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# West Nile virus envelope proteins: nucleotide sequence analysis of strains differing in mouse neuroinvasiveness

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**Several neuroinvasive and non-neuroinvasive West Nile (WN) viruses were characterized by nucleotide sequencing of their envelope (E) protein regions. Prolonged passage in mosquito cells caused loss of neuroinvasiveness and acquisition of an *N*-linked glycosylation site, which is utilized. Limited passage in cell culture also caused glycosylation but not attenuation, suggesting that glycosylation may not be directly responsible for attenuation and that a second mutation ($L_{68} \rightarrow P$) may also be involved. A monoclonal antibody-neutralization escape mutant with a substitution at residue 307, a site common to other flavivirus escape mutants, was also attenuated. A partially neuroinvasive revertant regained the parental E sequence, implying that determinants outside of the E region may also influence attenuation. Data suggest that the neuroinvasive determinants may be similar to those for other flaviviruses. Also, sequence comparison with the WN virus (Nigeria) strain revealed considerable divergence of the E protein at the nucleotide and amino acid levels.**

West Nile (WN) virus, a member of the Japanese encephalitis (JE) serogroup of the genus *Flavivirus*, family *Flaviviridae*, is distributed widely throughout Africa, the Middle East, parts of Europe (Camargue, France), the former Soviet Union, India and Indonesia (Hayes, 1989). The virus is classified into African and Middle Eastern subtypes based on antigenic variation of the envelope (E) protein (Hammam *et al.*, 1965; Price & O'Leary, 1967; Gaidamovich & Sokhey, 1973). WN virus causes an acute self-limited illness in humans (West Nile fever) (Hayes, 1989). However, central nervous system disease can occur (Pruzanski & Altman, 1962; Flatau *et al.*, 1981; George *et al.*, 1984). Many strains are neuroinvasive in mice (Smithburn *et al.*, 1940; Weiner *et al.*, 1970; Umrigar & Pavri, 1977) and a model of acute encephalitis has been developed (Halevy *et al.*, 1994). Using this model, it appears that loss of neuroinvasiveness correlates with *N*-linked glycosylation of the E protein, suggesting its importance for attenuation (Halevy *et al.*, 1994). For other flaviviruses, including JE virus, Murray Valley encephalitis (MVE) virus, tick-borne encephalitis (TBE) virus and louping ill (LI) virus, specific amino acid substitutions within the E protein are associated with loss of neuroinvasiveness (Cecilia & Gould, 1991; Hasegawa *et al.*, 1992; Sumiyoshi *et al.*, 1995; McMinn *et al.*, 1995*a*, *b*; Holzmann *et al.*, 1990; Jiang *et al.*, 1993), making it clear that this protein is important for neuropathogenesis.

To further characterize the molecular basis of WN virus neuroinvasiveness, we determined the nucleotide sequences of the E proteins of four previously reported viruses (Halevy *et al.*, 1994) and two new variants. The six viruses were as follows: (1) WNI, a parental strain of WN virus isolated in Israel; (2) WNI-25, a mosquito cell-passaged strain of WNI; (3) WNI-25A, a monoclonal antibody-neutralization escape mutant of WNI-25; (4) WNI-25R, recovered from the brain of a SCID mouse inoculated with WNI-25; (5) WNI-568, an early cell culture passage of the WNI parent; and (6) WNI-567, recovered from the brain of a mouse inoculated with WNI-568. The derivation of these viruses is indicated in Fig. 1(*a*).

C6/36 and Vero cells and growth of WN virus in these cells have been previously described (Halevy *et al.*, 1994). Plaque-purified viruses were amplified on C6/36 cells prior to RNA extraction or mouse inoculation. Reverse transcription was carried out with a 3′ anchoring primer corresponding to either nucleotides 2656–2669 or 2455–2474 of the reported sequence of the Nigerian strain of WN virus (WNN) (Wengler

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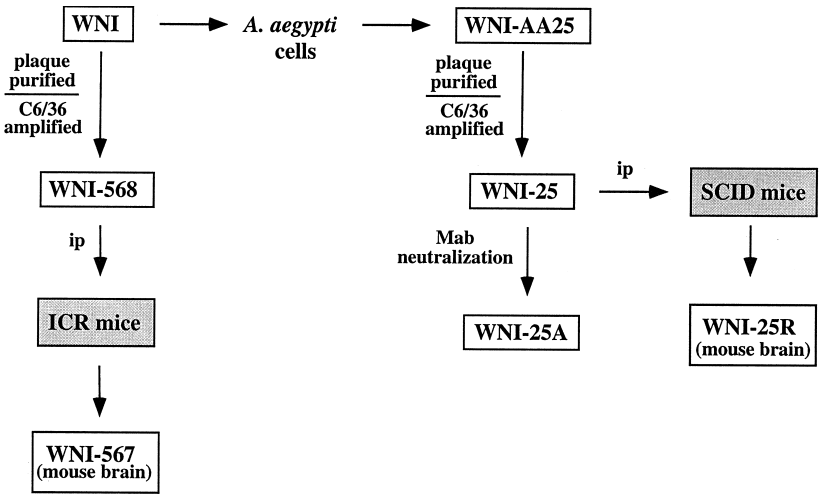
(*a*)




(*b*)

| Virus | Neuroinvasive | Neurovirulent | E-68‡ | E-154 to E-157‡ | Glycosylation§ | E-307‡ |
|---|---|---|---|---|---|---|
| WNI | Yes* | Yes* | L | SYST | No | K |
| WNI-568 | Yes† | Yes† | L, P, F | NYST | Yes | K |
| WNI-567 | Yes† | Yes† | L | NYST | Yes | K |
| WNI-25 | No* | Yes* | P | SNST | Yes | K |
| WNI-25A | No* | Yes* | L | SNST | Yes | E |
| WNI-25R | Partial* | Yes* | L | SYST | No | K |

* Neuroinvasiveness and neurovirulence are for normal adult mice (Halevy *et al.*, 1994). The respective p.f.u./$LD_{50}$ for WNI, WNI-25 and WNI-25A were 4.3, >$3.4\times10^6$ and >$2.0\times10^7$ by intraperitoneal inoculation, and 1.1, 10 and 13 after intracranial inoculation. For WNI-25R, p.f.u./$LD_{50}$ for intraperitoneal and intracranial inoculation were $1.4\times10^3$ and 1.5, respectively.

† WNI-568 and WNI-567 were neuroinvasive and neurovirulent in normal adult mice based on lethal doses of 15 p.f.u. given intraperitoneally or intracranially. The p.f.u./$LD_{50}$ for these viruses have not been determined.

‡ Predicted amino acid sequence (single letter code) at the indicated positions of the E protein. Heterogeneity was observed at position 68 in WNI-568 (see text). The *N*-linked oligosaccharide acceptor sites are underlined. E-307 is the site of the neutralization escape mutation.

§ Glycosylation refers to presence of an *N*-linked oligosaccharide acceptor site (underlined in preceding column) as determined by nucleotide sequence determination and utilization of the site for addition of carbohydrate, as indicated by immunoblot analysis (Halevy *et al.*, 1994; Fig. 3).

Fig. 1. (*a*) Passage history and derivation of WNI virus strains. WNI represents the parental WN virus originally obtained from the blood of a human case of West Nile fever in Israel, characterized by fever, headache and generalized weakness lasting several days (Goldblum *et al.*, 1954). The virus was passaged an unknown number of times in mouse brain. WNI-568 was isolated from the brain of an outbred mouse infected with WNI, and was subsequently propagated on Vero cells, plaque-purified three times on BHK cells and amplified on C6/36 cells. WNI-567 was isolated from the brain of a mouse which had received intraperitoneal inoculation (ip) with WNI-568. WNI-AA25 was derived by persistent infection of *Aedes aegypti* cells with WNI for a total of 25 subcultures. WNI-25 was derived by plaque-purification of WNI-AA25 twice on BHK cells and amplification on C6/36 cells. WNI-25A was derived by selection of a monoclonal antibody-neutralization-resistant variant of WNI-25 as described previously (Halevy *et al.*, 1994). WNI-25R was isolated from the brain of a SCID mouse which had received intraperitoneal inoculation with WNI-25, as described previously (Halevy *et al.*, 1994). (*b*) Properties of West Nile Israel viruses.

*et al.*, 1985) using Superscript reverse transcriptase (Gibco BRL). Single-stranded cDNA was amplified by PCR using the same 3′ primers, a 5′ primer corresponding to nucleotides 881–898 and Vent DNA polymerase (New England Biolabs). The primers used for PCR reactions contained either *Bam*HI or *Hin*dIII restriction sites to facilitate cloning of the products into pBluescript KS(+) (Stratagene). Sequencing was done by the dideoxy chain-termination method using Sequenase (USB). The entire E protein cDNA was analysed for two or more independent clones of each virus. Both the sense and antisense strands were sequenced for the parental virus (WNI). Immunoblotting of the E protein of WNI viruses and inoculation of outbred mice with WNI-567 and WNI-568 viruses was done as previously described (Halevy *et al.*, 1994).

Fig. 1(*b*) summarizes the sequence differences among the E proteins and the virulence properties of the six viruses in mice. Fig. 2 indicates the deduced amino acid sequences of the E proteins. The Nigerian strain, WNN, the only other strain for which a complete E protein sequence is available, is included for comparison. WNI and the Nigerian strain differ by approximately 20 and 5% at the nucleotide and amino acid levels, respectively. Substitutions were observed throughout

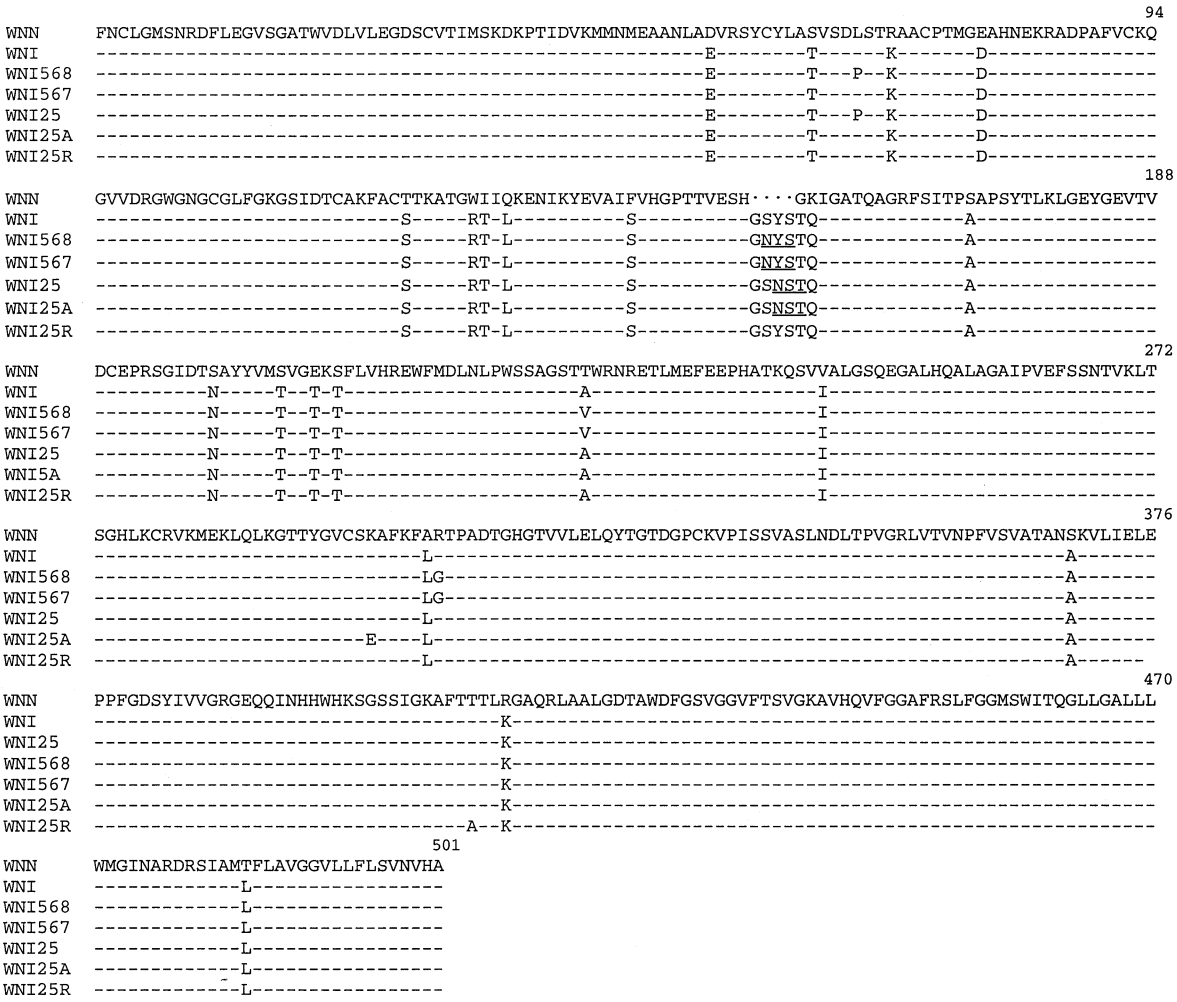



Fig. 2. Deduced amino acid sequences of the envelope proteins of the six WN virus strains WNI, WNI-568, WNI-567, WNI-25, WNI-25A and WNI-25R. WNN indicates the sequence of the Nigerian isolate of WN virus (Wengler *et al.*, 1985). The single letter code for amino acids is used. Dashed lines indicate identity between sequences. Proline at position 68 is indicated for WNI-568 because this was the most frequent clone-specific residue detected (see text and Fig. 1 *b*). The position of the conserved *N*-linked glycosylation site is underlined.

the E protein but were more frequent in certain areas, corresponding to regions of high sequence variability among flaviviruses (Gritsun *et al.*, 1995). These include the central and dimerization domains [domains I and II of the TBE virus model (Rey *et al.*, 1995)], where there are both cross-reactive and subtype-specific flavivirus epitopes (Mandl *et al.*, 1989; Rey *et al.*, 1995). The most notable difference was the presence of four extra amino acids in WNI (residues 153–156) relative to WNN. This occurs at the *N*-linked glycosylation site which is conserved among flaviviruses (residues 154–156 for TBE virus).

Comparison of WNI with the attenuated derivatives WNI-25 and WNI-25A revealed a restricted number of substitutions. A single base substitution (T → A) at nucleotide position 463 predicted the substitution of asparagine for tyrosine at residue 155 ($Y_{155}$ → N), and the introduction of an *N*-linked glycosylation site (N-S-T) at the conserved position in both WNI-25 and WNI-25A. This is consistent with electrophoretic analysis of their E proteins, which demonstrated *N*-linked glycosylation (Halevy *et al.*, 1994). This site is apparently acquired during passage in *Aedes aegypti* cells or possibly during amplification in C6/36 cells. WN virus is reported both to contain and not contain carbohydrate residues (Wright & Warr, 1985; Winkler *et al.*, 1987) and it is likely that differences in strains and passage histories influence glycosylation status. Studies on Kunjin and St Louis encephalitis viruses suggest the same conclusion (Adams *et al.*, 1995; Vorndam *et al.*, 1993). It appears that the WNI strain resembles these viruses rather than WNN in terms of potential glycosylation at the conserved site.

WNI-25 also contained a single base substitution at nucleotide position 203 (T → C), predicting replacement of proline for leucine at residue 68 ($L_{68}$ → P). Comparison of WNI-25 with WNI-25A, its monoclonal antibody-neutralization escape variant, revealed a nucleotide substitution (A →

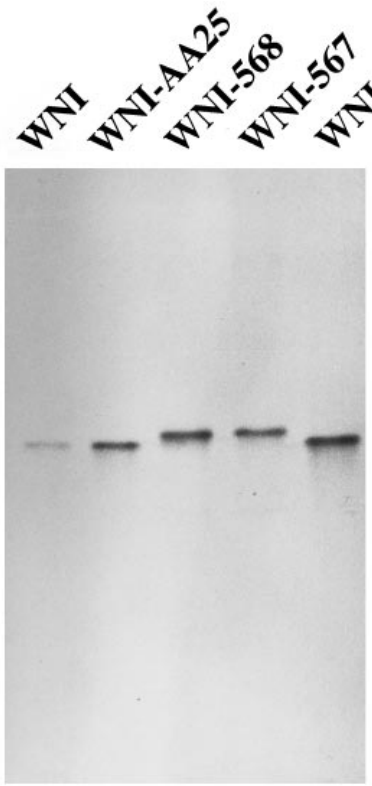



Fig. 3. Immunoblot of the E protein from the indicated virus strains after SDS–PAGE.

G) at position 919, predicting replacement of glutamate for lysine at amino acid residue 307 ($K_{307} \rightarrow E$). This substitution occurs at the site of neutralization escape mutants of both LI virus (residues 308 and 310; Jiang *et al.*, 1993) and dengue 2 virus (residue 307; Lin *et al.*, 1994), suggesting that an epitope broadly conserved in position among flavivirus E proteins is involved. WNI-25A unexpectedly contained leucine at position 68, which is characteristic of the parental WNI virus rather than WNI-25. This result was confirmed in repeated experiments. Reversion at this position presumably occurred during either selection for neutralization resistance or plaque purification of WNI-25A. Heterogeneity at position 68 was also observed in the WNI-568 virus after plaque purification in Vero cells and amplification in C6/36 cells (see Fig. 1*b*) suggesting that residue 68 may be under selective pressure in cell culture. The association of the $L_{68} \rightarrow P$ substitution with neuroinvasiveness is discussed further below. The role of the $K_{307} \rightarrow E$ substitution in attenuation of neuroinvasiveness is currently unclear. It is notable, however, that residue 307 is known to be a site involved in attenuation of LI virus escape mutants (Jiang *et al.*, 1993).

To further investigate the association of *N*-linked glycosylation with attenuation of neuroinvasiveness, two additional viruses derived from the WNI parent and exhibiting *N*-linked glycosylation of the E protein were also studied (WNI-568 and WNI-567). Both viruses were neuroinvasive [lethal dose < 15 p.f.u. after intraperitoneal injection (Fig. 1*b*)]. Both WNI-567 and WNI-568 also appear to have glycosylated E proteins (Fig. 3). The position of the *N*-linked glycosylation site differed from that of WNI-25 and WNI-25A by one residue (position 154–156 vs 155–157; Fig. 1). Multiple samples of PCR-derived clones of both WNI-568 and WNI-567 were examined for the $L_{68} \rightarrow P$ substitution. Three of the eight clones of WNI-568 contained leucine, four contained proline and one contained phenylalanine at position 68. All of the nine clones of WNI-567 contained leucine at position 68. None of the clones of either the WNI-568 or WNI-567 virus lacked an *N*-linked acceptor site at the conserved position. WNI-567 may represent a neuroinvasive variant within the population of WNI-568 which is polymorphic at position 68. Because the amount of WNI virus required for neuroinvasion is very small [< 5 p.f.u. (Halevy *et al.*, 1994)], WNI-568 may still exhibit a neuroinvasive phenotype despite this heterogeneity. Although the difference in the position of the glycosylation site may influence the properties of the E protein, data are consistent with the hypothesis that glycosylation at or adjacent to the conserved site may not directly cause loss of neuroinvasiveness. WNI-568 and WNI-567 also differed from the other WNI viruses at two additional positions ($A_{232} \rightarrow V$ and $R_{313} \rightarrow G$; Fig. 1*a*). It remains possible that these residues independently affect neuroinvasiveness.

Because the mutations in WNI-25 and WNI-25A occur in the background of a highly mosquito cell-passaged strain of WNI, it is possible that determinants outside of the E protein are also involved in loss of neuroinvasiveness. Sequence analysis of WNI-25R yielded data supporting this hypothesis. WNI-25R contained reversions to the parental sequence at nucleotide positions 203 and 463, predicting a leucine at amino acid position 68 and loss of the glycosylation site at position 155. The E protein of this revertant virus is known to be unglycosylated (Halevy *et al.*, 1994). Only one other substitution ($T_{410} \rightarrow A$) was observed during analysis of multiple independent clones of WNI-25R relative to the WNI-25 and WNI-25A viruses. We emphasize that WNI-25R is 325-fold less neuroinvasive than WNI (see Fig. 1*b*). Although the $T_{410} \rightarrow A$ substitution could be responsible for this difference, the determinant(s) may also be encoded outside of the E protein region. A similar observation was made in a study of the E proteins of MVE viruses which have been attenuated for mouse neuroinvasiveness by passage in cell culture (McMinn *et al.*, 1995*a*).

This study reports additional sequence data for WN virus, which has undergone limited molecular characterization (Castle *et al.*, 1985, 1986; Mathiot *et al.*, 1990; Porter *et al.*, 1993). The cloning strategy employed here may facilitate further studies of wild and laboratory strains of this virus. Several features of the WNI virus E protein are worth mentioning. Growth in cell culture is associated with amino acid substitutions at two predominant positions, residue 68 and the conserved flavivirus glycosylation site. We realize that definitive experiments

demonstrating the role of these positions in neuroinvasiveness will require the use of an infectious clone. However, some evidence has been gathered that the attenuation process may be more complicated than mere acquisition of *N*-linked carbohydrate, as previously suggested (Halevy *et al.*, 1994). The proximity of position 68 and the conserved glycosylation site to the contact surface of domains I and II of the E protein may influence structural transitions accompanying its low pH-induced conformational change and reorganization of the two domains (Rey *et al.*, 1995). Flavivirus mutants which exhibit a higher pH threshold for acid-induced fusion map near the conserved glycosylation site (Guirakhoo *et al.*, 1993) and mutations associated with loss of flavivirus virulence have also been localized in this region (Rey *et al.*, 1995). Conceivably therefore, substitutions at position 68 of the WNI virus E protein require glycosylation to stabilize their effects on the protein, which may be deleterious for functional activities associated with the process of neuroinvasion. This would be consistent with the known effects of carbohydrate on maintaining the antigenic properties of epitopes within domain I (formerly domain C) of the E protein (Guirakhoo *et al.*, 1989). It should be emphasized, however, that mutations within other regions of the viral genome may also affect the neuroinvasive phenotypes of these WNI virus strains.

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