Immunization of Mice Against West Nile Virus with Recombinant Envelope Protein[1]

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West Nile (WN) virus is a mosquito-borne flavivirus that emerged in the United States in 1999 and can cause fatal encephalitis. Envelope (E) protein cDNA from a WN virus isolate recovered from *Culex pipiens* in Connecticut was expressed in *Escherichia coli*. The recombinant E protein was purified and used as Ag in immunoblot assays and immunization experiments. Patients with WN virus infection had Abs that recognized the recombinant E protein. C3H/HeN mice immunized with E protein developed E protein Abs and were protected from infection with WN virus. Passive administration of E protein antisera was also sufficient to afford immunity. E protein is a candidate vaccine to prevent WN virus infection. *The Journal of Immunology,* **2001, 167: 5273–5277.**

Human West Nile (WN)[3] virus infection, a mosquito-borne flavivirus, was described in Uganda in 1937 and occurs in parts of Africa, Asia, the Middle East, and Eastern Europe (1–5). An outbreak of encephalitis that centered on New York City in the late summer of 1999 was determined to be due to the first cases of WN virus in the Western Hemisphere (6, 7). Since then, WN virus has been documented in mosquitoes, birds, and other animals in the northeastern U.S., has persisted over the winter months, and has been responsible for sporadic cases of fatal human disease (6–14). Treatment is largely supportive, and preventive measures include spraying to reduce the mosquito population and the use of insect repellents.

WN virus infection has been studied in several animals, including mice, rats, hamsters, and monkeys, thereby facilitating studies on immunity (15–22). WN virus causes a systemic murine infection and invades the CNS, resulting in death within 1–2 wk (15, 23). Infection of C3H/He, CD-1, BALB/c, Swiss, and C.B.-17 SCID mice has been documented, and, for the most part, all the animals suffer fatal infection (15, 23, 24). Strains of WN virus that lack neuroinvasiveness have been developed, and infection of CD-1 mice with these attenuated variants is sufficient to afford protection against challenge with WN virus (15, 23). Studies of related viruses may also provide some insight into the Ags that elicit immunity against WN virus. The Ab response to the envelope (E) protein of several flaviviruses, including dengue virus and Japanese encephalitis virus, may either contribute to immunity or potentially exacerbate disease (25–31). Infection of hamsters or macaques with dengue virus or Japanese encephalitis virus is partially protective against WN virus infection, and some mAbs to the WN virus E protein have neutralizing activity in vitro (16, 19, 23, 32, 33). Moreover, the i.m. injection of DNA encoding the WN virus premembrane and E proteins afforded immunity against WN virus infection (34). Therefore, the WN virus E protein could potentially serve as a candidate Ag for a WN virus vaccine.

Materials and Methods

Cloning, expression, and purification of the WN virus E protein

RNA was extracted from passage 2 of WN virus isolate 2741 from *Culex pipiens* cultivated in Vero cells at 37°C (6). Infected Vero cells (10^6) were scraped from the bottom of a T25 culture flask and centrifuged at 4500 × *g* for 10 min, and the supernatant was discarded. RNA was extracted from the pellet using RNeasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA (3 μg) was resuspended in a final volume of 40 μl RNase-free water. RT-PCR was used to amplify the gene encoding the E protein, using the Superscript one-step RT-PCR system (Life Technologies, Gaithersburg, MD). Sixty nanograms of RNA was used as the template. The 5′ primer sequence was TTCAACTGCCTTGGAATGAGC, and the 3′ primer sequence was AGCGTGCACGTTCACGGGAGAG. A total of 1503 nucleotides were amplified under the following conditions: 45 min at 50°C for cDNA synthesis and then 30 cycles with 1 min at 94°C for denaturation, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. The resulting DNA fragment was separated by electrophoresis on a 1% agarose gel, excised, and purified.

The E protein was expressed as recombinant fusion proteins in two *Escherichia coli* systems. The PCR product was first subcloned into the pBAD/ThioFusion expression vector (Invitrogen, Carlsbad, CA). The cloning reaction products were transformed into *E. coli* (One-Shot Top 10 competent cells, Invitrogen). Positive transformants were identified by PCR. The recombinant E protein was then expressed with thioredoxin (16 kDa) fused to the amino-terminus and a carboxyl-terminal polyhistidine tag to facilitate purification. Cells were harvested by centrifugation at 4000 × *g* for 20 min and then lysed by overnight freezing at −20°C and subsequent sonication for 5 min. The cell lysate was then centrifuged at 4°C for 15 min at 4000 × *g*, and the recombinant fusion protein was purified from the supernatant fraction. ThioBond resin (Invitrogen) was used to purify the recombinant protein according to the manufacturer's instructions. Fifty micrograms of recombinant fusion protein was purified from each 250-ml culture of bacteria. Thioredoxin control protein was expressed in *E. coli* from the pBAD vector and was purified from lysed cells in an identical manner. The recombinant E protein-thioredoxin fusion protein and recombinant thioredoxin were used to generate antisera in mice.

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[3] Abbreviations used in this paper: WN, West Nile; E, envelope; MBP, maltose-binding protein.

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To increase the solubility and yield of recombinant protein, a different expression vector was used, and the hydrophobic distal carboxyl-terminal region of the E protein was removed from the fusion protein. The nucleotides encoding the initial 80% of the gene (nt 1–1218) encoding the E protein were amplified by PCR using primers flanked by *Eco*RI and *Pst*I restriction enzyme digestion sites to facilitate subcloning. The primers were 5′-GAATTCTTCAACTGCCTTGGAATGAGC-3′ and 5′-CTG CAGTTATTTGCCAATGCTGCTTCC-3′. The DNA fragment was then digested with *Eco*RI and *Pst*I and inserted into the pMAL-c2X vector (pMAL protein fusion and purification system, New England Biolabs, Beverly, MA), downstream of the *E. coli malE* gene that encodes maltose-binding protein (MBP). This resulted in the expression of a recombinant fusion protein (MBP-E). Transformed cells (DH5α, Life Technologies) were grown to a concentration of 2×10^8 cells/ml. Isopropyl-D-thiogalactopyranoside was added (final concentration, 0.3 mM), and the culture was incubated at 37°C for 4 h. Cells were harvested by centrifugation at 4000 × *g* for 20 min and then lysed by overnight freezing at 20°C and subsequent sonication for 10 min. Expression in *E. coli* produced a soluble 82-kDa fusion protein (MBP-E) on a Coomassie blue-stained SDS-PAGE gel, and the MBP-E protein was then purified using a maltose affinity column according to the manufacturer's instructions (New England Biolabs). Three milligrams of protein was purified from a 250-ml cell culture. Recombinant MBP (control) was expressed and purified in an identical fashion. The recombinant MBP-E protein was used in the immunoblot studies to detect Abs in patient sera and in the active immunization studies.

Immunoblot, IFA, and ELISA

Recombinant MBP-E and MBP (control) proteins were boiled in SDS-PAGE sample buffer (Bio-Rad, Hercules, CA) containing 2% β-ME. The proteins (2 μg/gel) were separated by SDS-PAGE (10% gel) and transferred to nitrocellulose membranes using a semidry electrotransfer apparatus (Fisher Scientific, Pittsburgh, PA). The nitrocellulose membranes were then probed with sera from individuals with confirmed WN virus infection or sera from normal persons. The patient sera were from cases of WN virus infection in the New York City area. All patient sera tested in this West Nile ELISA were collected in the convalescent stage of infection, from 1 to 8 mo after the onset of illness. Diagnosis of WN virus infection was based upon clinical history, serologic testing including a positive IFA, capture IgM ELISA, or a 4-fold increase in WN virus-specific neutralization activity in the convalescent sera, performed at the Centers for Disease Control or the New York State Department of Health. The membranes were incubated with sera (1/100 dilution) at room temperature for 1 h, followed by three washes in TBST. An alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St. Louis, MO) at a dilution of 1/1000 was then used to detect the primary Ab. Blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate from Kirkegaard & Perry (Gaithersburg, MD).

Indirect immunofluorescence (IFA) and ELISA were used to detect Abs specific for WN virus or an E protein peptide. IFA was performed using WN virus-infected Vero cells that had been fixed to glass slides with 2% paraformaldehyde containing 0.1% Triton X-100 in PBS. To first block nonspecific binding, the fixed cells were incubated with PBS containing 2% BSA at room temperature for 1 h. Sera (1/10 to 1/640 dilution) from mice immunized with recombinant Ag or mice infected with WN virus were applied for 1 h at room temperature. The cells were then washed three times with PBS with Tween 20 (PBST). Goat anti-mouse IgG-FITC conjugate was then applied at a dilution of 1/2500 (Kirkegaard & Perry Laboratories) for 1 h at room temperature. After another washing in PBST, the slides were mounted and examined using a confocal microscope (×400 magnification).

To further identify Abs specific for the WN virus E protein, a peptide corresponding to the E protein aa 288–301 (CRVKMEKLQLKGTT) was synthesized. This protein was chosen because an examination of the E protein structure suggests that this region may be surface exposed and therefore accessible to Ab; certainly many other epitopes could have similar properties. As a control, a peptide that contained the same amino acids in a random sequence (CQLLMREVKTGTKK) was also generated. Peptides were prepared on a Rainin Symphony (Woburn, MA) instrument (50 μmol scale), purified by reverse phase HPLC, and analyzed by MALDI mass spectroscopy. The peptide synthesis, purification, and analysis were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). The peptides were conjugated to the carrier protein using Imject maleimide-activated OVA. Microtiter plates were coated with conjugated peptide overnight at 4°C at 100 ng/well in coating buffer (0.015 M Na_2CO_3, 0.03 M $NaHCO_3$, and 0.003 M NaN_3, pH 9.6). Sera from immunized mice were diluted from 1/50 to 1/10,000 in PBS with 2% BSA, added to the duplicate wells, and incubated for 1 h at room temperature. Plates were washed three times with PBST. Alkaline phosphatase-conjugated goat anti-mouse IgG at a dilution of 1/1,000 in PBST (Sigma) was added for 1 h at room temperature. After washing three times with PBST, color was developed with *p*-nitrophenyl phosphate for 10 min and read at an adsorbance of 405 nm using a spectrophotometer.

Neutralization assay

Serum samples were heat inactivated by incubation in a 56°C water bath for 30 min and were serially diluted in PBS with 5% gelatin from 1/10 to 1/2560. WN virus was diluted in PBS with 5% gelatin so that the final concentration was 100 PFU/well. We then mixed 75 μl virus with 75 μl serum in a 96-well plate at 37°C for 1 h. Aliquots of serum-virus mixture were inoculated onto confluent monolayers of Vero cells in a six-well tissue culture plate. The cells were incubated at 37°C for 1 h, and the plates were shaken every 15 min. The agarose overlay was then added. The overlay was prepared by mixing equal volumes of a solution consisting of 100 ml 2× MEM (Life Technologies) with sterile 2% agarose. Both solutions were placed in a 40°C water bath for 1 h before adding the overlay. The cells were incubated for 4 days at 37°C in a humidified 5% CO_2-air mixture. A second overlay with an additional 4% neutral red was added on day 5. Virus plaques were counted 12 h later.

Infection and immunization

Groups of five C3H/HeN mice were challenged with an i.p. inoculation (100 μl) of between 10^6 and 10^{-2} PFU of WN virus isolate 2741. The WN virus isolate was stored at −70°C, and serial 10-fold dilutions of the virus were prepared in PBS with 0.5% glucose, 30% rabbit serum, antibiotics, and antimycotics. Mice were monitored daily for morbidity, including lethargy, loss of appetite, disorientation, and difficulty in moving. The time of death, usually 6–10 days, was recorded. All surviving mice were euthanized at 15 days after inoculation and examined for infection by culture of selected tissues or PCR. Ten percent suspensions of brain tissue sections were prepared in 1.5 ml PBS by triturating with a mortar and pestle. Alundum was added to facilitate homogenization of the tissue. Suspensions were centrifuged at 520 × *g* for 10 min. Supernatant was then passed through a 0.22-μm filter, and 100 μl was added to a monolayer of Vero cells (10^7 cells/T25 flask). Cells were grown and examined for cytopathologic effect.

C3H/HeN mice were immunized with 20 μg recombinant E protein (fused to either thioredoxin or MBP) or control Ag (recombinant thioredoxin or MBP) in 200 μl CFA in the back, and they were then boosted with 20 μg Ag in 200 μl IFA at 2 and 4 wk. Ten days after the final immunization, mice were phlebotomized, and the E protein antisera were stored at −20°C until use. In the passive immunization studies, mice were intradermally injected with 150 μl antiserum (diluted 1/5 in PBS) pooled from five mice that had been actively immunized with E protein. A group of mice was also given thioredoxin antisera (control). The animals were challenged with WN virus (~10^1 or 10^6 PFU) 24 h after the Ab transfer. In the active immunization studies, mice were challenged with WN virus 10 days after the final immunization.

Results

We first determined whether Abs to the WN virus E protein developed during the course of infection. The gene encoding WN virus E protein was cloned as cDNA from an isolate of WN virus, designated 2741, that was recovered from *Culex pipiens* in Connecticut during the recent U.S. outbreak (6). WN virus 2741 E cDNA was amplified by PCR and expressed in *E. coli* as a recombinant fusion protein (Fig. 1, *A* and *B*). Sera from all six individuals with confirmed WN virus infection had Abs that bound to the recombinant E protein in immunoblot (Fig. 1*C*, lanes 13–18), demonstrating that the E protein was recognized during WN virus infection.

Groups of five C3H/HeN mice (6 wk old) were then inoculated with WN virus 2741 to determine the course of infection. Mice challenged with 10^6 PFU of virus by i.p. injection died within 10 days, and virus could be cultured from the blood and brain (Fig. 2). Mice administered as little as 10^0 PFU also uniformly died within 10 days, demonstrating the virulence of the 2741 WN virus isolate. Lower doses of virus were not sufficient to consistently infect the mice, as virus was not evident in any of the animals that survived at 2 wk. These data demonstrated that a level of 10^0 PFU, as

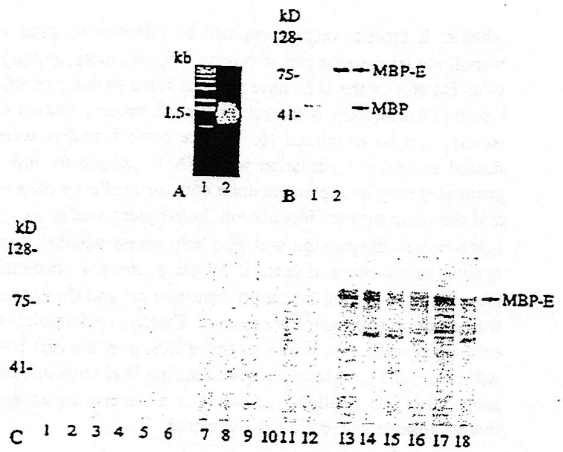

FIGURE 1. Patients with WN virus infection develop Abs to the E protein. *A*, Amplification of the gene encoding the E protein from WN virus isolate 2741 (*lane 2*). *Lane 1*, Molecular mass markers. *B*, Purified recombinant WN virus E protein (*lane 2*), expressed as a fusion protein with MBP. The 82-kDa recombinant fusion protein is designated MBP-E. *Lane 1*, Recombinant MBP. *C*, Sera from six patients with confirmed WN virus infection (*lanes 13–18*) had Abs that recognized the recombinant MBP-E fusion protein in immunoblot. Sera from six healthy subjects (*lanes 1–6*) did not recognize the MBP-E protein (control). As an additional control, the sera from six patients with WN virus infection were used to probe MBP in an immunoblot (*lanes 7–12*).

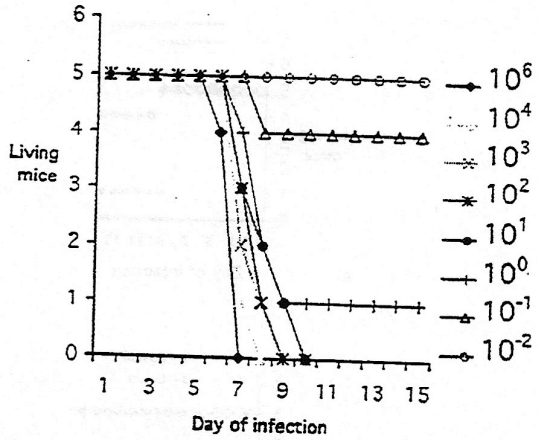

FIGURE 2. Infection of C3H/HeN mice with WN virus 2741. Groups of five mice were challenged with an i.p. injection of WN virus, ranging from 10^6 to 10^{-2} PFU, as determined in a Vero cell PFU assay. The time to death was recorded. Mouse survival was monitored daily. All mice that were alive at 15 days were euthanized and cultured for evidence of viral infection. Experiments were repeated three times.

determined in a Vero cell PFU assay, was sufficient to kill the majority of the animals (approximate LD_{50}).

C3H/HeN mice were then immunized to determine whether a humoral response to the recombinant E protein could be elicited. Groups of five mice were immunized with 20 μg recombinant E protein, expressed as a fusion protein with thioredoxin, in CFA and then boosted with the same amount of Ag in IFA at 14 and 28 days. Control mice were immunized with the recombinant carrier protein (thioredoxin) in an identical fashion. The recombinant Ags are shown in Fig. 3*C*. At 10 days following the final boost, the animals were phlebotomized, and the sera were pooled and examined for Abs specific for the WN virus. Abs in the murine sera (1/10 to 1/160 dilution) reacted with WN virus-infected Vero cells in indirect immunofluorescence, indicating that the Abs to the recombinant E protein recognized the native virus (Fig. 3*A*). Moreover, the antisera (1/100 dilution) reacted with a peptide that was specific for the E protein (Fig. 3*B*) in ELISA. Abs were readily detected at a sera dilution of up to 1/1000 (not shown). These data show that mice developed a strong humoral response to the recombinant E protein.

To explore the role of Abs to the WN virus E protein in viral killing, in vitro neutralization studies were performed. E protein antisera had neutralization activity that could be detected in a plaque formation assay. Neutralization activity was evident when the antisera used up to a dilution of 1/1280, demonstrating the functional significance of these Abs. To then directly determine the role of Ab in immunity against infection, five mice were passively administered 150 μl murine E protein antisera 24 h before challenge with approximately 10^1 PFU WN virus (Fig. 4*A*). Control mice were administered antisera to the carrier protein. Four of five control animals died after 2 wk, while only one of five mice immunized with E protein antisera died ($p < 0.05$). All the living mice were euthanized on day 15; WN virus was not recovered from any of the animals by culture or PCR. Two additional experiments yielded similar results. These studies demonstrate the protective effect of E protein antisera against murine WN virus infection.

Active immunization studies then used recombinant E protein fused to MBP, which we were able to express and purify in greater quantities than the recombinant E protein linked to thioredoxin (see *Materials and Methods*). Mice vaccinated with the E protein

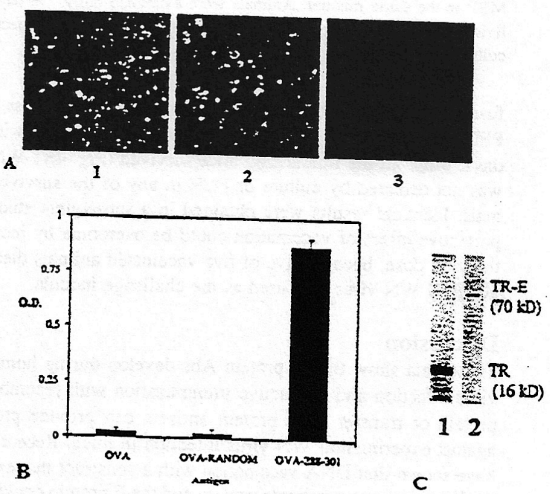

FIGURE 3. Humoral response of mice immunized with the recombinant E protein. *A*, Sera from mice infected with WN virus (*panel 1*, positive control), immunized with the recombinant E fusion protein (*panel 2*), or immunized with the carrier protein (*panel 3*, negative control) were used to probe WN virus-infected Vero cells in an indirect immunofluorescence assay. The presented panels show results at a serum dilution of 1/80. *B*, Sera from mice immunized with the recombinant E protein were used to probe a peptide corresponding to the E protein aa 288–301 (CRVKMEKLQLKGTT), linked to $OVA_{288-301}$ in an ELISA. OVA and OVA linked to the amino acids contained in the 288–301 peptide in a random order (CQLLMREVKTGTKK; OVA-RANDOM) served as controls. *C*, Recombinant Ags used to immunize the mice. *Lane 1*, Thioredoxin (TR); *lane 2*, thioredoxin-E protein (TR-E). Results are shown for a serum dilution of 1/100.

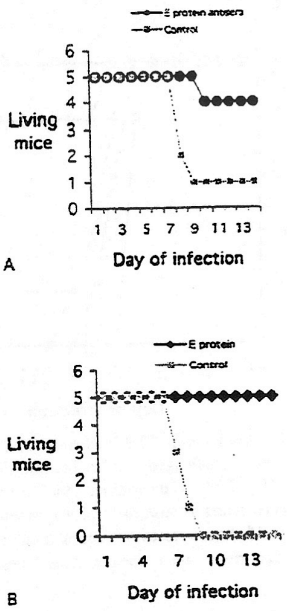

FIGURE 4. Vaccination against WN virus infection. *A*, Mice were passively administered E protein antiserum (raised against E protein fused to thioredoxin, see *Materials and Methods*) and challenged with WN virus (10^1 PFU). Control mice were given thioredoxin antiserum in an identical fashion. Animals were examined daily until death. On day 15 all remaining animals were euthanized and cultured for evidence of WN virus infection. *B*, Active immunization with WN virus E protein. Mice were immunized with recombinant E protein (fused to MBP, see *Materials and Methods*) and challenged with 10^1 PFU WN virus. Control mice were immunized MBP in the same manner. Animals were examined daily. On day 15 all living animals were sacrificed and examined for WN virus infection by culture and PCR.

fused to MBP were fully protected from a challenge dose of 10^1 PFU WN virus ($p < 0.001$). All the control mice died within 10 days, while all the immunized mice survived (Fig. 4*B*). WN virus was not detected by culture or PCR in any of the surviving animals. Identical results were obtained in a subsequent study. The protective effect of vaccination could be overcome by increasing the viral dose, because five of five vaccinated animals died when 10^6 PFU WN virus was used as the challenge inocula.

Discussion

These data show that E protein Abs develop during human WN virus infection and that active immunization with recombinant E protein or transfer of E protein antisera can provide protection against experimental WN virus infection in mice. Recent studies have shown that DNA vaccination with a construct that expresses the WN virus premembrane protein and the E protein could protect horses and mice from infection (34). It was implied that the premembrane protein was necessary for immunity, either for the genesis of a protective response directed against the (pre)membrane protein or by stabilizing the E protein. Our data demonstrate the recombinant E protein vaccination affords full protective immunity by itself, and moreover, that E protein Abs are sufficient for partial immunity. In addition, the recombinant E protein MBP used for active immunization lacked the distal carboxyl terminus of E protein, thereby showing that the initial 406 aa (80%) of the E protein can generate a protective immune response.

Although WN virus infection in mice has some similarities with human disease, such as neuroinvasion, it remains to be determined whether E protein vaccination will be effective in other experimental model systems and in humans. Furthermore, although WN virus isolates in the U.S. have demonstrated striking genetic similarities, vaccination with recombinant E protein against diverse isolates can be examined (6, 7). The current studies were conducted using i.p. inoculation with WN virus, and the efficacy of protection may be dependent upon both the challenge dose of virus and the route of viral inoculation. Subsequent studies using mosquito-borne transmission will also help assess whether protection against vector-borne disease is effective, thereby accounting for the viral dose during mosquito transmission and the influence of the vector on pathogen transmission. Finally, epidemiological and ecological studies on WN virus in the U.S. over the next few years will determine the overall risk of acquiring WN virus infection and subsequent encephalitis, and whether a vaccine-based approach toward disease prevention is warranted.

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