Co-circulation of West Nile Virus Variants, Arizona, USA, 2010

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West Nile virus (WNV) emerged in the Americas in 1999 after an outbreak of neuroinvasive disease in humans, birds, and horses in New York, New York. The virus spread rapidly across North America and was detected in Arizona in 2003. In 2004, Arizona experienced a large outbreak (214 neuroinvasive cases and 15 deaths, the largest number of cases for a state in that year), followed by ≈50–60 neuroinvasive cases per year during 2005–2008.

An outbreak in 2010 resulted in 107 neuroinvasive cases and 15 deaths, the largest number of cases for a state that year. WNV activity in Maricopa County, which includes the city of Phoenix and surrounding municipalities, where numerous human cases were reported in the town of Gilbert, was investigated by a team from the Centers for Disease Control and Prevention (Fort Collins, CO, USA) working with local and state public health officials. Epidemiologic and entomologic findings from those investigations have been reported (1,2). We describe the molecular and phenotypic characterization of WNV isolates obtained from that outbreak.

The Study

As part of the Centers for Disease Control and Prevention investigation, Vero cell culture isolates of WNV were obtained during a 2010 outbreak in Maricopa County, Arizona, USA, demonstrated co-circulation of 3 distinct genetic variants, including strains with novel envelope protein mutations. These results highlight the continuing evolution of WNV in North America and the current complexity of WNV dispersal and transmission.

Molecular analysis of West Nile virus (WNV) isolates obtained during a 2010 outbreak in Maricopa County, Arizona, USA, demonstrated co-circulation of 3 distinct genetic variants, including strains with novel envelope protein mutations. These results highlight the continuing evolution of WNV in North America and the current complexity of WNV dispersal and transmission.

The presence of the E-312 coding mutation was of particular interest. Most sequences for lineage 1 WNV strains encode Leu at E-312, whereas lineage 2 strains encode Val or Ala. E-312 lies in an exposed loop of EIII, where it may contribute to the antigenic and/or putative receptor binding activities of the domain (6). To assess the effects of the Leu→Ile mutation and tolerance for alternative amino acid

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substitutions at this site, we engineered 5 E-312 mutants by using an NY99 infectious clone (NY99ic) encoding alternative amino acids that are each only a single nucleotide substitution away from the wild-type Leu codon (CUU) (Table 2). (Although Phe also requires only a single nucleotide change from the Leu codon, it occurs naturally in some lineage 1 and 2 WNV strains and was not included in this analysis.) Mutagenesis, in vitro ligation, and transcription of genome equivalent RNA and virus recovery were performed as described (11). All mutant viruses were readily recovered from transfected Vero cells and grew to peak titers comparable to the parental NY99ic virus, and the introduced mutations were stable through 3 additional Vero cell passages.

Virulence of AZ10-75 and AZ10-581 (cluster A), AZ10-91 (cluster B), AZ10–892 (cluster C), and the recovered NY99ic E-312 mutants was compared with wild-type NY99ic after intraperitoneal inoculation of 3- to 4-week-old female Swiss Webster mice (Table 2) as described (11). The AZ10 strains and all E-312 mutants had 50% lethal doses (LD$_{50}$s) and average survival times comparable with that of NY99ic, with the exception of the L312P mutant, which was markedly attenuated (630 PFU/LD$_{50}$ vs. 0.3 PFU/LD$_{50}$, and prolonged survival time). Antigenic characteristics of viruses encoding L312I mutations were also compared by assessing their neutralization by monoclonal antibodies 7H2 and 5H10 and a polyclonal rabbit antiserum against the EIII region as described (5). AZ10-75, AZ10-581 and all E-312 variants were effectively neutralized by the monoclonal antibodies and antiserum (Table 2).

**Conclusions**

Detection of a Leu→Ile mutation at residue 312 in EIII and its apparent persistence since first detection in the 2005 South Dakota isolate were major findings given the variable nature of this residue in other WNV lineages and the presumed importance of EIII in antigenicity and receptor binding activity of E protein. Although other parameters that could contribute to the selection of E-312 variants in nature remain to be explored, analysis of engineered E-312 mutants suggested that most nonsynonymous single nucleotide mutations at this site, including the Leu→Ile substitution in some AZ10 isolates, have no major effect on virulence of NY99-derived WNV in mice and were not associated with major changes in antigenicity.

The 2010 epidemic of WNV disease in the Maricopa County area was associated with co-circulation of 3 distinct WNV variants. The high mouse virulence of all strains tested suggests that signature nucleotide and amino acid changes associated with the different genotypes involved (4,10) were probably not linked to major changes in virulence for mammalian hosts, and that all 3 variants might have contributed to human disease in the 2010 outbreak. Some nonstructural protein mutations have been shown to influence virulence in avian hosts (12), and that phenotype remains to be determined for these strains or other recently identified WNV variants.

Detection of multiple sequence variants has been associated with outbreaks in the United States in as early as 2002 (13), but co-circulation of variants in relatively narrow spatial and temporal contexts, such as that observed in
Maricopa County, has been a feature of recent investigations, including WNV transmission in El Paso, Texas, and Ciudad Juarez, Mexico, during 2010 (14) and during the 2012 outbreak in Dallas, Texas (15). These findings highlight the current complexity and dynamic nature of WNV transmission in the United States and suggest that co-circulation of multiple variants, with continued introduction or reintroduction of variants into disease-endemic areas, will be a major feature of future outbreaks.

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Table 1. Nucleotide divergence for open reading frame sequences between representative West Nile virus strains and other closely related strains from North America, Arizona, USA, 2010*

| Strain   | NY99 | Cluster A (%) | Cluster B (%) | Cluster C (%) |
|----------|------|---------------|---------------|---------------|
| AZ10–581 | 0.58 | 0.25–0.52     | 0.66–0.85     | 0.61–1.10     |
| AZ10–91  | 0.66 | 0.74–1.04     | 0.49–0.52     | 0.69–1.16     |
| AZ10–892 | 0.85 | 0.84–1.18     | 0.92–1.04     | 0.27–0.62     |

*Strains used for analysis in each cluster are those color coded with representative AZ10 strains and shown in Figure 2, panel B. For cluster A: BSL2–2005 (SD 2005; GenBank accession no. DQ666452), BSL2–09 (NV 2009, JF957175), BSL4–11 (AZ 2011, JQ700438); cluster B: v4530 (NM 2005, HM756677), 144WG–AZ06PI (AZ 2006, GQ507482); cluster C: v4798 (NY 2004, HM756671), BSL3–10 (AZ 2010, JF957186), BSL2–10 (AZ 2010 JF957185), BSL23–11 (AZ 2011, JQ700440).
Table 2. Mouse virulence and antigenic characteristics of selected isolates of West Nile virus and NY99ic-derived E-312 variants, Arizona, USA, 2010

| Strain/variant   | Mouse neuroinvasiveness | Neutralization indices ± SD           |
|------------------|-------------------------|---------------------------------------|
|                  | ip LD₅₀, PFU           | AST ± SD (days)                       | 7H2      | 5H10    | α-EIII   |
| NY99ic           | 0.3                     | 8.7 ± 1.8                             | 1.5 ± 0.2| 1.5 ± 0.2| 2.4 ± 0.3|
| AZ10–75          | 0.5                     | 8.7 ± 2.2                             | 1.3 ± 0.5| 1.3 ± 0.3| 2.1 ± 0.3|
| AZ10–581         | 0.8                     | 9.6 ± 2.4                             | 1.2 ± 0.2| 1.3 ± 0.1| 2.5 ± 0.2|
| AZ10–91          | 0.5                     | 7.8 ± 0.9                             | ND       | ND      | ND       |
| AZ10–892         | 0.3                     | 8.6 ± 2.0                             | ND       | ND      | ND       |
| NY99–312F        | 1.3                     | 9.8 ± 1.9                             | 1.5 ± 0.4| 1.5 ± 0.4| 2.5 ± 0.4|
| NY99–312H        | 0.8                     | 8.5 ± 1.9                             | 2.0 ± 0.1| 1.7 ± 0.3| 2.9 ± 0.0|
| NY99–312I        | 0.3                     | 8.5 ± 1.4                             | 1.8 ± 0.8| 1.4 ± 0.3| 2.5 ± 0.4|
| NY99–312P        | 630                     | 13.0 ± 0.0                            | 2.2 ± 0.1| 2.0 ± 0.1| 2.6 ± 0.2|
| NY99–312R        | 2.0                     | 8.5 ± 2.2                             | 1.8 ± 0.2| 1.7 ± 0.1| 2.7 ± 0.0|

*Average survival time (AST) for each strain/variant was determined on the basis of animals in all dose groups that did not survive. Values significantly different (p<0.05 by Student’s t-test) from NY99ic are indicated in boldface. ip, intraperitoneal; LD₅₀, 50% lethal dose; ND, not determined.

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