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A potent anti-West Nile virus (anti-WNV)-neutralizing humanized monoclonal antibody, hE16, was previously shown to improve the survival of WNV-infected hamsters when it was administered intraperitoneally (i.p.), even after the virus had infected neurons in the brain. In this study, we evaluated the therapeutic limit of hE16 for the treatment of WNV infection in hamsters by comparing single-dose peripheral (i.p.) therapy with direct administration into the pons through a convection-enhanced delivery (CED) system. At day 5 after infection, treatments with hE16 by the peripheral and the CED routes were equally effective at reducing morbidity and mortality. In contrast, at day 6 only the treatment by the CED route protected the hamsters from lethal infection. These experiments suggest that hE16 can directly control WNV infection in the central nervous system. In support of this, hE16 administered i.p. was detected in a time-dependent manner in the serum, cerebrospinal fluid (CSF), cerebral cortex, brain stem, and spinal cord in CSF. A linear relationship between the hE16 dose and the concentration in serum was observed, and maximal therapeutic activity occurred at doses of 0.32 mg/kg of body weight or higher, which produced serum hE16 concentrations of 1.3 μg/ml or higher. Overall, these data suggest that in hamsters hE16 can ameliorate neurological disease after significant viral replication has occurred, although there is a time window that limits therapeutic efficacy.

Since patients infected with West Nile virus (WNV) often present for medical attention with symptoms that suggest possible central nervous system (CNS) infection (9), therapies for WNV neurological disease should work even after the virus has infected the CNS. One possible therapy, immune immunoglobulin G (IgG), is being evaluated in a phase IIIB clinical trial (NIH identifier NCT00068055) that assesses safety and efficacy in patients with known or suspected WNV infection. However, the product (Omr-Ig-Gam) was generated from pools of nonimmune and immune serum from Israeli donors and has a relatively low neutralizing activity against the strains of WNV that currently circulate in North America (2, 6).

A mouse monoclonal antibody (MAb), E16, specific for domain III (DIII) of the envelope protein, has been identified to have potent WNV-neutralizing activity (7, 19, 20). This MAb engaged 16 residues positioned on four loops of DIII and has potent WNV-neutralizing activity (7, 19, 20). This MAb has retained its antigen specificity, avidity, and neutralizing activity when infectious virus was identified in homogenized mouse brain.

Studies with a hamster model of WNV infection subsequently confirmed that hE16 is effective after the virus had infected neurons in the CNS (16). This conclusion was based on the observation that WNV RNA and WNV antigen-positive neurons were present in the brain when hE16 was administered intraperitoneally (i.p.) at 5 days after infection. Moreover, individual hamsters with WNV in their cerebrospinal fluid (CSF) at 5 days postinfection (dpi) were protected from death by hE16 treatment on that day. The goals of the current study were (i) to determine how long hE16 systemic or intracerebral treatment could be delayed without losing efficacy, (ii) to define the effective dose limit of hE16, (iii) to measure the serum and CSF concentrations of hE16 at various time points after administration, (iv) to assess the concentration of hE16 in homogenized neurological tissues, and (v) to establish the timing of hE16 treatment in relation to the endogenous production of WNV-neutralizing antibody in the serum and CSF. Our studies suggest that hE16 in the CNS ameliorates neurological disease after significant viral replication has occurred. In the hamster, a survival benefit is gained up through day 6 after infection.

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MATERIALS AND METHODS

Animals and virus. Adult female Syrian golden hamsters (Charles River Laboratories) greater than 7 weeks of age were used. The animals were randomized to treatment groups. Animal use was in compliance with the Utah State University Institutional Animal Care and Use Committee, and the animals were kept in an AAALAC-accredited facility. Prototypic WNV strain NY’99 (11, 12) was propagated in MA-104 cells and diluted appropriately in minimal essential medium immediately prior to injection.

Antibody. The humanized MAb (IgG1) specific for WNV (MAb hE16) (16, 19) was obtained from MacroGenics, Inc. (Rockville, MD). Upon arrival, the material was immediately stored in a refrigerator. Palivizumab (Synagis; MedImmune, Gaithersburg, MD), a humanized IgG1 MAb used to prevent respiratory syncytial virus disease in at-risk infants, was used as a control.

Collection of CSF from hamsters. CSF was collected from the cisterna magna of live hamsters (16). The animals were anesthetized with ketamine HCl and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA) with the neck maximally flexed to fully expose the atlanto-occipital fossa. Anesthesia was maintained through the remainder of the procedure by the use of isoflurane inhalation (2% isoflurane, 1 liter O2/min). A 5- to 7-mm incision was made from the shoulders to the dorsal aspect of the skull. CSF was collected with a 30-gauge needle attached to one end of a short length of Tygon microbore tubing (diameter, 0.01 in.; Saint-Gobain Corporation, Valley Forge, PA) and with a syringe attached to the other end. The needle in the arm of the stereotaxic device was inserted into the neck 4 mm ventral of the crest of the skull and on the midline. With the needle tip being just above the syringe, the needle was slowly advanced until fluid was observed to be entering the tube. CSF (30 to 70 µl) was collected from each animal. Red blood cells were counted to determine the extent of blood contamination and to eliminate contaminated CSF from the data set.

Implantation of cannula into the pons. Anesthesia was induced by the i.p. administration of ketamine-HCl at a dose of 100 mg/kg body weight and was maintained throughout the duration of the procedure by using isoflurane inhalation anesthesia (2% isoflurane, 1 liter O2/min). A 5- to 7-mm incision was made from the shoulders to the dorsal aspect of the skull. CSF was collected with a 30-gauge needle attached to one end of a short length of Tygon microbore tubing (inner diameter, 0.01 in.; Saint-Gobain Corporation, Valley Forge, PA) and with a syringe attached to the other end. The needle in the arm of the stereotaxic device was inserted into the neck 4 mm ventral of the crest of the skull and on the midline. With the needle tip being just above the syringe, the needle was slowly advanced until fluid was observed to be entering the tube. CSF (30 to 70 µl) was collected from each animal. Red blood cells were counted to determine the extent of blood contamination and to eliminate contaminated CSF from the data set.

Viral assays. Quantitative reverse transcriptase PCR (RT-PCR) was used to determine the number of WNV RNA genomes present in the fluid extracted from cells, as reported previously (10, 16, 25). Serum or CSF was added to TRizol RNA purification reagent (Sigma-Aldrich Chemical), and linear acrylamide (10 to 20 ng; Ambion) and total normal mouse RNA (10 ng) were added as carriers. The RNA was purified with the TRizol reagent (Invitrogen) and used in the RT-PCRs. Primer pair TCACTTACTTCTCCACAAAG (forward primer) and GGGCTCGACAGTGTGATG (reverse primer) and the TaqMan probe (5′–6-carboxyfluorescein–TCCGGACATGGAGAACCTC–6′–carboxy-N,N′,N′,N′-tetramethylrhodamine–3′) (QIAGEN, Valencia, CA) were used. Mouse glyceraldehyde phosphate dehydrogenase (mgAPDH) was detected by using primer pair GTTACCAGGGCTGCCTTCTC (forward primer) and GGGTTCCTCTATGAC (reverse primer) and the TaqMan probe (5′–hexachlorofluorescein–AAGCGGACATCGAGTCGAG–BHQ-5′) (26). The one-step FullVelocity quantitative RT-PCR master mix (Stratagene, La Jolla, CA) was used for real-time amplification of mgAPDH and mGAPDH RNA in a duplex reaction. Samples were run on a DNA Engine Opticon 2 apparatus (MJ Research Inc, Waltham, MA). REVERSE transcription of the RNA was performed for 30 min at 50°C, followed by PCR, which consisted of 30 cycles of 15 s at 95°C and 60 s at 60°C. Control WNV RNA transcripts were obtained from an in vitro transcription reaction of the cloned PCR product in a linearized plasmid. The mean threshold cycle value for each sample was used to obtain the WNV RNA values by using a standard curve. The data are reported as WNV genome equivalents/ml for serum or CSF samples and WNV transcript equivalents/gram for all other tissues.

WNV plaque assay. The neutralizing activities of serum or CSF antibodies were determined by a plaque reduction assay (13). For infection at 56°C, serial dilutions of the serum or CSF samples were mixed and incubated with stock WNV (100 PFU). A volume of 50 µl was added to each well of a six-well plate, and the plate was incubated for 1 h with periodic rocking. A 2-ml agarose overlay was added. Four days later, the cells were stained with neutral red vital stain and the plaques were counted. The inverse of the dilution that caused a 90% reduction was reported as the plaque reduction neutralization titer. The titer of infectious virus was similarly determined, except that no serum was added to the sample. Serial dilutions of stock virus in the assay were used to calculate the numbers of PFU per gram of tissue or ml of serum or CSF.

Detection of MAb hE16. The MAb hE16 concentrations in hamster serum or CSF were determined by enzyme-linked immunosorbsent assay (16). Dilutions of serum were incubated in wells coated with 100 ng goat anti-human IgG (Fc specific; Jackson ImmunoResearch Laboratories). After incubation and washing of the plates, bound hE16 was detected with alkaline phosphatase-conjugated goat anti-human kappa chain (dilution, 1/10,000; Jackson) and then developed by using the fluorogenic substrate 4-methylumbelliferyl phosphate (Sigma). The plates were then read with a fluorometric plate reader, and the concentrations were calculated from a standard curve by using a nonlinear four-parameter fit. The limit of detection of hE16 in serum or CSF was 0.06 µg/ml.

Statistical analysis. Survival data were analyzed by log rank survival analysis (SP software, the Statistical Discovery software; SAS Institute, Inc.).

RESULTS

Treatment delay limitations. To determine how long hE16 treatment could be delayed without losing efficacy, hamsters that had been challenged s.c. with WNV were treated with MAb hE16, which was delivered either by i.p. injection or by use of a convection-enhanced delivery (CED) system (Fig. 1). As observed previously (16), hE16 is effective in preventing death when a single 32-mg/kg dose is administered i.p. at 5 dpi, a time when WNV had already infected CNS neurons (P < 0.01) (Fig. 1D). Efficacy was lost if the administration of hE16 by i.p. injection was delayed until 6 dpi (Fig. 1E), at which time the survival curves were essentially the same as those for vehicle-treated animals. Likewise, hE16 administration by the CED route was effective when treatment was initiated at 5 dpi (P < 0.01). In contrast, treatment by the CED route was also effective if it was initiated at 6 dpi (P ≤ 0.01) (Fig. 1A and B). However, treatment with hE16 by the CED route was not effective if it was initiated at 8 dpi (Fig. 1C).

Timing of hE16 treatment with virus infection and production of antibody. To better understand the relationship between the emergence of endogenously produced WNV-specific antibody, virus infection, and the timing of effective hE16 treatment, neutralizing antibody and WNV titers were measured in the serum and CSF of untreated, WNV-infected hamsters over time. Infectious virus was detected in serum by viral plaque assay beginning at day 2, and the virus levels decreased thereafter through day 5; infectious virus was no longer detected at day 6 after infection (Fig. 2A). On the basis of the findings from studies with mice (5), we postulated that virus reductions in serum should coincide with the appearance and accumulation of WNV-neutralizing antibody. Indeed, beginning on day 4 (Fig. 2B), we detected significant amounts of neutralizing antibody in the sera of some hamsters. Infectious virus was detected in the CSF on days 4 and 5 after infection (Fig. 2C), and WNV RNA was observed between days 3 and 6.
Presumably, since RT-PCR detects both viable and nonviable virus and the plaque assay detects viable virus, the virus was more detectable by the RT-PCR assay. The levels of infectious virus or viral RNA in the CSF declined significantly on day 6. Overall, there was an \( \frac{1}{2} \)-day lag in the appearance of WNV in the CSF compared to the time of appearance of WNV in serum. The decline in WNV levels in the CSF also correlated with the appearance of detectable levels of neutralizing antibody in the CSF (Fig. 2E).

**Effective dose and serum concentrations of MAb hE16.** To determine an effective dose for MAb hE16 treatment at 5 days after infection, a dose-response analysis was performed. Serial dilutions of hE16 (100 to 0.32 mg/kg) were administered to the hamsters i.p. at 5 days after infection and survival was monitored. The maximal percent survival (80%) occurred with a dose of 3.2, 10, or 32 mg/kg; the rate of survival was 67% with a dose of 0.32 or 1 mg/kg, and the rate of survival was 33% for the saline control group (data not shown).

Since significant protection was still observed at the lowest dose tested (0.32 mg/kg), the experiment was repeated with doses as low as 0.010 mg/kg. The percent survival was plotted against the hE16 dose (Fig. 3A). The maximal percent survival (62.5%) was observed at doses of 0.32, 1.0, and 100 mg/kg. The percent survival for the saline control group was 0%. By using linear regression to fit an equation to a line, half of the maximum effective dose (MED\(_{50}\)) of hE16 was calculated to be 0.13 mg/kg (Fig. 3A). The serum hE16 levels measured 1 day after i.p. injection correlated linearly (\( R^2 = 0.99 \)) with the hE16 dose administered (Fig. 3B). On the basis of this result, the half-maximal effective concentration (MEC\(_{50}\)) of hE16 in serum is 0.44 \( \mu \)g/ml. A plot of the percent survival against the serum hE16 concentration demonstrates that maximal therapeutic efficacy oc-
curs with serum hE16 concentrations 1.2 μg/ml or higher (Fig. 3C).

Serum and CSF MAb hE16 concentrations over time. To better understand the pharmacokinetics of antibody accumulation and/or consumption, we measured the concentrations of MAb hE16 in the serum and CSF of WNV-infected or sham-infected hamsters at 7, 12, and 30 dpi after treatment with a single i.p. injection of hE16 (32 mg/kg) at 5 dpi. Interestingly, although the concentrations of hE16 in serum (Fig. 4A) and CSF (Fig. 4B) declined over time, there was no statistically significant difference between the results for the WNV- and sham-infected hamsters. Even at day 30 postinfection, hE16 was detectable in all 15 serum samples (concentration range, 13.4 to 35.9 μg/ml) and in 7 of 15 CSF samples (concentration range, 0.07 to 0.15 μg/ml). However, hE16 penetration into the CSF was relatively poor, as the mean concentrations of hE16 in serum were 330- to 630-fold higher than those in CSF at each of the three time points at which they were tested. Nonetheless, because the concentrations of hE16 in serum and CSF were not significantly different between uninfected and infected animals, the majority of the antiviral antibody remains circulating and is not sequestered by phagocytic cells as virus-immune complexes.

MAb hE16 concentrations in neurological tissues. We assessed the MAb hE16 levels in tissue homogenates to determine if the levels in serum were a reliable indicator of the hE16 concentration in other relevant CNS tissues (Fig. 5). In hamsters treated with 32 mg/kg hE16 i.p. at 5 dpi, the hE16 concentrations in serum at 7 dpi correlated with the hE16 levels in CNS tissues and CSF at 7 dpi. Data from the WNV-infected and sham-infected hamsters were combined, because there were no statistically significant differences in the tissue hE16 concentrations between the groups (data not shown). There was a generally linear trend between increased serum and tissue hE16 levels, as the R² values between the levels in serum and the levels in CSF, cerebral cortex, brain stem, and thoracic/lumbar spinal cord were 0.47, 0.45, 0.57, and 0.17, respectively. Thus, with the exception of the thoracic/lumbar spinal cord, the serum concentrations of hE16 were indicators of other neurological tissue concentrations.

DISCUSSION

As established previously (16), MAb hE16 administered i.p. as a single injection at 5 dpi improved the survival of hamsters infected s.c. with WNV. In this study, we demonstrated that when hE16 treatment i.p. was delayed to day 6, it was no longer effective at reducing mortality. In contrast, if hE16 was directly administered into the pons of the brain by the CED route at day 6, it enhanced survival. CED administration was not performed with the anticipation that it might be eventually used in human patients. It was designed to show that hE16, in part, acts directly in the CNS to prevent neuronal infection and injury.

Our pharmacokinetic analyses demonstrate that the CSF MAb hE16 concentrations were approximately 500-fold lower than the serum hE16 concentrations. Similarly low CSF concentration/serum concentration ratios have been observed for other peripherally administered MAbs in mice, rats, and humans (1, 8, 14, 21, 23, 24). Despite encephalitis and CNS inflammation in the hamsters, only a small fraction of the peripherally administered hE16 efficiently crosses the blood-brain barrier and inhibits and/or controls infection. The CED route of administration, however, enhanced the hE16 levels in the brain and, accordingly, extended the therapeutic window by at least 1 day. Nonetheless, the delivery of hE16 directly into the brain by the CED route at day 8 after WNV infection did not reverse the course of disease, as no survival benefit was observed. We speculate that this limit was due to the overwhelming infection of neurons that was not reversed by hE16 treatment, irreversible neuronal injury that had occurred prior to therapeutic intervention, or theoretically, the emergence of
escape variants that were not neutralized by hE16. However, for unclear reasons, despite the sequencing of over 30 independent isolates from the brains of E16-treated WNV-infected moribund mice, we have been unable to identify a single virus that had mutations at any of the E16 contact residues, as defined by X-ray crystallography (T. Oliphant and M. S. Diamond, unpublished data).

The latest time at which MAb hE16 i.p. treatment effectively reduced mortality was 5 days after viral challenge, which in untreated animals coincided with the decline of WNV titers in the serum and CSF and the emergence of endogenous neutralizing antibody in serum. It is possible that endogenous antibodies may have been affected by the administration of hE16, but it is more likely that MAb hE16 provides a protective function up to day 5 that endogenously produced neutralizing antibody does not provide. For example, the findings of preliminary studies with human MAbs against WNV derived from B cells from infected patients suggest that the majority of the antibody response is nonneutralizing and that the antibody recognizes epitopes on domain II of the E protein (27). As a DIII-specific potently neutralizing antibody, hE16 IgG was effective at day 5, even though endogenously produced neutralizing antibody emerged in serum beginning at 4 days. One possible explanation is that the MAb hE16 IgG may cross the blood-brain barrier more effectively than neutralizing antibody, which is almost exclusively of the IgM isotype, endogenously produced early (5). Indeed, there was a further 2-day delay in the appearance of endogenously produced neutralizing antibody in the CSF. The detection of hE16 in neurological tissues and CSF indicates that at least some of it crosses the blood-brain barrier. Unfortunately, we were not able to detect the distribution of low levels of hE16 in tissues using immunohistochemical techniques (data not shown). It remains to be determined whether the administration of hE16 directly into the CSF could enhance the distribution of the antibody in CNS and thus enhance the protective function. Future preclinical studies with animals will test whether intrathecal or intraventricular dosing of hE16 could extend the time of treatment.

FIG. 3. Dose-response of MAb hE16 for survival of WNV-infected hamsters and concentrations in serum. Five days after s.c. injection of WNV (10^{5.1} 50% cell culture infectious doses), one-half log doses (100, 1.0, 0.32, 0.10, 0.032, 0.01, 0 mg/kg) of hE16 were administered i.p. Fifteen animals were included in each group except the 1.0-mg/kg group, which had 20 animals. On the next day, sera were obtained by bleeding of the orbital sinuses of anesthetized hamsters and were assayed for human IgG. (A) Percent survival plotted against hE16 dose. (B) Serum hE16 concentration plotted against hE16 dose. The dosage of hE16 that yielded half-maximal survival (MED_{50}), depicted in panel A, was calculated by using a linear equation. (C) The dosage of 0.13 mg/kg was extrapolated to derive the serum hE16 concentration reflecting half-maximal therapeutic activity (MEC_{50}) of 0.44 μg/mL.

FIG. 4. Concentrations of MAb hE16 in serum and CSF at 7, 12, and 30 dpi for WNV- or sham-infected hamsters treated i.p. at 5 dpi with 32 mg/kg hE16. The animals were injected s.c. with 10^{7.1} 50% cell culture infectious doses of WNV or were sham treated. Six animals each were used in the WNV-infected and sham-infected groups. Dashed line, limit of detection (0.6 μg/ml).
beyond 5 days in the hamster model. Such a route of administration is at least feasible in human patients. In these studies with hamsters, the i.p. dose of MAb hE16 that maximally improved survival was 0.32 mg/kg or higher, and the serum hE16 concentrations at 24 h associated with the maximal therapeutic effect were 1.2 μg/ml or higher. Based on the linear relationship between antibody dose and serum concentration, the hE16 MED50 and the MEC50 in serum were determined to be 0.13 mg/kg and 0.44 μg/ml, respectively. These data support a minimum serum target concentration for efficacy in humans of 4.4 μg/ml, which is 10-fold the MEC50. The dose required to achieve this level in the serum of humans may be estimated from data for palivizumab, an anti-respiratory syncytial virus humanized IgG1 MAb that is structurally similar to hE16. Intravenous administration of palivizumab at 15 mg/kg produced a concentration in serum of 228 μg/ml after 1 day (3); this level greatly exceeds the minimum target concentration in serum defined above. By using a linear relationship between administered dose and concentration in serum, hE16 doses of 0.3 mg/kg may be sufficient to produce concentrations in serum of approximately 4 μg/ml in humans after 1 day. Assuming that hE16 has a serum half-life in humans similar to that of palivizumab (~22 days), calculations predict that therapeutic levels of hE16 in a human subject should be sustainable for weeks to months following the administration of a single dose. Since the CED route is not likely to be used for the administration of hE16 in human patients, the concentration of hE16 was not determined in serum or CSF of hamsters that received hE16 by the CED route.

The concentration of MAb hE16 in the serum and CSF were not statistically significantly different between WNV-infected and sham-infected hamsters. Since other viral infections often cause increased permeability of the blood-brain barrier (22, 28), the WNV-infected animals might have been expected to have had higher levels of hE16 in the CSF; however, that was not the case. The hE16 concentrations in serum also correlated with the hE16 levels in CNS tissues, which were also similar in uninfected and infected animals. Thus, the majority of hE16 was not sequestered or destroyed after uptake of virus-immune complexes by phagocytic cells. Likely, after hE16-opsonized virus entered cells, hE16 was recirculated to the extracellular compartment after binding to the neonatal Fc receptor FcRn (4). Thus, the serum hE16 concentration was a marker of the concentration in other tissues of WNV-infected animals, with the possible exception of the spinal cord. Such data may be important in the planning of human clinical trials.

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FIG. 5. Correlation of hE16 concentration in serum with hE16 concentrations in neurological tissues and CSF at 7 dpi for WNV- and sham-infected hamsters treated i.p. at 5 dpi with 32 mg/kg hE16. The animals were injected with 10^7 50% cell culture infectious doses WNV or were sham treated. The results for the WNV-infected and sham-infected hamsters were combined because there was no difference in the hE16 concentration between these groups. Dashed lines, limit of detection (0.06 μg/ml).
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