Development of Resistance to Passive Therapy with a Potently Neutralizing Humanized Monoclonal Antibody against West Nile Virus

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Previous studies have established the therapeutic efficacy of humanized E16 (hE16) monoclonal antibody against West Nile virus in animals. Here, we assess the potential for West Nile virus strains encoding mutations in the hE16 epitope to resist passive immunotherapy and for the selection of neutralization escape variants during hE16 treatment. Resistance to hE16 in vivo was less common than expected, because several mutations that affected neutralization in vitro did not significantly affect protection in mice. Moreover, the emergence of resistant variants after infection with fully sensitive virus occurred but was relatively rare, even in highly immunocompromised B and T cell–deficient RAG mice.

West Nile virus (WNV) is a mosquito-borne flavivirus that has emerged as the primary cause of epidemic encephalitis in the United States. Although case numbers have declined somewhat since the peak of activity in 2002 and 2003, virus transmission continues throughout the continental United States, and thousands of cases of neurological disease have been reported each year since 2004. At present there is no WNV vaccine or antiviral therapy approved for use in humans, although promising treatment results have been observed in case reports with purified immunoglobulin derived from Israeli donors [1, 2].

The potential of passive immunotherapy for the treatment of WNV infection has led to the development and evaluation of potently neutralizing human or humanized mouse monoclonal antibodies (MAbs) [3, 4]. One therapeutic candidate, humanized E16 (hE16), binds to a highly conserved epitope on the upper lateral ridge of WNV envelope (E) protein domain III (E-DIII) and neutralizes WNV at low stoichiometric occupancy, apparently by inhibiting conformational changes in E that are required for fusion of the virus with host cell membranes [3, 5, 6]. However, mapping studies by several groups have identified mutations in the hE16 epitope that significantly reduce or abolish the binding of this and other neutralizing WNV MAbs [3, 7–9]. A few of these mutations are found in WNV strains isolated in the field [8], suggesting that the efficacy of hE16 could be limited. Furthermore, flaviviruses have error-prone genome replication, resulting in significant genetic diversity within any individual isolate. Indeed, this property has been used as the basis for in vitro selection of variants resistant to a particular selective pressure, such as that imposed by antiviral inhibitors or neutralizing MAbs. The potential for in vivo selection of MAb-resistant variants of flaviviruses has not been examined in detail.

In the present study, we assessed (1) the potential for WNV strains encoding engineered mutations in the hE16 binding site to resist passive immunotherapy with hE16 in 2 mouse models of WNV neuroinvasive disease and (2) the potential for neutralization-resistant variants to be selected in vivo during hE16 treatment.

Methods. All WNV strains and infectious clone-derived variants used in the present study (table 1 and Results) were grown and plaque titered on Vero cells. Neutralization assays were performed on Vero or BHK-21 cells, as described elsewhere [3]. RNA extractions, reverse-transcription polymerase chain reaction, and nucleotide sequencing of the premembrane and E coding regions of WNV genomes were performed using protocols and primers that have been described elsewhere [7].

Swiss Webster mice (female; 3–4 weeks of age) were obtained from Harlan Laboratories, and wild-type and congenic RAG C57BL/6 mice (female; 5 weeks of age) were obtained from the Jackson Laboratory. All mice were housed in animal biosafety level 3 facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International,
and experiments were conducted under protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch or the Washington University School of Medicine. Details of individual passive-protection experiments are described below.

**Results.** Previous crystallographic and epitope mapping studies have suggested that hE16 has key contacts at residues 307, 330, and 332 of the WNV E protein [3, 5]. The ability of hE16 to neutralize selected WNV strains and NY99 infectious clone–derived variants encoding single amino acid changes at these residues was initially assessed by a plaque-reduction neutralization assay on Vero cells. These viruses have been shown to variably escape neutralization by other anti–WNV E-DIII–specific neutralizing MAbs [7, 8]. Notably, only the mutation T332K resulted in a substantial loss of hE16 neutralizing activity, whereas other mutations (K307R, T330I, and T332A/M) showed only modest changes in neutralization compared with the wild-type lineage 1 NY99 virus (figure 1A). Lineage 2 South African strain H442 (SA58), isolated in 1958 from a human patient, normally encodes a lysine at residue 332 and has been reported to be resistant to neutralization by several E-DIII–reactive antibodies raised against NY99 [7]; this virus was also resistant to neutralization by hE16, whereas an SA58 variant encoding threonine at 332 [7] was efficiently neutralized (figure 1B).

Two independent mouse challenge models were used to assess the protection provided by hE16 against the neutralization-sensitive and neutralization-resistant WNV strains and variants. Groups of outbred Swiss Webster mice, which are highly susceptible to peripheral challenge with neuroinvasive WNV strains, were given 100-μg doses of hE16 or phosphate-buffered saline only and challenged 24 h later with 1 × 10^2 pfu of each WNV strain or variant (equivalent to ∼100 median lethal doses in each case). Alternatively, groups of inbred C57BL/6 mice, which are more resistant to WNV NY99 and have been used in previous evaluations of hE16 [3, 10], were challenged with 1 × 10^2 pfu of each virus and treated 2 days after infection with 100 μg of hE16 or phosphate-buffered saline only and challenged 24 h later with 1 × 10^2 pfu of each WNV strain or variant (equivalent to ∼10 median lethal doses in each case). Notably, only the mutation T332K resulted in a substantial loss of hE16 neutralizing activity, whereas other mutations (K307R, T330I, and T332A/M) showed only modest changes in neutralization compared with the wild-type lineage 1 NY99 virus (figure 1A). Lineage 2 South African strain H442 (SA58), isolated in 1958 from a human patient, normally encodes a lysine at residue 332 and has been reported to be resistant to neutralization by several E-DIII–reactive antibodies raised against NY99 [7]; this virus was also resistant to neutralization by hE16, whereas an SA58 variant encoding threonine at 332 [7] was efficiently neutralized (figure 1B).

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Figure 1. Neutralization by humanized E16 (hE16) monoclonal antibody of lineage 1 West Nile virus (WNV) strain NY99ic and the K307R, T330I, and T332A/K/M variants (A); lineage 2 WNV strain SA58 and the K332T variant (B); and K307E escape variants selected in vivo from hE16-treated RAG mice (C). Data are an average of values from 2–4 independent experiments performed in triplicate on Vero (A and B) or BHK21-15 (C) cells.

MAbs, we questioned whether this commonly occurred under selective pressure during the course of treatment. To assess the potential for selection of resistant variants in vivo during MAb treatment, Swiss Webster mice were given 100 µg of hE16 and then exposed to a high dose (1 × 10⁵ pfu) of NY99 virus. This challenge dose was chosen on the basis of the known frequency of MAb escape variants selected from this viral population during earlier experiments [7] and because this represents a virus dose reliably introduced during feeding by Culex mosquitoes [11]. All untreated control mice died by day 9 after infection (average survival time, 7.4 days; standard deviation, 1.0 days). Two of 10 treated mice showed signs of neuroinvasive disease and were euthanized on days 8 and 9. Virus was isolated from the brain of each mouse, and the isolates were found to encode single-nucleotide changes in E coding for the mutation K307E or T332M. Both isolates retained the highly neuroinvasive phenotype of the parental NY99 virus (data not shown).

Immune dysfunction is a significant risk factor for the development of severe WNV disease (reviewed in [12]), suggesting that therapeutic antibodies and other treatments may be especially important for immunodeficient patients. To assess the potential for the emergence of resistant variants during treatment of an immunocompromised host, a group of 30 B and T cell–deficient RAG mice was exposed to a low dose (1 × 10³ pfu) of strain WNV-NY2000 (3000-0259), which has an E gene sequence identical to that of NY99 [13], and then treated with 500-µg doses of hE16 administered 1 day after infection and at 14-day intervals thereafter. Although 24 mice remained healthy over a period of several months, 6 mice became ill and were either found dead or euthanized on days 18 (2 mice), 20 (2 mice), 32 (1 mouse), and 34 (1 mouse). Brains recovered from the latter 2 mice each yielded WNV encoding the K307E E protein mutation.

Consistent with the findings of a previous study [3], the recovered K307E variants were resistant to in vitro neutralization (figure 1C) or in vivo protection (table 1) by hE16, whereas the recovered T332M variant was only modestly resistant to E16 neutralization in vitro (data not shown) and in vivo (table 1), similar to the NY99ic T332M variant.

Discussion. The use of polyclonal or monoclonal immunotherapy is currently being explored for the treatment of infections caused by many families of viruses and clearly offers a promising approach for postexposure treatment of flavivirus infections. In the case of hE16 for treatment of WNV infection, the presence of naturally occurring mutations in some WNV strains at the MAb-binding site suggests that treatment coverage will not be universal, as evidenced by the resistance of lineage 2 strain SA58 in vitro and in vivo. Nonetheless, hE16 still afforded strong protection against variants encoding other mutations that increase resistance to neutralization but that do not abolish binding. Analysis of >650 derived amino acid sequences in the National Center for Biotechnology Information protein database representing human, avian, equine, or mosquito North American WNV isolates during 1999–2005 identified none with amino acid variation at positions 307, 330, or 332, which could impair hE16 recognition and neutralization.

The present experiments have also demonstrated the potential for in vivo selection of MAb escape variants. Although surprisingly infrequent, in vivo resistance may occur via selection of a preexisting mutant subpopulation after exposure to higher doses of virus, a phenomenon that has also been reported recently in passively immunized monkeys exposed to 1 × 10⁴ pfu of dengue virus type 2 [14]. Alternatively, resistance can develop through the emergence of variant viruses during prolonged treatment.
The length of time required for the emergence of the resistant K307E variants after challenge with $1 \times 10^2$ pfu in RAG mice (up to 34 days), compared with the rapid selection of variants after challenge with $1 \times 10^3$ pfu in Swiss Webster mice (8–9 days), was somewhat surprising. Given the inherent error rate of the flavivirus RNA polymerase (∼1 mutation per genome replication), the occurrence of resistant mutations at the genetic level should be expected to occur rapidly. The delay in the emergence of resistance in RAG mice could be explained by packaging of early variant genomes in virions that also contain levels of wild-type E protein sufficient to allow neutralization by hE16. This is possible because flavivirus RNA can be encapsidated by structural proteins in trans [15] and because complete neutralization (>99%) of WNV virions by hE16 requires occupancy of <50% of the available epitopes [6]. Although additional studies are necessary, high levels of circulating hE16 with a long half-life may adequately neutralize virions composed of wild-type and resistant E protein variants to prevent the rapid or eventual emergence of pathogenic escape variants, even in a highly immunocompromised subject.

Overall, these findings suggest that resistance to neutralizing anti-WNV antibody therapy can occur in vivo, although at frequencies that are perhaps lower than would be anticipated on the basis of analogous studies of resistance to antiviral drugs that target viral replication. However, our data suggest that clinical evaluation of hE16 and other MAb therapeutics for flavivirus infection should monitor for the selection or emergence of resistant variants, especially in immunocompromised patients, who are a likely target population for the treatment of severe WNV infection. Such studies should include characterization of viral populations for detection of resistance and possibly the use in some patients of combinations of neutralizing antibodies that recognize distinct epitopes.

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References