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Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus

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Two New York (NY) strains of the West Nile (WN) virus were plaque-purified and four variants that had different amino acid sequences at the *N*-linked glycosylation site in the envelope (E) protein sequence were isolated. The E protein was glycosylated in only two of these strain variants. To determine the relationship between E protein glycosylation and pathogenicity of the WN virus, 6-week-old mice were infected subcutaneously with these variants. Mice infected with viruses that carried the glycosylated E protein developed lethal infection, whereas mice infected with viruses that carried the non-glycosylated E protein showed low mortality. In contrast, intracerebral infection of mice with viruses carrying either the glycosylated or non-glycosylated forms of the E protein resulted in lethal infection. These results suggested that E protein glycosylation is a molecular determinant of neuroinvasiveness in the NY strains of WN virus.

INTRODUCTION

The West Nile (WN) virus is a mosquito-borne flavivirus of the Japanese encephalitis (JE) serocomplex group that causes lethal encephalitis in humans and horses. Up until 1999, the WN virus was distributed geographically in Africa, the Middle East, western and central Asia, India and Europe (Anderson *et al.*, 2001; Hamman *et al.*, 1965; Hubálek & Halouzka, 1999). Since the outbreak in New York City, USA (NY) of WN encephalitis in humans and horses in late August 1999, the WN virus endemic has spread throughout the United States (Garmendia *et al.*, 2001). Currently, there is no effective therapy or vaccine against WN virus for use in humans. Therefore, the prevention of WN virus outbreaks is an important public health concern in regions where the virus is prevalent.

The WN virus endemic in NY was characterized by large-scale mortality in wild birds, a phenomenon that had not been observed in other areas prior to the NY outbreak (Garmendia *et al.*, 2001). Therefore, the pathogenicity of the WN virus strain that was isolated in NY (NY strain) appears to differ from that of previously isolated strains. Elucidation of the pathogenesis of the NY strain is important for the development of new vaccines and therapies; however, studies of this type have been limited to date.

The flavivirus envelope (E) protein is an important structural protein in virus–cell interactions and is a major target of host antibody responses (Seligman & Bucher, 2003; Wang *et al.*, 2001a). The E proteins of many flaviviruses have one or two potential *N*-linked glycosylation sites (Chambers *et al.*, 1990). Glycosylation of the Japanese encephalitis virus E protein, for example, is essential to the native conformation of the epitopes in this protein (Lad *et al.*, 2000). Of the many WN virus sequences that are listed in GenBank, some viruses, including the NY strain, contain the *N*-linked glycosylation motif (N-Y-T/S) at residues 154–156 of the E protein, whereas others lack the glycosylation site due to amino acid substitutions (Table 1). Halevy and coworkers isolated variants WN25 and WN25A from the WN virus strain that was isolated in Israel in 1952 and found that these variants had glycosylated E proteins and were not neuroinvasive in mice that were infected intraperitoneally (i.p.) (Chambers *et al.*, 1998; Halevy *et al.*, 1994). The authors concluded that E protein glycosylation was not directly responsible for viral attenuation, because other glycosylated variants of the same virus strain were neuroinvasive when administered i.p. to mice. However, Scherret *et al.* (2001) reported that the glycosylated clone of the Kunjin virus, which is a subtype of WN virus, produced 10- to 100-fold more virus in cell culture than non-glycosylated Kunjin virus. Therefore, the relationship of E protein glycosylation with WN virus pathogenicity remains controversial.

In this study, we performed plaque purification of two NY strains and isolated four variants. Two of the variants contained glycosylated E proteins, whilst the others contained

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this paper are AB185914–AB185917.

Table 1. *N*-linked glycosylation sites of the sequenced WN virus strains

GenBank accession no.	Name	Year	Location	Origin	Glycosylation site	
Lineage 1						
AF260967	NY99-equi	1999	USA (New York)	Horse	+	NYS
AF196835	NY99-flamingo-382-99	1999	USA (New York)	Flamingo	+	NYS
AF206518	Connecticut2741	1999	USA (Connecticut)	*Culex pipiens*	+	NYS
AF404753	WN MD 2000-crow265	2000	USA (Maryland)	Crow	+	NYS
AF404754	WN NJ 2000-MQ5488	2000	USA (New Jersey)	*Culex pipiens*	+	NYS
AF404755	WN NY 2000-grouse	2000	USA (New York)	Grouse	+	NYS
AF404756	WN NY 2000-crow3356	2000	USA (New York)	Crow	+	NYS
AF481864	IS-98	1998	Israel	Stork	+	NYS
AF260969	RO97-50	1996	Romania	*Culex pipiens*	+	NYS
AF404757	WN Italy 1998-equine	1998	Italy	Horse	+	NYS
AY268132	PaAn001	2000	France	Horse	+	NYS
AY268133	PaH001	1997	Tunisia	Human	+	NYS
AY278441	Ast 99-901	1999	Russia (Astrakhan)	Human	+	NYS
AY278442	LEIV-Vlg00-27924	2000	Russia (Volgograd)	Human	+	NYS
AY262283	KN3829	1998	Kenya (Rift Valley)	*Culex univittatus*	+	NYS
AF260968	Eg101	1950	Egypt	Human	−	NYP
AF317203	VLG-4	1999	Russia (Volgograd)	Human	−	KYS
AY277252	LEIV-Vlg99-27889	1999	Russia (Volgograd)	Human	−	KYS
D00246	Kunjin (MRM61C)				−	NYF
Lineage 2						
M12294	FCG				−	Deleted
NC_001563	Unknown				−	Deleted

non-glycosylated E proteins. To elucidate the relationship between E protein glycosylation and pathogenicity of the NY strain, we compared the virulence characteristics of the glycosylated and non-glycosylated variants in a murine infection model.

METHODS

Viruses. Two NY strains of WN virus (NY99-6922 and BC787) were kindly provided by Dr Duane Gubler, Centers for Disease Control and Prevention (Fort Collins, CO, USA). The NY99-6922 strain was isolated from mosquitoes and BC787 was isolated from an infected horse; both strains were isolated in NY in 1999. The two parent viruses were subjected to three (for plaque-purified variants 6-LP, 6-SP and B-LP) or six (for plaque-purified variant B-SP) rounds of plaque purification on BHK cells. The descendent variants were propagated once in the brains of suckling mice as described previously (Shirato *et al.*, 2003) and working stocks of the viruses were propagated once in C6/36 cell cultures. GenBank accession numbers for the sequences of the plaque-purified virus variants were as follows: 6-LP, AB185914; 6-SP, AB185915; B-SP, AB185916; B-LP, AB185917.

Sequence analysis. Viral RNA was extracted by using a MagExtractor Viral RNA Purification kit (Toyobo), according to the manufacturer's instructions. First-strand cDNA was synthesized by using M-MLV reverse transcriptase (TaKaRa Bio). In preparation for DNA sequencing, the full-length viral sequence was divided into seven regions and amplified by using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen) and specific primers. The amplified DNA fragments were purified by using a QIAquick PCR Purification kit (Qiagen) and then sequenced directly in both directions or cloned into pT7Blue-2 T vector (Novagen) before subsequent sequencing.

The 3′ non-coding region (NCR) was sequenced by using a previously reported method (Kolykhalov *et al.*, 1996; Yun *et al.*, 2003). Briefly, ddATP was incorporated into the 5′-phosphorylated primer (T: 5′-CCAGTGTTGTGGCCTGCAGGGCGAATT-3′) with terminal deoxynucleotidyltransferase (TdT) to prevent intramolecular and intermolecular ligation of the primers. The following protocol was used: 500 pmol T primer was mixed with 15 U TdT (TaKaRa Bio), 40 U RNase inhibitor (TaKaRa Bio), 20 nmol ddATP (TaKaRa Bio), 2 µl 10 mM $CoCl_2$, 2 µl 0·1 % BSA and 4 µl 5× TdT buffer; diethyl pyrocarbonate (DEPC)-treated water (Ambion) was added to give a final volume of 20 µl. The mixture was incubated at 37 °C for 1 h and the T primer was extracted with phenol, precipitated with ethanol and resuspended in DEPC-treated water. The modified T primer was ligated into the 3′ end of the viral genomic RNA by mixing viral genomic RNA with the modified T primer, 40 U T4 RNA ligase (TaKaRa Bio), 5 µl 10× T4 RNA ligase buffer, 3 µl 0·1 % BSA, 40 U RNase inhibitor and 12·5 µl 50 % PEG 6000 (Wako Pure Chemical Industries); DEPC-treated water was added to give a final volume of 50 µl. The mixture was incubated at 16 °C for 12–16 h and then used directly for first-strand cDNA synthesis. Reverse transcription was performed with the TR15 primer (5′-AATTCGCCCTGCAGG-3′) and M-MLV reverse transcriptase. PCR amplification was performed with the forward primer (5′-AAATGGAGTGACGTCCCATA-3′, nt 10189–10208 of WN virus strain NY99-6922) and TR20 primer (5′-AATTCGCCCTGCAGGCCACAACA-3′) by using Platinum *Taq* High Fidelity DNA polymerase. The amplified fragment was purified by using a QIAquick PCR purification kit, ligated into the pT7Blue-2 T vector and sequenced.

All sequencing analyses were performed by using a BigDye Terminator cycle sequencing kit and an ABI PRISM 310 genetic analyser (Applied Biosystems).

To examine the influence of substitutions in the 3′ NCR, RNA structure models of the 3′ terminus were constructed by using GENETYX-WIN software (GENETYX).

Western blot analysis. BHK cell monolayers were infected with each of the virus variants. After incubation for 48 h, supernatants were collected and mixed with lysis buffer (1 % Triton X, 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA). The supernatants of the extracts were immunoprecipitated with the anti-flavivirus mAb D1-4G2 (ATCC) and protein G–Sepharose (Amersham Biosciences). The samples were then separated by SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane (Millipore). To detect the glycoproteins, the membranes were treated with biotin–ConA (Honen Corporation) and then with peroxidase-conjugated streptavidin (Sigma). To confirm that the D1-4G2 antibody had precipitated the WN virus protein, the membranes were probed by using D1-4G2 as the primary antibody and peroxidase-conjugated anti-mouse IgG as the secondary antibody (Sigma). The membranes were developed with diaminobenzidine.

Virus infection and sample collection. To assay virus growth *in vitro*, BHK cell monolayers were inoculated with each of the virus variants at an m.o.i. of 1. Supernatant samples were collected at the indicated times and titrated as described below.

For the *in vivo* analysis, 6-week-old male or female BALB/c mice (Japan SLC) were used. To investigate the viral virulence and distribution in mice, the indicated doses of viruses were inoculated subcutaneously (s.c.) into the axilla or injected intracerebrally (i.c.) into the left hemisphere and the survival rates of the mice were monitored. Virus-inoculated mice were euthanized by ether overdose and tissue samples were collected at the indicated number of days post-infection (p.i.). Tissue samples were homogenized in minimum essential medium (MEM; Nissui Pharmaceutical) to yield a 10 % suspension and then centrifuged at 5000 r.p.m. for 15 min at 4 °C. Supernatants were collected and stored at −80 °C until used.

Viral titration. Viruses in the working stocks and collected samples were titrated by plaque assay on BHK cells. BHK cell monolayers were grown in 12-well plates and inoculated with serial dilutions of the virus solutions. After 60 min virus adsorption, the virus solution was aspirated and the cells were washed twice with PBS. An overlay consisting of MEM containing 1·5 % carboxymethylcellulose (CMC; Wako) and 2 % fetal calf serum (CMC-MEM) was added to the cells and the plates were incubated at 37 °C in a CO_2 incubator. After 4 days cell culture, the CMC-MEM was aspirated, the cells were washed twice with PBS and then fixed and stained with a solution of 0·1 % crystal violet and 10 % formalin in PBS under UV light. After staining for 2 h, the cells were washed with water and dried and plaques were counted. The virus titre was calculated from the virus dilution that produced 10–100 plaques per well and expressed as p.f.u.

Statistical analysis. Statistically significant differences were determined by using the unpaired *t*-test or Mann–Whitney test. To determine the statistical significance of the survival curves, the log-rank and generalized Wilcoxon tests were performed. Values of $P<0·05$ were considered to be statistically significant.

RESULTS

Virus cloning by plaque purification

We found initially that the parental virus stocks produced plaques of two different sizes. Both parental viruses produced large (>1 mm) and small (<1 mm) plaques, but the stock of strain NY99-6922 exhibited many large plaques [69·3±5·5 %, $n=8$; Fig. 1a(i)] and the BC787 strain exhibited fewer large plaques [15·6±5·5 %, $n=8$; Fig. 1a(ii)]. This difference in the rate of production of large plaques between the two virus strains was statistically significant ($P<0·0001$). We then sequenced the E protein regions of the two parental viruses and found that the sequences of their glycosylation sites differed. Based on these two findings, we subjected the two parental viruses to plaque purification using BHK cells. The plaque-purified variant of strain NY99-6922 that exhibited large-plaque morphology was designated 6-LP [Fig. 1b(i)] and the variant that exhibited small-plaque morphology was designated 6-SP [Fig. 1b(ii)]. The small-plaque variant of strain BC787 was designated B-SP [Fig. 1b(iii)] and the large-plaque variant was designated B-LP [Fig. 1b(iv)].

Sequencing analysis

To analyse the viral genomic sequences, full-length clones of the viral genomes were sequenced. The sequences of the cloned variants were compared with those of the prototype NY strain, NY99-flamingo 382-99 (382-99 strain; Table 2). The two parental viruses had different origins: the NY99-6922 strain was isolated from a mosquito and the BC787 strain was isolated from a horse. The translated amino acid sequence of 6-LP was identical to that of strain 382-99; however, in the small-plaque variant 6-SP, a single nucleotide change (T→C) at position 1432 caused an amino acid change at position 156 of the E protein, which resulted in loss of the glycosylation site. The sequences of both B-SP and B-LP, derived from strain BC787, had several amino acid changes relative to the sequence of strain 382-99 (Table 2): M→I at position 11 (nt 498) of the preM protein, S→P at position 557 (nt 6280) of the NS3 protein and T→A at position 165 (nt 7408) of the NS4B protein. In addition, three amino acid differences were noted between the sequences of B-SP and B-LP. One amino acid change, A→T at position 287 (nt 5470) of the NS3 protein, was seen only in B-SP. Strain B-LP appears to be a virus mixture that contains two viral types with different amino acids at position 86 (nt 2725) of the NS1 protein. Finally, B-SP, but not B-LP, had a single nucleotide change at position 1433 (C→T) that resulted in an amino acid change (S→F) at position 156 of the E protein and loss of the glycosylation site.

In addition, some silent mutations were seen in the coding region of all the variants, as compared to the 382-99 strain. In the 3′ NCR, nucleotide substitutions at positions 10754 and 10851 were identified in both 6-LP and 6-SP and an additional nucleotide substitution at position 10956 was seen in 6-LP. The nucleotide substitution at position 10851 was also seen in the 3′ NCR of B-LP and B-SP. To examine the influence of these substitutions in the 3′ NCR, RNA structure models of the 3′ terminus were constructed by using GENETYX-WIN software and compared with published data. The results showed that the substitutions had no

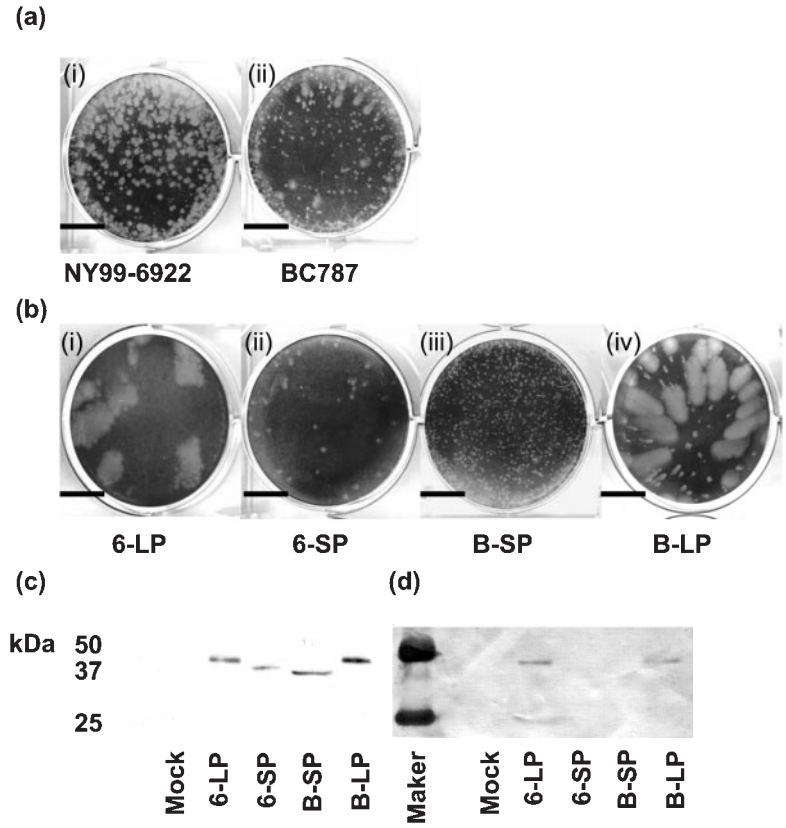

Fig. 1. (a, b) Plaque morphologies of the parental viruses and the cloned variants. Plaques were formed on BHK cell monolayers grown in six-well plates. After 5 days incubation, the cells were washed, fixed and stained with crystal violet. Bars, 10 mm. (c, d) Verification of the glycosylation status of the viral E proteins of the plaque-purified variants. BHK cells were infected with the viruses and, after 48 h incubation, the supernatants were collected and immunoprecipitated with D1-4G2 mAb. Western blot analysis was performed on samples electrophoresed in 10% SDS-PAGE gels under non-reducing conditions. (c) Detection of viral proteins by sequential incubations with the D1-4G2 mAb and peroxidase-conjugated anti-mouse IgG. (d) Detection of glycosylation by sequential incubations with biotin–ConA and peroxidase-conjugated streptavidin.

Table 2. Sequence differences between the cloned variants and strain NY99 flamingo-382-99 (GenBank accession no. AF196835)

All variants were sequenced twice.

Nucleotide position	Difference (amino acid change)				Region	Amino acid position
	6-LP*	6-SP*	B-SP*	B-LP*		
498			G→A (M→I)	G→A (M→I)	preM	11
1432–1433		CC (S→P)	TT (S→F)		E	156
2725				[C→T (L→F)]†	NS1	86
3702		C→T (silent)		C→T (silent)	NS2A	
5469				G→A (silent)	NS3	
5470			G→A (A→T)		NS3	287
5709	C→T (silent)	C→T (silent)			NS3	
6280			T→C (S→P)	T→C (S→P)	NS3	557
7015	C→T (silent)	C→T (silent)	C→T (silent)	C→T (silent)	NS4B	
7275	C→T (silent)	C→T (silent)			NS4B	
7408			A→G (T→A)	A→G (T→A)	NS4B	165
8307		C→T (silent)			NS5	
8811	T→C (silent)	T→C (silent)	T→C (silent)	T→C (silent)	NS5	
9841	T→C (silent)	T→C (silent)			NS5	
10341	C→T (silent)	C→T (silent)			NS5	
10754	G→T	G→T			3′ NCR	
10851	A→G	A→G	A→G	A→G	3′ NCR	
10956	T→C				3′ NCR	

*GenBank accession numbers are as follows: 6-LP, AB185914; 6-SP, AB185915; B-SP, AB185916; B-LP, AB185917.
†Mixed sequences were present.

effect on the predicted structure of the 3′ terminus (data not shown).

Western blotting

Western blotting analysis was performed to verify the glycosylation status of the viral E proteins. The WN virus proteins were immunoprecipitated by the mAb D1-4G2 (Fig. 1c). As predicted by the sequencing results, the 6-LP and B-LP variants contained glycosylated E proteins and the 6-SP and B-SP variants did not contain glycosylated E proteins (Fig. 1d). To avoid contamination of the membranes with the heavy chain of IgG, which was used for the immunoprecipitation, the electrophoresis procedure was performed under non-reducing conditions; therefore, the precise molecular masses of the viral proteins could not be deduced. However, the non-glycosylated E proteins migrated faster than the glycosylated forms.

Virus replication in tissue culture

The kinetics of virus replication were examined by using BHK cells (Fig. 2). The 6-LP and 6-SP variants exhibited similar kinetics; however, the titre of 6-LP was 10-fold higher than that of 6-SP at 24 h p.i. ($P<0{\cdot}05$). Variant B-SP replicated slowly, whereas B-LP replicated very rapidly. The titre of B-LP was approximately 1000-fold higher than that of B-SP at 24 h p.i. ($P<0{\cdot}01$). Thus, the titres of the glycosylated viruses tended to be higher than those of the non-glycosylated viruses during the early period of replication *in vitro*.

Virus virulence in mice

We first examined the virulence of the parental viruses in mice. To simulate the natural mode of infection, we used the s.c. route of inoculation. Six-week-old BALB/c mice were infected s.c. with 10^5 p.f.u. virus and then monitored daily for clinical signs and mortality. Mice infected with strain NY99-6922 had a mortality rate of 80 % and those infected with strain BC787 had a mortality rate of 30 % (Table 3); this difference between the groups was statistically significant ($P<0{\cdot}05$). Thus, strain NY99-6922, which produced mostly large plaques on BHK cell monolayers, produced high mortality in mice.

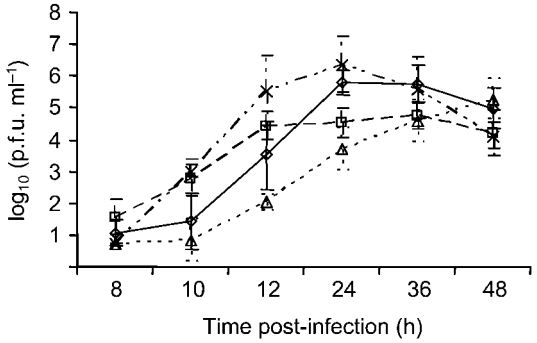

Fig. 2. *In vitro* replication kinetics of the 6-LP (◇), 6-SP (□), B-SP (△) and B-LP (×) strains. BHK cell monolayers were infected with the viruses at an m.o.i. of 1. Supernatants were collected at the indicated times p.i. and virus titres were determined by plaque assays using BHK cells ($n=4$).

We then examined the virulence of the plaque-purified descendent variants (Table 3). Mice were infected with 10^3–10^6 p.f.u. of each of the variants. Mice infected with either 6-LP or B-LP variants had high mortality rates, whereas those infected with variants 6-SP or B-SP showed low mortality. Most of the 6-LP- and B-LP-infected mice began to show clinical signs of disease at 5–7 days p.i.; some of these mice succumbed to infection, whereas others recovered from the disease. In contrast, mice infected with 6-SP or B-SP rarely showed clinical signs of disease. Although mice infected with either 6-LP or B-LP showed mortality, no dose-dependent increase in the mortality rate was seen; the lowest mortality rate in mice infected with the large-plaque variants was seen in infection with 10^5 p.f.u. virus. However, even at this dose, there were statistically significant differences ($P<0{\cdot}05$) in mortality rates between the 6-LP- and 6-SP-infected mice, and between the B-SP- and B-LP-infected mice.

Changes in body weight of the mice after infection were also determined (Fig. 3). Body weights of the infected mice began to decrease when the mice showed clinical signs of disease; in mice infected with either 6-LP or B-LP, weight loss began 6 days p.i. In contrast, body weights of mice infected with either 6-SP or B-SP increased during the time-course of infection. Eight days after infection, differences in the body weights between the 6-LP- and 6-SP-infected animals and between the B-SP- and B-LP-infected animals were statistically significant ($P<0{\cdot}05$).

We also examined the kinetics of virus replication in the spleen, brains and blood of virus-infected mice after s.c. infection. Viruses were detected in blood samples and spleens from mice infected with each of the variants (Fig. 4a and b, respectively); however, the titres of the virus variants with glycosylated E protein were higher than those of the non-glycosylated variants ($P<0{\cdot}05$). The 6-LP or B-LP variants were detected initially in the brains of infected mice on day 5 p.i. (Fig. 4c). In contrast, no virus was detected in the brains of mice infected with either 6-SP or B-SP. Plaque morphologies and the sequence of the E protein region of the virus recovered from the animals did not differ, as compared with those of the stocks of the variants (data not shown).

We also examined the viral neurovirulence of the variants following i.c. infection and determined the LD_{50} values for this route. Dose-dependent outcomes were seen following i.c. infection of mice with each of the variants (Table 3). The LD_{50} values for i.c. infection of mice with the 6-LP, 6-SP, B-LP and B-SP variants were 1·6, 1·4, 0·44 and 0·08 p.f.u., respectively. The LD_{50} values of 6-LP and 6-SP were similar

Table 3. Neuroinvasiveness and neurovirulence of parent viruses and variants of WN virus NY strains in mice

Virus strain	s.c. infection			i.c. infection			
	Infectious dose (p.f.u.)	No. surviving mice*	Mortality (%)	Infectious dose (p.f.u.)	No. surviving mice*	Mortality (%)	LD_{50} (p.f.u.)
Parent viruses							
NY99-6922	10^5	3/15	80·0				
BC787	10^5	7/10	30·0				
Isolated variants							
6-LP	10^6	2/10	80·0	10^1	1/8	87·5	1·6
	10^5	9/15	40·0	10^0	5/8	62·5	
	10^4	3/9	66·7	10^{-1}	5/5	0	
	10^3	1/10	90·0				
6-SP	10^6	10/10	0	10^1	2/8	75·0	1·4
	10^5	10/10	0	10^0	2/5	60·0	
	10^4	8/10	20·0	10^{-1}	5/5	0	
	10^3	10/10	0				
B-LP	10^6	4/10	60·0	10^1	0/8	100·0	0·08
	10^5	9/15	40·0	10^0	0/5	100·0	
	10^4	2/9	77·8	10^{-1}	3/5	40·0	
	10^3	0/10	100·0				
B-SP	10^6	8/10	20·0	10^1	0/6	100·0	0·44
	10^5	10/10	0	10^0	2/6	66·6	
	10^4	10/10	0	10^{-1}	5/6	16·7	
	10^3	10/10	0				

*Data are expressed as 'no. surviving mice/total no. inoculated mice'. Total numbers of mice used in the s.c. infection experiment were as follows: 10^6, for all variants, $n=10$ (six and four mice, in two experiments); 10^5, for variants 6-LP and B-LP, $n=15$ (five mice per experiment, in three experiments); 10^5, for variants 6-SP and B-SP, $n=10$ (five mice per experiment, in two experiments); 10^4, for all variants, $n=9$ or 10 (five or six mice and four mice, in two experiments); 10^3, for all variants, $n=10$ (six and four mice, in two experiments).

to each other. However, the LD_{50} value of B-LP was about fivefold higher than that of B-SP. Thus, the outcome of infection with large- or small-plaque variants differed when the mice were infected s.c., whereas all of the variants induced lethality when administered by the i.c. route.

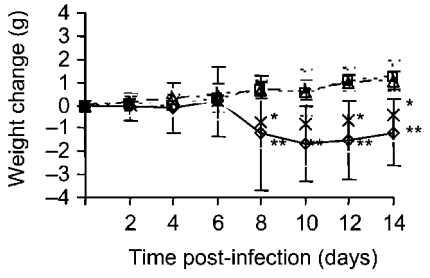

Fig. 3. Changes in body weight of mice infected with the variants. Mice were infected with 10^5 p.f.u. virus injected s.c. into the axilla and weighed daily [6-LP (◇) and B-LP (×), $n=15$; 6-SP (□) and B-SP (△), $n=10$]. *, The difference between B-SP and B-LP was statistically significant ($P<0·05$); **, the difference between 6-SP and 6-LP was statistically significant ($P<0·05$).

DISCUSSION

The aim of this study was to elucidate the relationship between glycosylation of the E protein and pathogenicity of the NY strains of WN virus. The results of this study suggest that glycosylation of the E protein is a molecular determinant of neuroinvasiveness for the NY strains of WN virus; when the variants were administered by the s.c. route, only those mice infected with the variants with glycosylated E protein developed lethal infections and had virus isolated from the brain.

We isolated the 6-LP and 6-SP strains from NY99-6922 and the B-SP and B-LP strains from BC787 by plaque purification. Sequence analysis detected an amino acid difference between 6-LP and 6-SP; this difference was in the glycosylation site of the E protein. On the other hand, in addition to a difference in the amino acid sequence at the glycosylation site, two other amino acid differences between B-LP and B-SP were detected. A nucleotide change from A to T at position 287 (nt 5470) of the NS3 protein was seen in B-SP. The NS3 protein of flaviviruses is multifunctional and includes serine proteinase, RNA helicase and nucleoside triphosphatase activities (Falgout *et al.*, 1991; Li *et al.*, 1999). The RNA helicase region is

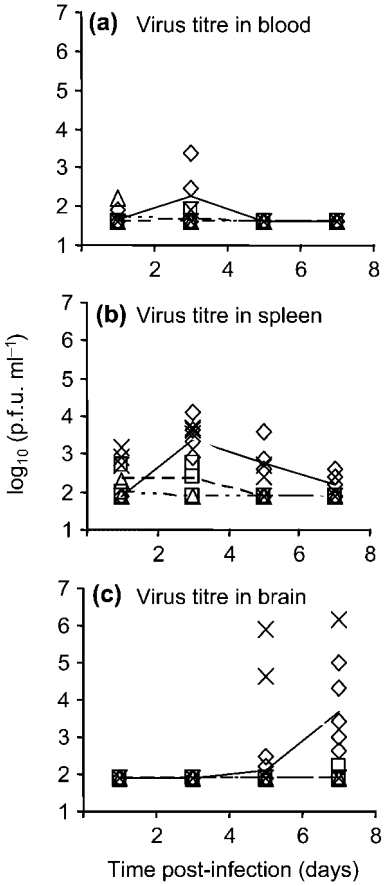

Fig. 4. Virus titres in the blood (a), spleens (b) and brains (c) of mice infected with 6-LP (◇), 6-SP (□), B-SP (△) and B-LP (×). Mice were infected with 10^5 p.f.u. virus injected s.c. into the axilla and tissues were collected on the indicated days post-infection. Virus titres were determined by plaque assay using BHK cells ($n=4$). Data are presented as individual p.f.u. values; mean values are connected by a line. Detection limits were 80 p.f.u. g^{-1} in spleen and brain and 40 p.f.u. ml^{-1} in blood.

classified into subfamilies, based on the sequence of motif II (Matusan *et al.*, 2001), and the 'DExH' motif exists at positions 285–288 of the NS3 protein of WN virus. The substitution detected in the NS3 protein of B-SP was in the 'x' position in the 'DExH' motif, which suggested that this amino acid substitution would not affect the function of the NS3 protein. The B-LP variant stock contained two viral types with different amino acids at position 86 of the NS1 protein (substitutions at nt 2725). The precise role of the flavivirus NS1 protein remains unclear; however, it is strongly immunogenic (Timofeev *et al.*, 2004; Xu *et al.*, 2004). Therefore, a mutation at this position of NS1 may influence the host immune response, but the role of this mutation is unclear. In addition, two or three nucleotide substitutions were identified in the 3′ NCR of 6-LP and 6-SP and one substitution was seen in that of B-LP and B-SP, as compared with the 3′ NCR sequence of the 382-99 strain. The 3′ NCR of all sequenced flavivirus genomes can be folded into similar 3′-terminal structures that are formed by approximately 100 nt near the 3′ end of the genome, consisting of a large stem–loop (SL) followed by a smaller SL (Shi *et al.*, 1996; Tilgner & Shi, 2004). In addition, the conserved sequences (CS) CS1, CS2, repeated CS2 (RCS2), CS3 and RCS3 exist upstream of the 3′ SL structure of mosquito-borne flaviviruses (Lo *et al.*, 2003). These SL structures and the CS region are important in genomic RNA synthesis (Lo *et al.*, 2003; Shi *et al.*, 1996; Tilgner & Shi, 2004). In our isolated variants, the nucleotide substitutions at positions 10754 and 10851 were upstream of the 3′ SL structure, but were not in the CS region. Therefore, we considered that these substitutions would not have a major influence on pathogenicity. The nucleotide substitution at position 10956 of variant 6-LP was in the SL region. However, when we performed an analysis of the predicted RNA structure by using GENETYX-WIN software and compared the results with published data, we found that the SL structure of 6-LP was not altered at all by the substitution at position 10956. These findings suggested that none of the substitutions in the 3′ NCR that were identified in variants were critical to the known functions of the viral RNA.

As described above, a number of nucleotide substitutions were detected between the LP and SP variants. However, our analyses suggested that most of the nucleotide and amino acid substitutions were not critical and that the determining difference between the LP and SP variants was that the E protein of the LP variants was glycosylated, whereas that of the SP variants was not.

The kinetics of replication of the descendent variants were determined by using BHK cell cultures. Although the titres of the four variants were similar during the final stages of infection, glycosylated viruses exhibited more rapid replication than non-glycosylated viruses during the early stages of infection. Consistent with our results, Scherret *et al.* (2001) reported that a glycosylated clone of Kunjin virus produced 10- to 100-fold more virus than a non-glycosylated clone. Together, these findings indicate that glycosylation of the E protein leads to enhanced virus replication.

In our animal-infection studies, when mice were infected by the s.c. route, only viruses with glycosylated E protein were neuroinvasive, suggesting that glycosylation of the E protein is a molecular determinant of viral neuroinvasiveness. Virus with non-glycosylated E protein was detected in blood and spleen samples from mice infected via the s.c. route; however, replication of non-glycosylated virus in the peripheral organs was inefficient, as compared to that of glycosylated virus. This suggests that the efficiency of WN virus replication in peripheral organs may correlate with neuroinvasiveness.

Chambers *et al.* (1998) suggested that E protein glycosylation might not be directly responsible for the attenuation of WN viruses, as the non-glycosylated E protein

variant of the Israel strain of the WN virus showed neuroinvasiveness; however, our results differed from those findings, possibly due to differences in the inoculation routes. The authors of the previous study used i.p. injection as the peripheral infection route. In the present study, we used s.c. injection as the peripheral infection route, to simulate the natural mode of infection. In animal models of JE serocomplex virus infection, the outcome of peripheral infection with some viruses differs depending on the route of infection. In our studies, mice infected by the s.c. route with the WN variants exhibited mortality rates that were independent of dose. Similar results were shown for s.c. infection of 8- to 12-week-old C57BL/6 mice with a WN virus strain isolated in NY in 2000 (Diamond *et al.*, 2003). In addition, it has been reported that intravenous (i.v.) infection of mice with WN virus and Murray Valley encephalitis virus leads to dose-independent mortality (Licon Luna *et al.*, 2002; Wang *et al.*, 2003b). On the other hand, i.p. infection of mice with WN virus showed dose-dependent mortality and the LD_{50} value could be estimated (Beasley *et al.*, 2002; Wang *et al.*, 2001b, 2003a). The reason for this difference is unclear; however, the i.p. inoculation route differs from other peripheral infection routes in that the virus enters directly into the peritoneal tissue. Taking into consideration the natural route of WN virus infection, the s.c. inoculation route may be useful for the elucidation of the pathogenesis of WN virus infection. In addition, the results of experiments using the s.c. and i.v. routes suggest that the quantity of virus inoculated into the mouse does not correlate directly with eventual mortality; only the secondary virus replication in the peripheral organs was related to viral invasion of the central nervous system (CNS).

In this study, all four WN variants induced lethality when inoculated into mice by the i.c. route. This result suggests that E protein glycosylation is not a molecular determinant of neurovirulence for WN virus. In addition, the viruses that were isolated from infected horses, B-LP and B-SP, were strongly neurovirulent (as compared to the viruses that were isolated from mosquitoes). Three major amino acid differences [M→I at position 11 (nt 498) of the preM protein; S→P at position 557 (nt 6280) of the NS3 protein; and T→A at position 165 (nt 7408) of the NS4B protein] were detected in B-LP and B-SP when compared to the sequence of the type strain NY99-flamingo 382-99. The amino acid substitutions in NS3 and NS4B were also observed in strain NY99-equi (GenBank accession no. AF260967), which was isolated from a horse in NY in 1999. However, WN Italy 1998-equi (accession no. AF404757) and PaAn001 (accession no. AY268132), which were isolated from horses in Europe, did not have these mutations. Further studies are needed to elucidate the relationship between viral virulence and the substitutions detected in the NY strain isolated from a horse.

The results of this study suggest that glycosylation of the E protein is a molecular determinant of neuroinvasiveness of the NY strains of WN virus. However, additional factors involved in the pathogenesis of WN viruses remain to be elucidated. It has been reported that the initial target of infection for dengue virus, a mosquito-borne flavivirus, are dendritic cells (Wu *et al.*, 2000) and that dendritic cells migrate to the local lymph nodes following arbovirus infection (Johnston *et al.*, 2000). However, subsequent events leading to viral invasion of the CNS remain unclear. To further the development of effective therapies and new vaccines against WN virus infections, additional studies are needed to elucidate the pathogenesis of WN virus infection.

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