Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice

Huafang Lai[a], Michael Engle[b], Anja Fuchs[b], Thomas Keller[a], Syd Johnson[c], Sergey Gorlatov[c], Michael S. Diamond[b,d,e,2], and Qiang Chen[a,1]

[a]The Biodesign Institute, Arizona State University, Tempe, AZ 85287; Departments of [b]Medicine, [d]Molecular Microbiology, and [e]Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110; and [c]MacroGenics, Inc., Rockville, MD 20850

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Over the past decade, West Nile virus (WNV) has spread to all 48 of the lower United States as well as to parts of Canada, Mexico, the Caribbean, and South America, with outbreaks of neuroinvasive disease occurring annually. At present, no therapeutic or vaccine is available for human use. Epidemics of WNV and other emerging infectious disease threats demand cost-efficient and scalable production technologies that can rapidly transfer effective therapeutics into the clinical setting. We have previously reported that Hu-E16, a humanized anti-WNV mAb, binds to a highly conserved epitope on the envelope protein, blocks viral fusion, and shows promising postexposure therapeutic activity. Herein, we generated a plant-derived Hu-E16 mAb that can be rapidly scaled up for commercial production. Plant Hu-E16 was expressed at high levels within 8 days of infiltration in *Nicotiana benthamiana* plants and retained high-affinity binding and potent neutralizing activity in vitro against WNV. A single dose of plant Hu-E16 protected mice against WNV-induced mortality even 4 days after infection at rates that were indistinguishable from mammalian-cell-produced Hu-E16. This study demonstrates the efficacy of a plant-produced mAb against a potentially lethal infection several days after exposure in an animal challenge model and provides a proof of principle for the development of plant-derived mAbs as therapy against emerging infectious diseases.

flavivirus | mAb therapeutics | plant-made pharmaceuticals

West Nile virus (WNV) is a member of the *Flavivirus* genus of the Flaviviridae family. It is a neurotropic, enveloped virus with a single-stranded, positive polarity, 11-kilobase RNA genome and has circulated in Africa, Asia, the Middle East, and Europe (1). In 1999, WNV entered the Western Hemisphere as a point introduction in New York City. More than 29,000 human cases have been diagnosed with severe WNV infection in the continental United States during the past decade, and many more have been infected and remain undiagnosed. Advanced age is by far the greatest risk factor for severe neurological disease, long-term morbidity, and death (2), although a genetic basis of susceptibility also has been recently identified (3, 4).

Historically, there has been a lack of effective and specific antiviral treatment for infection by WNV or other flaviviruses (reviewed in ref. 5). Whereas several small molecule compounds have been described with antiviral activity against WNV in vitro, few have demonstrated efficacy in vivo (6, 7). IFN, which is used as part of combination therapy against the distantly related hepatitis C virus, inhibits flaviviruses including WNV when used as prophylaxis. However, its effect is attenuated once viral replication has commenced as flavivirus nonstructural proteins antagonize IFN signaling pathways (reviewed in ref. 8). New threats of WNV globally and lack of available treatments warrant studies to develop effective therapeutics and production technologies that can rapidly transfer the candidates into the clinical care settings in a cost-conscious manner.

We previously described a humanized murine mAb (Hu-E16) therapeutic candidate that binds to a highly conserved epitope on domain III (DIII) of WNV envelope (E) protein (9). This mAb is highly inhibitory because it blocks viral fusion at concentrations that result in low occupancy of accessible sites on the virion (10, 11). Hu-E16 has therapeutic activity in rodents even after WNV has entered the central nervous system (9, 12), in part because it can directly disrupt virus transmission between neurons (13). Despite the promise that Hu-E16 and other mAbs have as prophylactics and therapeutics for WNV or other infectious diseases, their application may be limited by the high production costs and scalability associated with the mammalian-cell culture production system. Moreover, if biological drugs are too costly to produce for resource-poor health care systems, their therapeutic potential may never be realized. As such, the development of production platforms that are cost-effective, scalable, and safe for biological therapeutics is urgently needed.

Plants can be engineered to produce proteins efficiently, with significantly lower manufacturing costs than mammalian-cell cultures (reviewed in ref. 14). Plants are also far less likely to introduce adventitious human or animal pathogens compared to mammalian cells or transgenic animals. Unlike bacterial and other prokaryotic systems, plants share a similar endomembrane system and secretory pathway with human cells (15). Thus, plant cells efficiently assemble multiple subunit proteins such as mAbs and perform necessary posttranslational modifications on transgenic proteins. Protein glycosylation in plant cells, however, is slightly different from that of animal cells. Plants have unique plant-specific β-1,2-xylose and core α-1,3-fucose residues on complex N-linked glycans (16). As such, mAbs produced by plants may have some functional differences from the native molecules. The impact of such differences on the activity of mAb therapeutics in vivo has not been evaluated. The low-cost, high-scalability, and safety characteristics of a plant production system offer an attractive alternative for both commercial pharmaceutical production and for manufacturing products for the developing world.

Herein, we further develop Hu-E16 as a possible therapeutic by applying technology that allows cost-saving scale-up capability through the use of plants. We produced 0.8 g of Hu-E16 per kilogram of fresh *Nicotiana benthamiana* leaves within 8 days of infiltration. Plant-produced Hu-E16 (pHu-E16) IgG1 was readily isolated to >95% purity by a simple three-step separation procedure. pHu-E16 retained potent neutralizing activity and showed strong pre- and postexposure therapeutic activity in mice that was virtually equivalent to that observed with

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Conflict of interest statement: M.S.D has consulting agreements with MacroGenics, a company that has licensed the Hu-E16 mAb from Washington University for possible commercial development. S.J. and S.G. are employees of MacroGenics.

[1]To whom correspondence may be addressed at: Arizona State University, Mail Zone 5401, 1001 S. McAllister Avenue, Tempe, AZ 85287. E-mail: qiang.chen.4@asu.edu.

[2]To whom correspondence may be addressed at: Washington University School of Medicine, Campus Box 8051, 660 S. Euclid Avenue, St. Louis, MO 63110. E-mail: diamond@borcim.wustl.edu.

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mammalian-cell culture-derived Hu-E16 (mHu-E16). This study provides a proof of principle for the rapid development and use of plant-derived mAb against human infectious diseases.

Results

Expression and Assembly of Hu-E16 mAb in Plants. As a first test of the feasibility of developing a plant-derived mAb therapeutic, we needed to demonstrate that plants could express and assemble Hu-E16. To ensure high-level expression in plants, the coding sequences of Hu-E16 light chain (LC) and heavy chain (HC) were optimized in silico with *N. benthamiana*-optimized codons (17), cloned into the 5′ modules of plant expression vectors of the MagnICON system (18), and transformed into *Agrobacterium tumefaciens*. To coexpress Hu-E16 LC and HC, *A. tumefaciens* strains harboring the LC and HC 5′ modules were codelivered into *N. benthamiana* leaves along with their respective 3′ modules and an integrase construct through vacuum infiltration (18). Western blot analysis after reducing or nonreducing gel electrophoresis confirmed that the LC and HC of pHu-E16 were produced in leaves with the expected molecular weights (Fig 1*A* and *B*) and that pHu-E16 mAb assembled into its tetrameric (2HC + 2LC) form (Fig 1*C*). The assembly of pHu-E16 was corroborated by an ELISA that detects the assembled form of E16 (HC capture, LC probe) (Fig 2*A*). ELISA results also indicated that pHu-E16 reached the highest level of production 7 days postinfiltration with *A. tumefaciens*, with an average accumulation of 8.1 mg/g leaf fresh weight. This level is greater than the highest expression level for mAbs in plants ever reported (18) and demonstrates that plants can rapidly express fully assembled pHu-E16 at high levels.

Purification and Scale-Up Production of pHu-E16. For plant-produced pHu-E16 to become a viable WNV therapeutic candidate, an efficient purification scheme must be developed. pHu-E16 was extracted and purified by a three-step purification protocol comprised of ammonium sulfate precipitation, protein A affinity, and DEAE-anion exchange chromatographies. Precipitation with ammonium sulfate removed the most abundant plant host protein, the photosynthetic enzyme RuBisCo, and other plant proteins (Fig 2*B*, lane 2). Protein A affinity chromatography removed the remaining contaminating proteins and enriched pHu-E16 to greater than 95% purity (Fig 2*B*, lane 5). In the presence of a reducing agent, purified pHu-E16 was detected as the HC and LC in the same stoichiometric ratio as the Hu-E16 produced in mammalian cells (Fig 2*B*, lanes 5 and 6). Under oxidizing conditions, purified pHu-E16 antibody assembled in its tetrameric form (Fig 2*B*, lane 7). For future clinical testing and cGMP

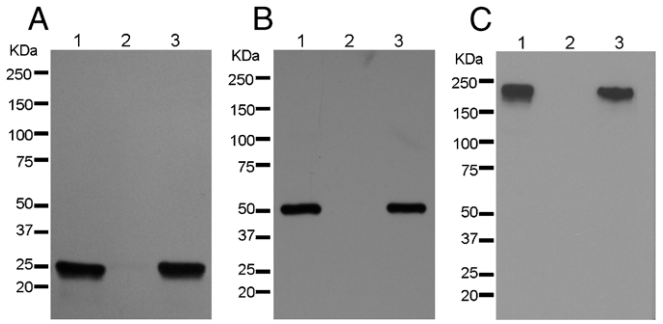


Fig. 1. Western blot analysis of pHu-E16. Leaf protein extracts were separated on 4–20% SDS-PAGE gradient gels under a reducing (*A* and *B*) or nonreducing (*C*) condition and blotted onto PVDF membranes. The membranes were incubated with a goat anti-human-kappa chain antibody or a goat anti-human-gamma chain antibody to detect LC (*A* and *C*) or HC (*B*). Lane 1, mHu-E16 as a reference standard; lane 2, protein sample extracted from uninfiltrated leaves; lane 3, extract from leaves coinfiltrated with Hu-E16 LC and HC constructs.

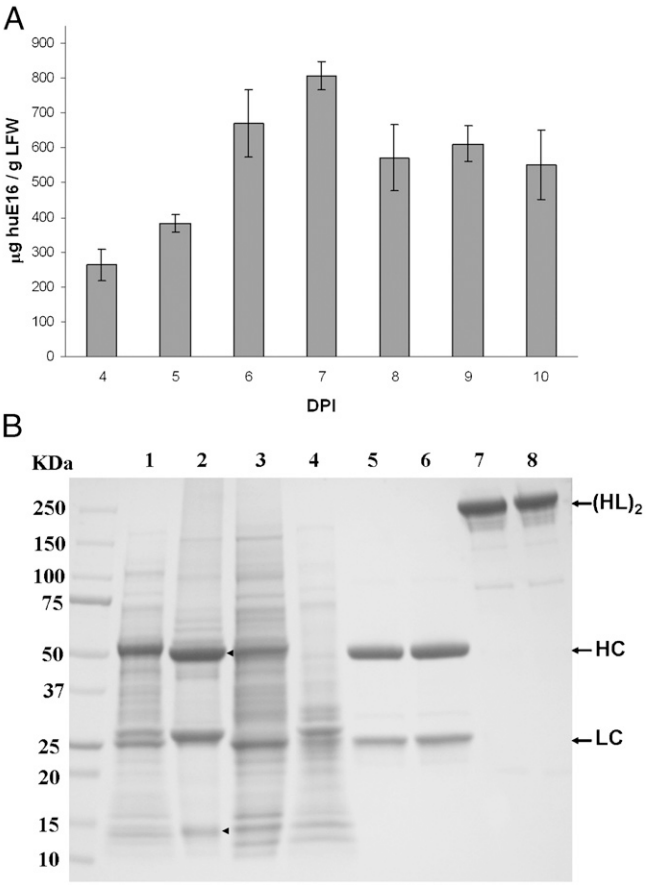


Fig. 2. Expression and purification of Hu-E16 mAb in *N. benthamiana* plants. *N. benthamiana* leaves were coinfiltrated with Hu-E16 LC and HC constructs. Leaf proteins were extracted on days 4–10 after agroinfiltration (*A*) or on day 7 after agroinfiltration (*B*). (*A*) Protein extracts were analyzed with an ELISA that detects the assembled form of pHu-E16 mAb. Mean ± SD of samples from three independent infiltration experiments are presented. (*B*) Leaf protein extract was purified and analyzed on a 4–20% SDS-PAGE gel under a reducing (lanes 1–6) or nonreducing (lanes 7 and 8) condition. Lane 1, clarified plant extract; lane 2, plant proteins removed by 25% ammonium sulfate precipitation; lane 3, 50% ammonium sulfate pellet fraction resuspended for protein A chromatography; lane 4, protein A flow-through fraction; lanes 5 and 7, purified pHu-E16 mAb in the protein A eluate; lanes 6 and 8, mHu-E16 as a reference standard. ◂: RuBisCo large and small subunits; ←: LC, HC, and assembled form $(HL)_2$ of Hu-E16 mAb.

production, an ion exchange chromatographic step was added to eliminate residual DNA, endotoxin, and protein A from the final purified product. Contaminants and/or impurities were efficiently removed by using this purification scheme so that levels in the final pHu-E16 product were below the Food and Drug Administration specifications for injectable human mAb pharmaceuticals (Table S1).

To validate the scalability of our purification protocol, we purified pHu-E16 at different scales of plant materials ranging from 10 to 5,000 g. Our protocol produced highly purified pHu-E16 from *N. benthamiana* plants with consistent recovery among batches of different scale (Table S1 and Fig. S1). In total, >5 g of Hu-E16 was purified from 16 kg of plant material for in vitro and in vivo studies.

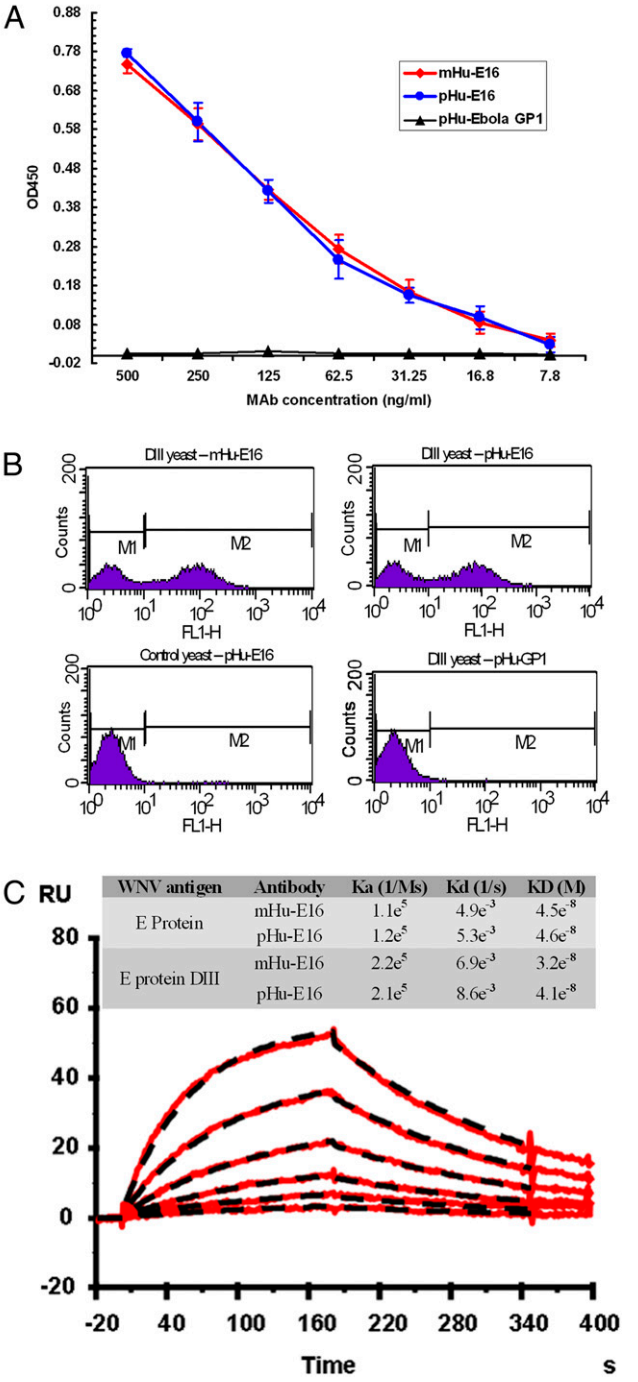
pHu-E16 Retains Antigen Binding Activity. To begin to establish a similarity of structural, biochemical, and functional properties between plant- and mammalian-cell-derived Hu-E16, we compared their recognition and binding kinetics for WNV E proteins or domains in three assays: (*i*) The binding of pHu-E16 to WNV E DIII was determined by ELISA in which DIII was immobilized

(19). pHu-E16 and mHu-E16 bound in a similar manner to DIII (Fig 3*A*). (*ii*) Recognition of pHu-E16 for DIII was examined in a binding assay with yeast that displayed DIII on their surface. Flow cytometric analysis showed that the percentage of positive yeast and the mean fluorescence intensity of binding by pHu-E16 and mHu-E16 were virtually identical (Fig 3*B*). (*iii*) To assess the binding of pHu-E16 more quantitatively, a surface plasmon resonance (SPR) assay was utilized with purified pHu-E16 or mHu-E16 immobilized on a BIAcore chip. Monomeric WNV E protein and E DIII were generated and flowed across the solid-phase Hu-E16 mAbs. pHu-E16 had almost identical binding affinity and kinetics for WNV E protein and DIII compared to its mHu-E16 counterpart (Fig 3*C*).

Neutralizing Activity of pHu-E16. To evaluate the neutralization potential of pHu-E16, we used a validated and quantitative flow cytometry-based neutralization assay (20) that measures antibody inhibition of infection with WNV reporter virus particles (RVPs). WNV RVPs were mixed with varying concentrations of pHu-E16 or mHu-E16 mAbs and then incubated with permissive Raji-DC-SIGN-R cells. Neutralization was monitored as a function of GFP fluorescence at 40 hours after infection. pHu-E16 neutralized WNV infection equivalently compared to mHu-E16 (Fig 4*A* and *B*). Recent studies have suggested that the complement component C1q augments the neutralizing potency of mHu-E16 (21). In the presence of purified C1q, the neutralization curve of mHu-E16 but not pHu-E16 showed a shift to the left, indicating greater inhibition at lower antibody concentrations. This shift suggests that slightly different carbohydrate modifications on the plant-derived mAb impaired an interaction with C1q as compared to mHu-E16. This effect was confirmed by an SPR assay because pHu-E16 showed lower binding to C1q (Fig 4*C*). Overall, the binding and neutralization studies in vitro suggest that pHu-E16 and mHu-E16 had similar but not identical functional properties.

pHu-E16 Protects Against Lethal WNV Infection. ***Prophylaxis studies.*** Although the functional studies suggested similar activity of the pHu-E16, it was essential to confirm this in vivo. Pretreatment studies were performed in 5 week-old wild-type C57BL/6 mice ($N > 20$, per group) to compare the concentrations of pHu-E16 and mHu-E16 that prevent severe WNV infection. Mice were infected with 10^2 pfu of WNV, which causes a baseline mortality of 80–90% (22). Increasing amounts (0.001–10 μg) of pHu-E16 or mHu-E16 were administered as a single dose on the day of infection. Mice were significantly protected when administered as little as 0.1 μg of pHu-E16 (Fig 5*A*, $P < 0.001$). More than 80% of mice were protected from lethal infection when 10 μg of pHu-E16 was administered ($P < 0.0001$). Protection against WNV lethality achieved by pHu-E16 was similar in magnitude to that observed with mHu-E16 (pHu-E16, $IC_{50} = 0.19$ μg, mHu-E16, $IC_{50} = 0.15$ μg, $P > 0.6$) (Fig 5*A* and *B*).

Therapeutic studies. Postexposure treatment studies were performed to confirm the therapeutic activity of pHu-E16. Mice were passively administered a single dose (4–100 μg) of pHu-E16 or mHu-E16 at day 2 after subcutaneous inoculation of 10^2 pfu of WNV (Fig 5*C* and *D*). Notably, 20 μg of pHu-E16 protected most mice from lethal infection when given 2 days after WNV inoculation, and a single injection of as low as 4 μg also prevented mortality; these results were similar to that observed in experiments with mHu-E16 performed in parallel. Because WNV spreads to the brain in mice by day 4 after infection (9), we also investigated the efficacy of pHu-E16 at this later time point (Fig 5*E*). A single administration of 50 μg of pHu-E16 protected up to 70% of mice from lethal infection, and a 90% survival rate was achieved with a single 500-μg dose, results that were equivalent in protection to



WNV antigen	Antibody	Ka (1/Ms)	Kd (1/s)	KD (M)
E Protein	mHu-E16	$1.1e^{5}$	$4.9e^{-3}$	$4.5e^{-8}$
	pHu-E16	$1.2e^{5}$	$5.3e^{-3}$	$4.6e^{-8}$
E protein DIII	mHu-E16	$2.2e^{5}$	$6.9e^{-3}$	$3.2e^{-8}$
	pHu-E16	$2.1e^{5}$	$8.6e^{-3}$	$4.1e^{-8}$

Fig. 3. Antigen recognition and binding kinetics of pHu-E16 for WNV DIII and E protein. (*A*) ELISA of pHu-E16 binding to WNV DIII. Serial dilutions of pHu-E16 were incubated on plates coated with WNV DIII and detected with a HRP-conjugated anti-human-kappa antibody. Dilutions of mHu-E16 were used as reference standards. A plant-produced humanized mAb against Ebola virus GP1 protein (pHu-Ebola GP1) was used as a negative control. The OD_{450} (mean ± SD) from three independent experiments are presented. (*B*) Binding of pHu-E16 to DIII of WNV E displayed on yeast cell surface. DIII displaying or negative control yeast cells were stained with pHu-E16, mHu-E16, or a negative control mAb (pHu-Ebola GP1) and processed by flow cytometry. Representative data from several independent experiments are shown. (*C*) SPR analysis of binding affinity and kinetics of pHu-E16 and mHu-E16 for WNV DIII and E protein. WNV DIII fragment or E ectodomain protein was injected over pHu-E16 or mHu-E16 immobilized to the CM-5 biosensor chip. Binding responses were normalized to the same level of immobilized antibody and analyzed by Langmuir 1:1 interaction fit (*Black Dashed Lines*). A representative set of SPR binding curves of pHu-E16 for WNV E protein from several independent experiments is shown.

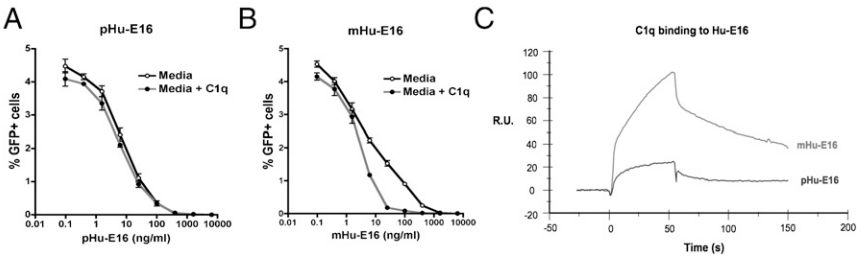


Fig. 4. The neutralizing activity of mHu-E16 and pHu-E16 and effect of C1q. Serial dilutions of (*A*) mHu-E16 or (*B*) pHu-E16 were incubated with WNV RVP in the presence of media or 50 μg/mL of purified human C1q prior to infection of Raji-CD-SIGN-R cells. Forty hours later, cells were fixed and analyzed by flow cytometry for GFP expression. Data are representative of at least three independent experiments performed in triplicate, and bars represent the standard error of the mean. (*C*) SPR analysis of C1q binding to mHu-E16 and pHu-E16. C1q (24 nM) was injected over captured antibody on an immobilized DIII fragment. Data are representative of several independent experiments.

mHu-E16. Overall, pHu-E16 appeared as potent as mHu-E16 in mice.

Discussion

Despite annual WNV outbreaks in North America, there is a lack of effective and specific antiviral treatment (reviewed in ref. 5). The high production costs and limited scalability associated with mammalian-cell culture production may restrict the use of therapeutic mAbs against WNV and other flaviviruses in resource-poor settings. Here, we investigated the feasibility of producing in plants a candidate mAb therapeutic against WNV infection. Our results show that (*i*) plant-derived mAb therapeutics have similar potency as their mammalian-cell counterparts, and (*ii*) generation of biological therapeutics in plants provides a platform that can address the cost and scalability issues associated with production. pHu-E16 retained high-affinity binding and potent neutralizing activity in vitro against WNV, and a single dose protected mice against WNV-induced mortality even 4 days after infection at rates that were indistinguishable from mHu-E16.

Transgenic plants are suitable for mAb production because they can be rapidly expanded in commercial production without the high-capital investment associated with traditional mAb bioreactor facilities (reviewed in ref. 14). pHu-E16 was expressed rapidly in *N. benthamiana* leaves within 4–8 days of infiltration and efficiently assembled into a native IgG form. Without any genetic optimization, pHu-E16 accumulated at an average of 0.8 mg/g of fresh leaf weight, greater than the highest expression level for mAbs in plants ever reported (18). The rapid high-level production and assembly of pHu-E16 and the availability of a scalable and cGMP-compliant processing scheme convincingly demonstrate the viability of this system for the more large-scale cost-effective production of mAbs.

Hu-E16 derived from mammalian cells is highly potent against almost all WNV strains because it binds a conserved epitope and blocks viral fusion (11). Compared to the parent mHu-E16, pHu-E16 showed equivalent binding kinetics and neutralization activity in vitro. However, pHu-E16 did not shift the neutralization curve to lower antibody concentrations in the presence of human C1q. C1q augments the neutralization potency of mHu-E16 IgG1 by ~3-fold (21). SPR studies confirmed that pHu-E16 bound less well to human C1q compared to mHu-E16. This impairment was likely caused by the slightly different carbohydrate modifications on plant-derived antibodies (see below). Overall, the functional studies in vitro suggest that pHu-E16 and mHu-E16 had similar but not identical properties.

Whereas plant-derived mAbs or mAb fragments are currently in clinical trials as a cancer vaccine or as topical treatment for tooth decay, and a mAb as postexposure rabies prophylaxis has been reported (23, 24), our results demonstrate the efficacy of a plant-produced mAb against a lethal infection several days after exposure. A single dose of pHu-E16 protected mice when administered 2 or 4 days after WNV infection. Because WNV has already disseminated to the brain by day 4 (9, 25), pHu-E16 improves survival after the virus has spread into the CNS. Although our in vitro results showed a decrease in the binding to human C1q and an absence of C1q-augmented WNV neutralization by pHu-E16, this effect did not alter potency in vivo in mice, which is likely because Hu-E16 binds mouse C1q less well than human C1q. Indeed, we previously did not observe a difference in protection of the mHu-E16 IgG1 between wild-type and $C1q^{-/-}$ mice and reported a smaller shift in the neutralization potency in vitro of mHu-E16 with murine C1q (21).

The N-linked glycosylation of proteins in plants is generally similar to that in mammalian cells. However, plants have unique plant-specific β-1,2-xylose and core α-1,3-fucose residues on complex N-linked glycans and lack terminal β1,4-Gal and N-acetylneuraminic acid (Neu5Ac) residues (16). The impact of such differences on the activity of mAb therapeutics in vivo has not been evaluated, although glycan variations in the Fc region of IgG modulate the binding and activation of C1q (26, 27). The difference between plant and mammalian glycosylation patterns raises concerns for the immunogenicity of plant-derived mAb therapeutics. The possibility of inducing plant-glycan specific antibodies could reduce therapeutic efficacy by accelerating clearance from plasma or cause potential adverse effects through immune complex formation. Immunization studies with plant glycoproteins in different animal models have yielded inconsistent results: Rats and rabbits develop antibodies to plant-specific xylose and α-1,3-fucose, yet mice generate no antibody response against these glycans (28, 29). Moreover, no adverse effects were observed in patients with topical application of plant-produced mAbs with plant unique carbohydrates (30, 31). To date, the immunogenicity of systemic-administered plant-produced mAbs has not been evaluated in humans.

To avoid problems associated with plant-specific glycans, "humanized" *N. benthamiana, Arabidopsis thaliana,* and *Lemna minor* plant lines have been generated (32–34). In these plants, enzymes for the biosynthesis of plant-specific glycans are inactivated, resulting in structurally equivalent mAbs as those derived in mammalian cells. Moreover, the glycan uniformity of mAbs produced by these optimized plant lines is better than those from mammalian-cell cultures. Indeed, an anti-human CD30 mAb produced from these genetically modified plants had only a single predominant N-glycan species and showed improved antibody-dependent cell-mediated cytotoxicity compared to the same mAb produced in mammalian cells (34). We speculate that the therapeutic utility of pHu-E16 can be improved by expression in such humanized *N. benthamiana* lines.

In summary, we demonstrate that plant-derived mAbs can function effectively as postexposure therapy against a potentially lethal infectious disease. Plants are an efficient platform to produce Hu-E16 with high-yield, speed, scalability, and cost effectiveness, satisfying all major metrics for a successful therapeutic candidate. This technology can be readily applied

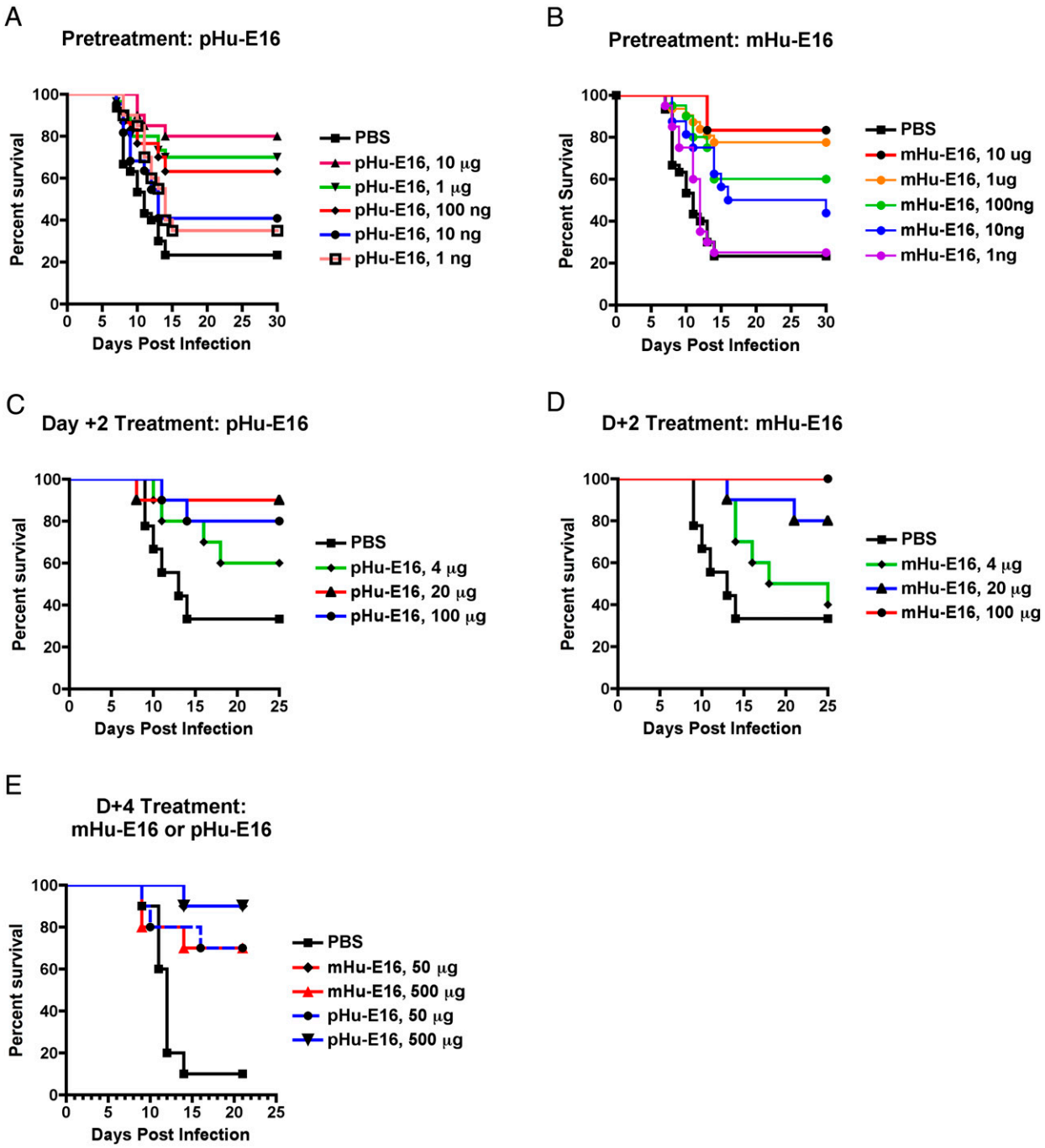


Fig. 5. pHu-E16 and mHu-E16 mediated protection in mice. (*A*)–(*B*) Five week-old C57BL/6 mice were passively transferred saline or serial 10-fold increases in dose (ranging from 0.001 to 10 μg, $N > 20$ per dose) of pHu-E16 (*A*) or mHu-E16 (*B*) via an intraperitoneal route on the same day as subcutaneous infection with 10^2 pfu of WNV. Survival data from at least two independent experiments were analyzed by the log-rank test, and IC_{50}s were calculated by nonlinear regression of survival percentage at each mAb dose. As indicated in the text, both pHu-E16 and mHu-E16 were highly protective; there was no significant difference in IC_{50} values ($P > 0.6$). (*C*)–(*E*) C57BL/6 mice were infected with 10^2 pfu of WNV and then given a single dose of the indicated doses of pHu-E16 or mHu-E16 via an intraperitoneal route at (*C* and *D*) day +2 or (*E*) day +4 after infection. Survival data from at least two independent experiments ($N = 20$ per dose) were analyzed by the log-rank test.

to antiviral antibodies against other emerging infectious disease threats and may be most useful in resource-poor settings such as the developing world.

Materials and Methods

Construction of pHu-E16 mAb Expression Vectors. The coding sequences of Hu-E16 mAb LC and HC (9) were optimized in silico with *N. benthamiana*-optimized codons by using an algorithm described in ref. 17. An 18-bp sequence coding for a Ser-Glu-Lys-Asp-Glu-Leu hexapeptide endoplasmic reticulum-retention signal was added to the C terminus of the HC gene. Optimized LC and HC sequences were synthesized (DNA 2.0) and cloned into the 5′ modules of plant expression vectors pICH21595 and pICH11599 of the MagnICON system as described previously (18).

Agroinfiltration of *N. benthamiana*. Plant expression vectors were individually transformed into *A. tumefaciens* GV3101 by electroporation as previously described (35). Wild-type *N. benthamiana* plants were grown in a greenhouse with a 16/8 hr light/dark cycle at 25 °C for 5 weeks. Plant leaves were coagroinfiltrated with GV3101 strains containing the LC and HC 5′ modules

along with their respective 3′ modules and an integrase construct as described previously (18).

Extraction of Total Protein from Plant Leaves. Agroinfiltrated *N. benthamiana* leaves were harvested 4, 5, 6, 7, 8, 9, and 10 days postinfiltration (dpi) for evaluating the temporal pattern of pHu-E16 mAb expression. For other protein analysis, plant leaves were harvested 7 dpi. Total leaf protein was extracted by homogenization with extraction buffer (PBS, 1 mM EDTA, 10 mg/mL sodium ascorbate, 10 μg/mL leupeptin, and 0.3 mg/mL PMSF) by using a FastPrep machine (Bio101) following the manufacturer's instruction. The crude plant extract was clarified by centrifugation at 14, 000 × *g* for 10 min at 4 °C.

SDS-PAGE and Western Blot Analysis. Protein samples were subjected to 4–20% gradient SDS-PAGE under reducing (5% vol/vol β-mercaptoethanol) or nonreducing conditions. Gels were either stained with Coomassie blue or used to transfer proteins onto PVDF membranes. HRP-conjugated antibodies against human-kappa LC or gamma HC (Southern Biotech) were used for Western blot analysis.

ELISA. An ELISA designed to detect the assembled form of mAb (with both LC and HC) was performed to quantify pHu-E16 expression (18). Plates were coated with a goat anti-human-gamma HC antibody (Southern Biotech). After incubation with plant protein extract, a HRP-conjugated anti-human-kappa LC antibody was used as the detection antibody. mHu-E16 was used as a reference standard (9).

The ELISA for examining the binding of pHu-E16 to WNV E DIII was performed as described (19). DIII (amino acids 296–415) of the New York 1999 strain of WNV purified from *Escherichia coli* (19) was immobilized on microtiter plates. An HRP-conjugated anti-human-kappa LC antibody was used as the detection antibody. The plates were developed with tetramethylbenzidine substrate (KPL Inc).

Purification of pHu-E16. pHu-E16 was purified from *N. benthamiana* leaves by a three-step purification protocol comprised of ammonium sulfate precipitation, protein A affinity, and DEAE-anion exchange chromatographies. A detailed purification scheme is provided in *SI Text*.

Cells, RVPs, and Antibody Neutralization. WNV RVPs were produced in HEK293T cells (10, 20). The neutralization potency of pHu-E16 or mHu-E16 was measured in the presence or absence of purified human C1q protein (Complement Technologies). Neutralization potency was calculated as a function of the concentration of antibody required to block 50% of the infection events by using nonlinear regression analysis (GraphPadPrism4). mHu-E16 was produced in CHO cells and purified by protein A affinity and size-exclusion chromatography as described (9).

Recombinant Protein Expression and Yeast Surface Display. The WNV E ectodomain (residues 1–404) and DIII (residues 296–404) bacterial expression constructs have been described (19). Proteins were expressed in *E. coli* and purified after an oxidative refolding protocol (19) and size-exclusion chromatography.

Yeast expressing WNV DIII were generated, stained with mAbs, and analyzed with a Becton Dickinson FACSCalibur flow cytometer as described (9).

SPR. Affinity measurement of mAb for DIII or the E ectodomain of WNV was performed by SPR. The binding of human C1q to mHu-E16 and pHu-E16 was also analyzed by SPR. Details of the SPR methods are provided in *SI Text*.

Efficacy of mAbs in Vivo. C57BL/6 mice were housed in a pathogen-free mouse facility. Studies were performed with approval from the Washington University School of Medicine Animal Safety Committee. Mice received a single dose of purified pHu-E16 or mHu-E16 by intraperitoneal injection the same day, 2 days after, or 4 days after footpad infection with 10^2 pfu of WNV strain 3000.0259. Kaplan–Meier analysis of survival data was performed by using the log-rank test. IC_{50} analyses were performed by nonlinear regression, and statistical significances were determined by using ANOVA and F tests.

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1. Hubalek Z, Halouzka J (1999) West Nile fever—A reemerging mosquito-borne viral disease in Europe. *Emerg Infect Dis*, 5:643–650.
2. Bode AV, et al. (2006) West Nile virus disease: A descriptive study of 228 patients hospitalized in a 4-county region of Colorado in 2003. *Clin Infect Dis*, 42:1234–1240.
3. Diamond MS, Klein RS (2006) A genetic basis for human susceptibility to West Nile virus. *Trends Microbiol*, 14:287–289.
4. Lim JK, et al. (2008) Genetic deficiency of chemokine receptor CCR5 is a strong risk factor for symptomatic West Nile virus infection: A meta-analysis of 4 cohorts in the US epidemic. *J Infect Dis*, 197:262–265.
5. Diamond MS (2009) Progress on the development of therapeutics against West Nile virus. *Antiviral Res*, 83:214–227.
6. Furuta Y, et al. (2009) T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Res*, 82:95–102.
7. Morrey JD, et al. (2008) Efficacy of orally administered T-705 pyrazine analog on lethal West Nile virus infection in rodents. *Antiviral Res*, 80:377–379.
8. Diamond MS (2009) Mechanisms of evasion of the type I interferon antiviral response by flaviviruses. *J Interferon Cytokine Res*, 29:521–530.
9. Oliphant T, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nat Med*, 11:522–530.
10. Pierson TC, et al. (2007) The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. *Cell Host Microbe*, 1:135–145.
11. Thompson BS, et al. (2009) A therapeutic antibody against West Nile virus neutralizes infection by blocking fusion within endosomes. *PLoS Pathog*, 5:e1000453.
12. Morrey JD, et al. (2006) Humanized monoclonal antibody against West Nile virus envelope protein administered after neuronal infection protects against lethal encephalitis in hamsters. *J Infect Dis*, 194:1300–1308.
13. Samuel MA, et al. (2007) Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. *Proc Natl Acad Sci USA*, 104:17140–17145.
14. Chen Q (2008) Expression and purification of pharmaceutical proteins in plants. *Biol Eng*, 1:291–321.
15. Vitale A, Pedrazzini E (2005) Recombinant pharmaceuticals from plants: The plant endomembrane system as bioreactor. *Mol Interv*, 5:216–225.
16. Gomord V, et al. (2004) Production and glycosylation of plant-made pharmaceuticals: The antibodies as a challenge. *Plant Biotechnol J*, 2:83–100.
17. Villalobos A, et al. (2006) Gene Designer: A synthetic biology tool for constructing artificial DNA segments. *BMC Bioinformatics*, 7:285–293.
18. Giritch A, et al. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc Natl Acad Sci USA*, 103:14701–14706.
19. Oliphant T, et al. (2007) Induction of epitope-specific neutralizing antibodies against West Nile virus. *J Virol*, 81:11828–11839.
20. Pierson TC, et al. (2006) A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection. *Virology*, 346:53–65.
21. Mehlhop E, et al. (2009) Complement protein C1q reduces the stoichiometric threshold for antibody-mediated neutralization of West Nile virus. *Cell Host Microbe*, 6:381–391.
22. Engle M, Diamond MS (2003) Antibody prophylaxis and therapy against West Nile Virus infection in wild type and immunodeficient mice. *J Virol*, 77:12941–12949.
23. Ko K, et al. (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proc Natl Acad Sci USA*, 100:8013–8018.
24. McCormick AA, et al. (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: Safety and immunogenicity in a phase I clinical study. *Proc Natl Acad Sci USA*, 105:10131–10136.
25. Diamond MS, et al. (2003) B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J Virol*, 77:2578–2586.
26. Raju TS (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr Opin Immunol*, 20:471–478.
27. Wang F, et al. (2006) Structural and functional characterization of glycosylation in an immunoglobulin G1 to Cryptococcus neoformans glucuronoxylomannan. *Mol Immunol*, 43:987–998.
28. Chargelegue D, et al. (2000) A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res*, 9:187–194.
29. Jin C, et al. (2008) A plant derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. *Glycobiology*, 18:235–241.
30. Zeitlin L, et al. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat Biotechnol*, 16:1361–1364.
31. Ma JK, et al. (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat Med*, 4:601–606.
32. Schahs M, et al. (2007) Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. *Plant Biotechnol J*, 5:657–663.
33. Strasser R, et al. (2008) Generation of glyco-engineered Nicotiana benthamiana for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol J*, 6:392–402.
34. Cox KM, et al. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. *Nat Biotechnol*, 24:1591–1597.
35. Huang Z, et al. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnol Bioeng* doi:10.1002/bit.22652.