in individual hamsters, using WNV in the CSF as an antemortem marker for CNS infection.** In human patients, it may be difficult to define the stage of WNV infection at clinical presentation by nonneuroinvasive means. One possible marker of disseminated CNS infection may be the presence of viral RNA in the CSF. To address whether hE16 retained therapeutic efficacy in animals after CSF samples had become positive for WNV, an additional prospective study was performed. CSF and serum samples were collected 2, 5, or 6 days after viral injection and were assayed for WNV RNA by quantitative reverse-transcription polymerase chain reaction. A, Cerebellum, cerebrum, brain stem with midbrain, cervical spinal cord, and the remainder of the thoracic with the lumbosacral spinal cord. B, CSF and serum.

To confirm that the WNV in the CSF did not come from viremic blood, RBCs were quantified to rule out possible blood contamination of CSF. RBC levels in the CSF ranged from 2 to 9000 cells/mm\(^3\) (data not shown), levels that are low given that the average RBC count in the rodents is \( 1 \times 10^7 \) cells/mm\(^3\) (Mouse Phenome Database; Jackson Laboratories). If viremic contamination had contributed to the WNV titer in the CSF, then the WNV RNA:RBC ratio in serum should have correlated with the WNV titer in CSF; in fact, there was no such correlation (data not shown). Finally, theoretical WNV titers in CSF contributed from blood contamination were calculated using the numbers of RBCs in the CSF and serum and the WNV titer in serum. Because the actual WNV titers 5 and 6 days after viral injection (mean \( \pm \) SD, log \( 6.1 \pm 1.2 \) ge/mL of CSF) were many logs higher, we conclude that the CSF WNV RNA levels did not result from contaminating blood via traumatic entry into the CSF compartment.
Figure 3. Confocal microscopy showing, 5 days after viral injection, staining of West Nile virus (WNV) envelope (red) with neuron-specific enolase (NSE) (green) in the cerebral cortex (A–C), with calbindin (green) in the cerebellum as a marker of Purkinje cells [24, 25] (D–F), with NSE in the cervical spinal cord (G–I), and with NSE in the midbrain/brain stem (J–L). Also shown is WNV envelope staining in the kidneys 3 and 5 days after viral injection as a positive tissue control (M–O), and WNV envelope staining 7 days after viral injection in the brains of WNV-infected hamsters treated intraperitoneally 5 days after the injection with 100 mg/kg hE16 (P) or placebo (Q). Arrows indicate cells that are stained with markers of neurons (NSE or calbindin) (green) and WNV envelope (red). The scale bar is 20 μm.
WNV RNA in the CSF was then used as an antemortem marker to verify that the individual surviving hE16-treated hamsters were actually infected in their brains 5 days after viral injection. After collection of CSF 5 days after injection, hamsters were treated ip with 32 mg/kg hE16 and were monitored for survival. As expected, hE16 at 32 mg/kg significantly improved overall survival (90% vs. 40%). Importantly, individual hamsters with viral RNA in their CSF were protected from death by treatment with hE16 5 days after viral injection. Three of the 4 hamsters that had the highest levels of WNV RNA (>5 log ge/mL) in their CSF survived after receiving hE16. Similarly, hamsters with low WNV RNA levels in their CSF also were protected. One hamster of 10 having a titer higher than 5.2 log ge/mL died, but death was delayed out to 21 days after viral injection. Collectively, our data suggest that hamsters with active CNS infection, as determined by the presence of WNV RNA in CSF, were protected from death by ip treatment with hE16.

**DISCUSSION**

Previous studies have demonstrated that, in rodent models, WNV-reactive Ab reduces mortality especially when administered before or soon after viral challenge [1–4]. Other studies of WNV-reactive Abs in mice have shown effective treatment even 5 days after viral injection [3, 6, 11], a time at which infectious virus is identified in homogenized mouse brain. These studies suggested that WNV-reactive Ab is effective even when administered after CNS infection. The possibility still existed, however, that neurons were not yet infected 5 days after viral injection and that nonneuronal tissue accounted for the titers in the homogenized brains. In the present study, we have established that hE16 reduces WNV-induced mortality even when administered after productive viral infection of neurons. Our conclusion is based on several findings: (1) WNV RNA was detected in homogenized hamster brains 5 days after infection when animals were treated with hE16; (2) WNV antigen staining was detected at several independent sites in the brain and spinal cord 5 days after infection; (3) WNV antigen staining colocalized with cells of neuronal origin; (4) WNV RNA was present in high levels in the CSF 5 days after infection; (5) levels of viral RNA in neurological tissues decreased between days 5 and 7 after administration of hE16; and (6) direct administration of hE16 into the brain 5 days after infection resulted in the improvement of clinical outcome.

Protection by hE16 via a peripheral route 5 days after injection suggests that hE16 enters the brain and directly limits viral spread or replication. hE16 may penetrate into the brain to neutralize virus, because of an altered blood–brain barrier associated with encephalitis. In mice, WNV infection induces cytokines, such as tumor necrosis factor–α, that can alter the permeability of the blood–brain barrier [7]. The precise mechanism by which hE16 enters the brain remains to be determined.

The ability to collect CSF from hamsters during WNV infection provided an opportunity to investigate its prognostic value for different stages of disease and for identifying times for therapeutic intervention. The presence of viral RNA before the appearance of native WNV Ab in CSF may suggest an important time for clinical intervention, such as with passive, WNV-specific neutralizing Ab [28]. Conversely, the detection of high titers of native neutralizing WNV Ab in CSF could signal a point in the course of the disease when the efficacy of passive Ab treatment may be diminished. WNV RNA has been detected in human patients [29, 30], and WNV-specific IgM in CSF has been used as a marker of CNS infection with WNV [28, 31]. By combining WNV RNA levels in CSF with other markers of neurological disease [32, 33], better prognostic information may be obtained as to the probable outcome. The ability to collect CSF from WNV-infected rodents should also allow pharmacokinetic studies to define drug distribution in neurological tissues. Indeed, in future studies, we plan to compare the levels of hE16 in CSF achieved after peripheral administration with clinical outcome.

The efficacy of hE16 therapy after WNV has entered the CNS in 2 independent species, hamster and mouse, increases the plausibility that hE16 treatment may be applied to other species, including humans. For WNV, the infection and treatment of 2 species of laboratory animals is especially important from a preclinical evaluation standpoint, because most nonhuman primates develop low levels of viremia without evidence of disease [34] or develop fatal neurological disease only after intracranial inoculation [35]. Similarly, other mammalian species (e.g., horses, cats, and dogs) develop low levels of viremia after WNV infection, but clinical signs of disease develop in only a small percentage of infected animals (such as 1 of 80 mosquito-infected horses) [36–39], which can increase the logistical difficulties of therapeutic studies. Nonetheless, there are differences between these 2 rodent models and human disease. The time course of CNS infection in the mouse and hamster models appears to be accelerated. In particular, WNV infection in humans is characterized by an asymptomatic incubation period of variable length that follows the viremic phase and the onset of neurological symptoms [28, 31], whereas the asymptomatic period in rodents appears to be shorter. Additionally, the mortality rate observed in humans is substantially less than that observed in rodents.

In summary, the present study establishes the utility of hE16 for treatment of WNV infection that has advanced to the CNS in a second animal species, the hamster. We show that detection of WNV RNA in CSF is an antemortem marker of CNS infection and is possibly an indication for therapeutic intervention. In addition, we demonstrate the utility of convexion-
enhanced delivery of drugs into the brains of hamsters to elucidate mechanisms for drug efficacy. Overall, these findings suggest that further study of hE16 as possible prophylaxis against or therapy for WNV infection in humans is warranted.

Acknowledgments

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