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- H. 知的財産権の出願・登録状況
1. 特許取得
なし
 2. 実用新案登録
なし
 3. その他
なし

II. 研究成果の刊行に関する一覧表

II. 研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・印刷

Glycosylation of the West Nile Virus Envelope Protein Increases *In Vivo* and *In Vitro* Viral Multiplication in Birds

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Abstract. Many West Nile (WN) virus isolates associated with significant outbreaks possess a glycosylation site on the envelope (E) protein. E-protein glycosylated variants of New York (NY) strains of WN virus are more neuroinvasive in mice than the non-glycosylated variants. To determine how E protein glycosylation affects the interactions between WN virus and avian hosts, we inoculated young chicks with NY strains of WN virus containing either glycosylated or non-glycosylated variants of the E protein. The glycosylated variants were more virulent and had higher viremic levels than the non-glycosylated variants. The glycosylation status of the variant did not affect viral multiplication and dissemination in mosquitoes *in vivo*. Glycosylated variants showed more heat-stable propagation than non-glycosylated variants in mammalian (BHK) and avian (QT6) cells but not in mosquito (C6/36) cells. Thus, E-protein glycosylation may be a requirement for efficient transmission of WN virus from avian hosts to mosquito vectors.

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. WN virus was first isolated in 1937 from the blood of a febrile patient in the WN district of Uganda.¹ WN virus has since been found to be endemic over a wide range of areas in Africa, the Middle East, Western Asia, and Australia.^{2–4} Outbreaks of various magnitudes occurred in Israel in 1941 and 1951–1954 and in Africa in 1974. After that, no large outbreaks were observed for 20 years; however, from 1994 to 2000, WN outbreaks occurred among humans and horses.⁵ Specifically, outbreaks occurred in Algeria in 1994, in Morocco in 1996, in Romania in 1996, in Tunisia in 1997, in the Czech republic in 1997, in the Congo in 1998, in Italy in 1998, in Israel from 1997 to 2000, in Russia in 1999, in France in 2000, and in the United States from 1999 to the present.⁶ In the early outbreaks of the 1990s, the WN virus was associated only with mild pathogenicity to avian and mammalian hosts.

However, during the latter half of the 1990s, new strains of WN virus emerged in Europe. Humans and horses infected with those strains frequently suffered from meningitis and encephalitis.⁵ Since the outbreak of WN encephalitis in humans and horses in New York City (NY) in late August 1999, the WN virus has spread throughout North America and has very rapidly expanded to South American countries. Endemic areas are still expanding. The WN virus endemic in North America was characterized by large-scale mortality in wild birds,⁷ particularly in corvids, a phenomenon that had not been observed before the outbreaks in NY and Israel.⁵ A single nucleotide change resulting in the T249P substitution in the NS3 helicase was reported to be associated with large-scale mortality in American crows.⁸

WN virus is maintained in nature through an enzootic transmission cycle between avian reservoir hosts and *Culex* mosquito vectors. Viremic levels of the avian host directly affect the infection rates of vector mosquitoes; birds with higher viremia generate more infected mosquitoes after blood feeding.⁹ Replication and dissemination characteristics of the virus within the mosquito vectors also affect transmission efficiency.

The flavivirus envelope (E) protein is an important structural protein in virus–cell interactions, and it is a major target of the host-antibody responses.¹⁰ All flaviviruses have one or two potential N-linked glycosylation sites on the E protein.¹¹ Some WN viruses contain the N-linked glycosylation motif (N-Y-T/S) at residues 154–156 of the E protein, whereas others lack this glycosylation site because of amino acid substitutions. It is interesting to note that many of the WN virus isolates associated with significant human outbreaks, including the recent North American epidemic, possess the glycosylation site on the E protein.¹²

In a previous study,¹² we isolated four variants from two WN virus NY strains using plaque purification on baby-hamster kidney (BHK) cells. Two of the variants contained glycosylated E proteins, whereas the others contained non-glycosylated E proteins. To determine the relationship between E-protein glycosylation and pathogenicity of the WN virus, mice were inoculated subcutaneously with these four variants. The glycosylated variants caused higher mortality than the non-glycosylated variants in mice, which suggests that E-protein glycosylation is a molecular determinant of neuroinvasiveness in the NY strains of WN virus. Other studies also established the importance of glycosylation of Flaviviruses E protein for viral assembly and infectivity *in vitro* and *in vivo*.^{12–14}

In the present study, we examined the effect of E-protein glycosylation on the interaction between WN virus and avian hosts and mosquito vectors. Using a young-chick infection model, we examined if the glycosylated and non-glycosylated variants exhibited differences in virulence and viremic level in the chicks. We also examined multiplication and dissemination of WN virus variants in mosquitoes *in vivo*. We then examined the multiplication characteristics of the variants in *in vitro*

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tissue-culture cells of mosquito, mammalian, and avian origin to establish how E-protein glycosylation affects WN virus multiplication in these different cell types. Using the young-chick model, we showed that E-protein glycosylation allows the virus to multiply in a heat-stable manner and therefore, has a critical role in enhanced viremic levels and virulence of the NY strain of WN virus.

MATERIALS AND METHODS

Viruses. Two NY strains of WN virus (NY99-6922 and BC787) were kindly provided by Dr. Duane Gubler at the Center for Disease Control and Prevention (CDC, Fort Collins, CO) in 2003. The NY99-6922 strain was isolated from mosquitoes, and BC787 was isolated from a horse; both strains were isolated in NY in 1999. The two parent viral strains were subjected to plaque purification on BHK cells. The descendent variants were propagated one time in the brains of suckling mice, as described previously, and working stocks of the viruses were propagated one time in C6/36 cell cultures. The plaque-purified variant of strain NY99-6922 that exhibited large-plaque morphology was designated 6-large plaque (LP), and the variant that exhibited small-plaque morphology was designated 6-small plaque (SP). The large-plaque variant of strain BC787 was designated B-LP, and the small-plaque variant was designated B-LP. In previous studies, we sequenced the variants and showed that the 6-LP and B-LP variants contain the N-linked glycosylation motif (N-Y-S) at residues 154–156 of the E proteins, whereas the 6-SP and B-SP variants do not. The 6-SP and B-SP variants had mutations at aa156; thus, residues 154–156 were NYP in 6-SP and NYF in B-SP.¹² GenBank accession numbers for the sequences of the plaque-purified virus variants are as follows: 6-LP is AB185914, 6-SP is AB185915, B-SP is AB185916, and B-LP is AB185917. Western blot analysis revealed that the 6-LP and B-LP variants contain glycosylated E proteins, and the 6-SP and B-SP variants do not contain glycosylated E proteins.

WN virus variants with or without N-linked glycosylation sites on the E protein were generated by infectious clone methodology.¹⁵ WN virus NY99-6922 was used to generate recNY/Gly+, which has a glycosylated E protein, and recNY/Gly-, which has a non-glycosylated E protein. The recNY/Gly+ variant produced large plaques in infected cell monolayers, and the recNY/Gly- variant produced small plaques. The amino acid sequence analysis of the full-length cDNAs of the recNY/Gly+ and recNY/Gly- variants showed that they differed only at the N-linked glycosylation site; although aa156 of the recNY/Gly+ variant was an S residue, it was a P residue in recNY/Gly-.

Cells. BHK cells (BHK-21, ATCC #CCL-10) were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 8% fetal calf serum (FCS), 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate. Quail fibroblast cells (QT6, ATCC #CRL-1708) were maintained in Minimal Essential Medium (MEM; Gibco, Invitrogen Co., CA) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. *Aedes albopictus* mosquito cells (C6/36, ATCC #CRL-1660) were maintained in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. BHK, QT6, and C6/36 cells were grown and maintained at the optimum temperature for each cell line, which was 37°C, 37°C,

and 28°C, respectively. Confluent monolayers of each cell line were prepared for infection by seeding on 12-well plates (BD, Falcon, NJ) in 1 mL of the appropriate medium and by incubation at the optimum temperature for 2 days. Confluent cell monolayers and virus-infected cells were maintained in the appropriate medium for each cell line and were supplemented with 2% FCS.

Viral growth *in vitro*. Twelve-well plates containing confluent monolayers of BHK, QT6, or C6/36 cells were infected with virus, in triplicate, at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU)/cell, based on the titer of BHK cells determined in the plaque assay. After 60 minutes of adsorption at various temperatures (BHKs: 34°C, 37°C, and 40°C; QT6s: 37°C, 40°C, and 42°C; C6/36s: 28°C and 34°C), the viral solution was aspirated, the cells were washed three times with PBS(-), 1 mL of the appropriate medium was added to each well, and the plates were returned to their respective optimum temperatures. Culture fluids were collected at the indicated times and stored at -80°C. All samples were titrated, in triplicate, by the plaque assay on BHK cells as described below, and viral growth curves were constructed using the mean virus titer for each time point. The detection limit of plaque assay was 100 PFU/mL.

The 6-LP and 6-SP variants were diluted in MEM containing 2% FCS without cells and were incubated at 37°C and 42°C, respectively, for 12, 18, or 24 hours. Virus titers in the culture medium were measured by plaque assay on BHK cells.

Viral titration. Viruses in the working stocks and collected samples were titrated by the plaque assay on BHK cells. BHK cell monolayers were grown in 6-well plates and inoculated with serial dilutions of the viral solutions. After 60 minutes of viral adsorption, the viral solution was aspirated, and the cells were washed three times with PBS(-). A 2-mL volume of overlay consisting of EMEM containing 1.5% carboxymethyl cellulose (CMC; Wako, Japan) and 2% FCS was added to the cells, and the plates were incubated at 37°C in a CO₂ incubator. After 5 days of cell cultivation, the CMC-EMEM was aspirated, and the cells were fixed and stained under ultraviolet (UV) light with a solution of 0.1% crystal violet and 10% formalin in PBS(-). After staining for 2 hours, the cells were washed with water and dried, and the plaques were counted. The viral titer was calculated from the viral dilution that produced 20–200 plaques per well, and it was expressed as PFU/mL.

Enzyme-linked immunosorbent assay. At various times post-inoculation, culture-cell lysates and supernatants were harvested, and the amounts of intracellular and extracellular E protein of the 6-LP and 6-SP variants were measured by antigen-detection enzyme-linked immunosorbent assay (ELISA). C6/36 cells were incubated at 28°C, and QT6 cells were incubated at 42°C. Culture fluids (0.5 mL) were treated with 1% Triton X-100 and added to the mouse MAb F6/16A-coated wells¹⁶ of 96-well microtiter ELISA plates that were blocked with 4× Block Ace (Snow Brand Milk Products Co., Tokyo, Japan). The viral antigens in the fractions were detected using biotinylated mouse MAb 4H8¹⁷ and Horseradish peroxidase (HRP)-conjugated streptavidin (Zymed, South San Francisco, CA). HRP activity was detected by adding 100 µL of *o*-phenylene-diamine dihydrochloride (Sigma Chemical Co., St. Louis, MO) to the fractions in the presence of 0.03% H₂O₂. The plates were read for optical density (OD) at 450 nm on a microplate reader. The non-infected cell lysates and supernatants removed at various time points were used as negative controls.

We designated a reaction as being positive if the OD of the sample was greater than that of the negative control. The non-infected cell lysate and supernatant at each of the time points were used as negative controls. OD values were obtained by correcting background values of negative controls. Negative control OD values were from 0.03 to 0.4 depending on samples at different hours post-inoculation. The percentage of expressed proteins were (OD values at each time point)/(OD values at 48 hours of LP in A to D or those at 96 hours of LP in E and F). Mean (\pm standard deviation [SD]) titers are from triplicate cultures.

Chicks. Young Boris-Brown chicks (Hokuren, Hokkaido, Japan) were housed in a biosafety level-3 (BSL-3) animal facility. The experiments were approved under the guidelines for using experimental animals of Hokkaido University. Two-day-old male chicks ($N = 4$) were inoculated with 100 PFU of WN virus (6-LP, 6-SP, B-LP, or B-SP variant) through subcutaneous injection into the femoral region. All variants were diluted in PBS(-) containing 10% FCS (10% FCS-PBS[-]). At various time points post-inoculation, chicks were euthanized by sevoflurane overdose, and blood samples were collected from the heart, held at room temperature for 60 minutes, and then held at 4°C overnight. The blood samples were centrifuged (4,000 rpm for 10 minutes), and sera were removed and stored at -80°C. The viral titers in sera were measured using the plaque assay on BHK cells.

To determine the differences of pathogenicity of the variants, 2-day-old male chicks ($N = 5$) were inoculated subcutaneously with 10^{-1} – 10^4 PFU of the 6-LP variant and with 10^2 – 10^5 PFU of the 6-SP variant, both diluted in 10% FCS-PBS(-). The survival rates of the chicks were scored daily, and tissue samples (brain, heart, spleen, liver, and kidney) were collected for viral titration.

Mosquitoes. Infection experiments using mosquitoes were performed in an arthropod containment level-3 facility under a regulation for animal experiment of Oita University. The *Culex pipiens pallens* colony (516th generations) was derived from mosquito larvae that were collected in drains of the Osaka prefecture in 1965. Six- to seven-day-old female mosquitoes ($N = 4$) were inoculated intrathoracically with 100 PFU of all variants. Mosquitoes were maintained in cartons at 27°C and 85% humidity, fed a 5% sucrose solution, harvested 0, 3, 5, and 7 days post-inoculation, and stored at -80°C. Individual mosquitoes were triturated in mosquito diluents (2% FCS-MEM containing 500 μ g/mL streptomycin and 500 U/mL penicillin) using a Mixer Mill apparatus (Qiagen, Valencia, CA), and the homogenates were centrifuged at 1,000 rpm for 10 minutes. Viruses in the supernatants were titrated on BHK cells using the plaque assay.

Five- to six-day-old female *Culex pipiens pallens* ($N = 19$ – 21) were fed a blood/virus suspension using an artificial feeding technique. A blood/virus suspension was prepared by mixing stock virus (the 6-LP or 6-SP variant), normal defibrinated human blood, and PBS(-). The mosquitoes were allowed to feed on the blood/virus suspension, containing 10^7 , 10^6 , or 10^5 PFU/mL of virus, over a 24-hour period. They were maintained at 27°C and 85% humidity, harvested 13 days post-inoculation, and stored at -80°C. Individual mosquito legs were triturated and titrated for virus using the plaque assay.

Pathology and immunohistochemistry. The young chicks were necropsied in a BSL-3 containment facility. Tissue samples, including samples from the liver, spleen, kidneys, heart, and brain, were fixed in 20% neutral buffered formalin

solution and embedded in paraffin. The tissue samples were sectioned at a thickness of 4 μ m and stained with hematoxylin-eosin (HE) stain for light microscopy. For the detection of WN virus antigens, serial sections were stained with the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO kit; Nichirei Co., Tokyo, Japan), using mouse anti-WN virus hyperimmune ascitic fluid at a dilution of 1:5,000 as the primary antibody. The sections were counterstained with hematoxylin.

RESULTS

Pathogenicity in chicks. We previously showed that the LP variant of the NY strain of WN virus, which has a glycosylated E protein, showed higher neuroinvasive virulence in mice than the SP variant, which has a non-glycosylated E protein.¹² To examine whether or not glycosylation of the E protein affects the pathogenicity of WN virus in avian hosts, young chicks were selected as the experimental infection model. Two-day-old chicks ($N = 5$) were inoculated subcutaneously with 10^{-1} – 10^4 PFU of the 6-LP variant or with 10^2 – 10^5 PFU of the 6-SP variant. The survival rates of chicks were recorded daily, and tissue samples were collected for observing histopathological changes. The survival rate of chicks inoculated with 10^0 – 10^4 and 10^{-1} PFU of the 6-LP was 0% and 20%, respectively (Figure 1A). The LD₅₀ value of the 6-LP variant in young chicks was $< 10^{-1}$ PFU. All chicks began to show disease symptoms by 3 days post-inoculation (dpi). Chicks exhibited depressed signs and wheezing, but neurological symptoms were not observed. In contrast, the 6-SP variant exhibited low virulence in young chicks and resulted in dose-independent death. Survival rates were 60% with a 10^2 , 10^4 , and 10^5 PFU inoculation and 40% with a 10^3 PFU inoculation (Figure 1B). The clinical symptoms of the chicks were mild. To confirm whether or not the mutation has occurred to 6-SP in young chicks, eight plaques were rescued from two 6-SP inoculated chicks (four plaques from a dead chick and four other plaques from a survived chick). These eight variants were subjected to a direct-sequence analysis of E-protein gene, and no difference in the E-protein gene was observed. This result is suggesting absence of reversions (data not shown).

Histopathological findings of organs of 6-LP inoculated chicks included severe necrosis in the liver (data not shown) and heart (Figure 2A). But the chicks inoculated with 6-SP showed only mild inflammation in these tissues (Figure 2C). WN virus antigens were detected in the myocytes of 6-LP inoculated chicks (Figure 2B) but not in 6-SP (Figure 2D).

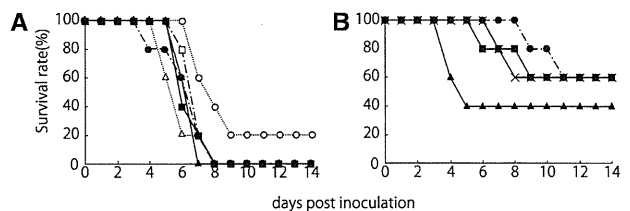


FIGURE 1. Survival curves of young chicks subcutaneously inoculated with 6-LP (A) and 6-SP (B) variants of WN virus. Two-day-old male chicks were inoculated with 10^{-1} (○), 10^0 (△), 10^1 (□), 10^2 (●), 10^3 (▲), or 10^4 (■) PFU of 6-LP and 10^2 (●), 10^3 (▲), 10^4 (■), or 10^5 (×) PFU of 6-SP. Chicks were observed daily for changes in their health. Five chicks of each variant were examined.

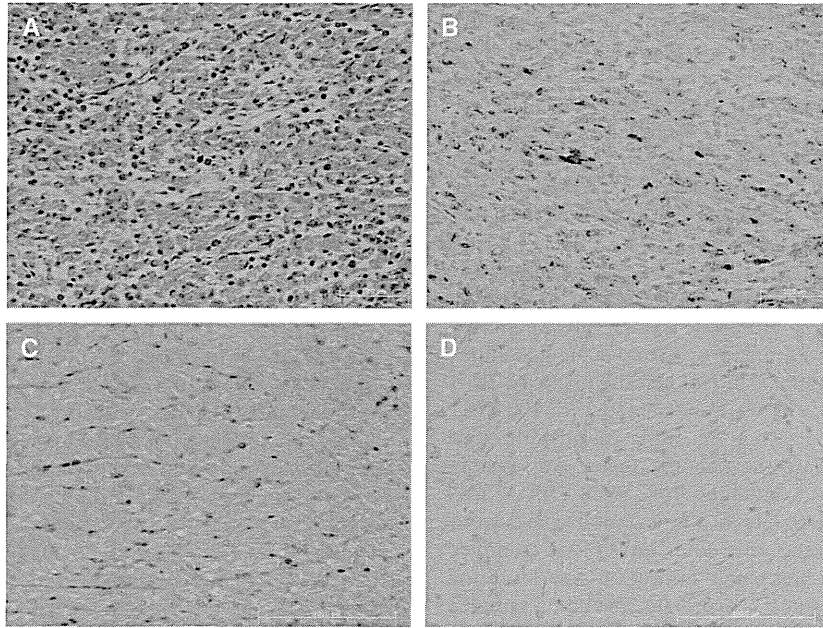


FIGURE 2. Histopathological and immunohistochemical characteristics of young chicks inoculated with WN virus variant 6-LP (A and B) and 6-SP (C and D). Myocytes were stained with HE stain (A and C) and immune stain (B and D). (A) Photomicrograph showing marked necrosis of myocytes from the heart of a young chick infected with WN virus, strain 6-LP. Preparation was stained with HE stain. (B) Myocytes of the heart are positively stained for WN virus antigen. (C) No histological changes were found. (D) Myocytes of the heart are negatively stained for the antigen.

These results suggest that LP variants of WN virus, which have glycosylated E proteins, are highly pathogenic to young chicks.

Viremia in chicks. To investigate whether or not glycosylation of the E protein influences multiplication of the NY strain of the WN virus in avian hosts, we compared viremia titers in chicks infected with LP and SP variants. Two-day-old chicks ($N = 4$) were subcutaneously inoculated with 100 PFU of both variants, blood samples were collected every 24 hours from 0–7 dpi, and viral titers in the serum were measured.

The viremia titers of the 6-LP variant in chicks were 10 or more times greater than those of the 6-SP variant during 1–7 dpi (Figure 3). The titers of the 6-LP variant exceeded 10^5 PFU/mL during 2–4 dpi, whereas those of the 6-SP variant did not exceed 10^4 PFU/mL during the 7-day observation period. The titer of the 6-SP variant was below the detection limit (100 PFU/mL) at 7 dpi. The viremia titers of B-LP were also found to be higher than those of B-SP. The titers of B-LP exceeded 10^5 PFU/mL during 2–4 dpi, whereas those of B-SP were mostly below 10^4 PFU/mL.

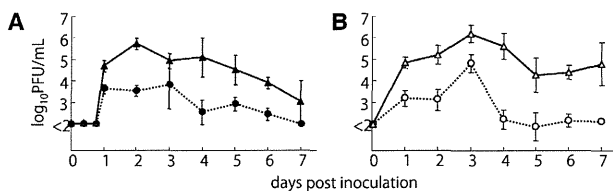


FIGURE 3. Viremic levels of young chicks subcutaneously inoculated with WN virus variants. Young chicks were inoculated with WN virus variants 6-LP (▲) and 6-SP (●) in experiment A and B-LP (Δ) and B-SP (○) in experiment B. Two-day-old chicks were inoculated with 100 PFU of the respective variant ($N = 4$). The viral titers in the sera were measured using the plaque assay on BHK cells. Mean (\pm SD) titers are from triplicate cultures.

These results suggest that glycosylation of the WN virus E protein enhances viremia levels in chicks.

Viral growth in *Culex pipiens pallens*. To establish whether or not glycosylation of the E protein influences multiplication of the WN virus in mosquito hosts, 7-day-old female *Culex pipiens pallens* ($N = 4$) were inoculated intrathoracically with 100 PFU of the 6-LP, 6-SP, B-LP, or B-SP variant. The mosquitoes were harvested 0, 3, 5, and 7 dpi, and the viral titers in their bodies were measured using the plaque assay. The viral titers in mosquitoes inoculated with LP variants were not significantly different from those inoculated with SP. The titers of all variants increased by up to about 10^5 PFU/mosquito by 3 dpi, and no differences in viral titer were observed between any combination of variants or harvested days (dpi; data not shown).

Furthermore, to investigate whether or not glycosylation of the E protein would affect disseminated infection of WN virus in mosquitoes, *Culex pipiens* were fed blood meals containing 10^5 – 10^7 PFU/mL of 6-LP or 6-SP variants. Mosquitoes were harvested 13 dpi, and mosquito legs were triturated and examined. The infection rates (Table 1) and virus titers (data not shown) were not significantly different between mosquitoes fed with 6-LP and 6-SP variants.

The results suggest that glycosylation of the E protein may not influence multiplication and dissemination of the WN virus in mosquitoes.

Viral growth *in vitro*. To determine the effect of glycosylation of the E protein on heat stability of the virus, the growth kinetics of the LP and SP variants of the NY strain of WN virus were examined in three kinds of cell cultures, namely mammalian (BHK), avian (QT6), and mosquito (C6/36) cell lines, at various incubation temperatures.

Mammalian BHK cells were inoculated with the virus and incubated at 34°C, 37°C, or 40°C, and the viral titers were

TABLE 1
Disseminated infection rates of *Culex pipiens* during peroral infection experiments with WN virus

WN virus variant	Virus dose (PFU)		
	107	106	105
6-LP	10/10*	9/9	5/6
6-SP	6/6	11/11	4/10

* Number of virus-positive mosquitoes/number of inoculated mosquitoes.

Mosquitoes were fed a blood-virus mixture and were maintained at 28°C for 13 days, harvested, and titrated for virus in a BHK cell-plaque assay.

determined in the culture fluids. When incubated at 34°C, there were no differences in viral titers between 6-LP and 6-SP or between B-LP and B-SP variants (Figure 4A and B). However, when BHK cells were incubated at 37°C and 40°C, viral titers of 6-SP and B-SP were lower than those of 6-LP and B-LP, respectively (Figure 4C–F).

Avian QT6 cells were also examined for the multiplication of the WN virus variants at different temperatures. When QT6 cells were incubated at 37°C, viral titers of the culture fluids did not differ between those inoculated with 6-LP and 6-SP or with B-LP and B-SP (Figure 5A and B). However, at 40°C and 42°C of incubation, viral titers of 6-SP and B-SP were significantly lower than those of 6-LP and B-LP, respectively (Figure 5C–F).

Next, the multiplication of the variants was tested in mosquito C6/36 cells at different temperatures. Viral titers of 6-LP and B-LP were not significantly different from those of 6-SP and B-SP when incubated at 28°C or 32°C (data not shown).

These results showed that the LP variants of WN virus, which have glycosylated E proteins, multiplied more efficiently in mammalian and avian cell cultures at high temperatures than did the SP variants, which lacked glycosylation of the E protein.

However, there was no difference in viral multiplication between the LP and SP variants in mammalian and avian cell cultures at low temperatures. The efficiency of viral multiplication did not differ between the LP and SP variants in mosquito C6/36 cells at different temperatures.

To examine if the thermostabilities of the variants accounted for the differences in viral multiplication observed in avian and mammalian cells at higher temperatures (i.e., 40°C and 42°C), the variants of WN virus were incubated at different temperatures in culture medium without cells, and the viral titers were determined (Figure 6). In culture medium, the viral titers declined more rapidly at 42°C than 37°C. However, there was no difference between the rates of reduction of 6-LP and 6-SP titers, either at 37°C or 42°C. These results suggest that the difference in viral multiplication among the variants at higher temperatures was not caused by the different thermostabilities of the variants.

To confirm that N-glycosylation of the E protein is indeed responsible for the above-described differences in multiplication characteristics of WN virus in different cell types and at various temperatures, WN virus strains with or without the N-glycosylated site were generated from infections cDNA clone, and their multiplication patterns were examined in C6/36 and QT6 cells (Figure 7). When C6/36 cells were infected with WN virus recNY/Gly+, which bears an E-protein N-glycosylation site, and WN virus recNY/Gly-, which lacks an E-protein glycosylation site (S156P), and were incubated at 28°C, we observed no difference in viral multiplication efficiency between the two strains (Figure 7A). When QT6 cells were infected with both strains and incubated at 40°C, the WN virus recNY/Gly+ strain multiplied more efficiently than WN virus recNY/Gly- (Figure 7B). This result confirmed that N-glycosylation of the E protein is responsible for

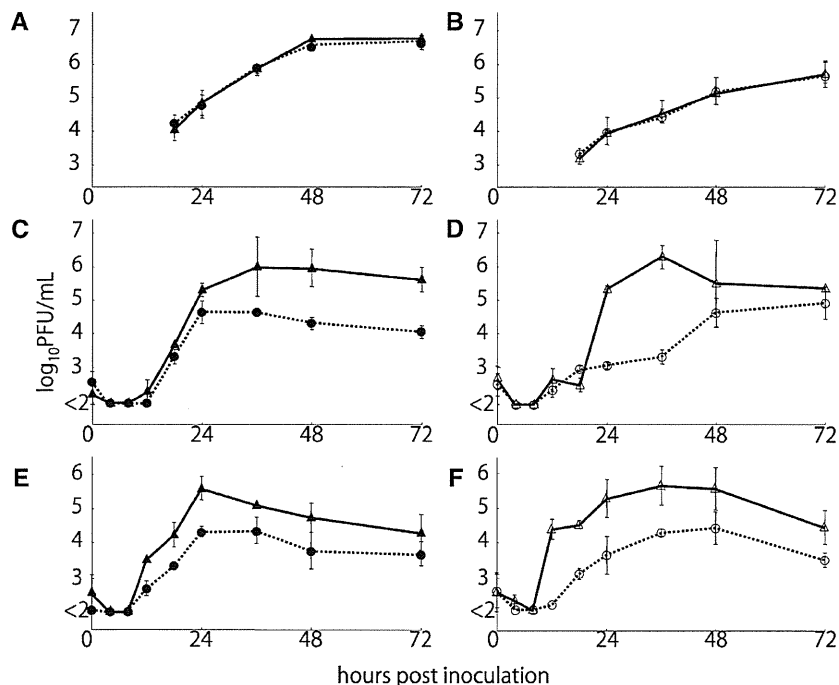


FIGURE 4. *In vitro* replication of WN virus variants in hamster (BHK) cells. BHK cells were inoculated with the 6-LP (▲), 6-SP (●), B-LP (Δ), or B-SP (○) variant at an MOI of 0.01. BHK cells were incubated at 34°C (A and B), 37°C (C and D), or 40°C (E and F). The viral titers in culture fluids were measured using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (\pm SD) titers are from triplicate cultures.

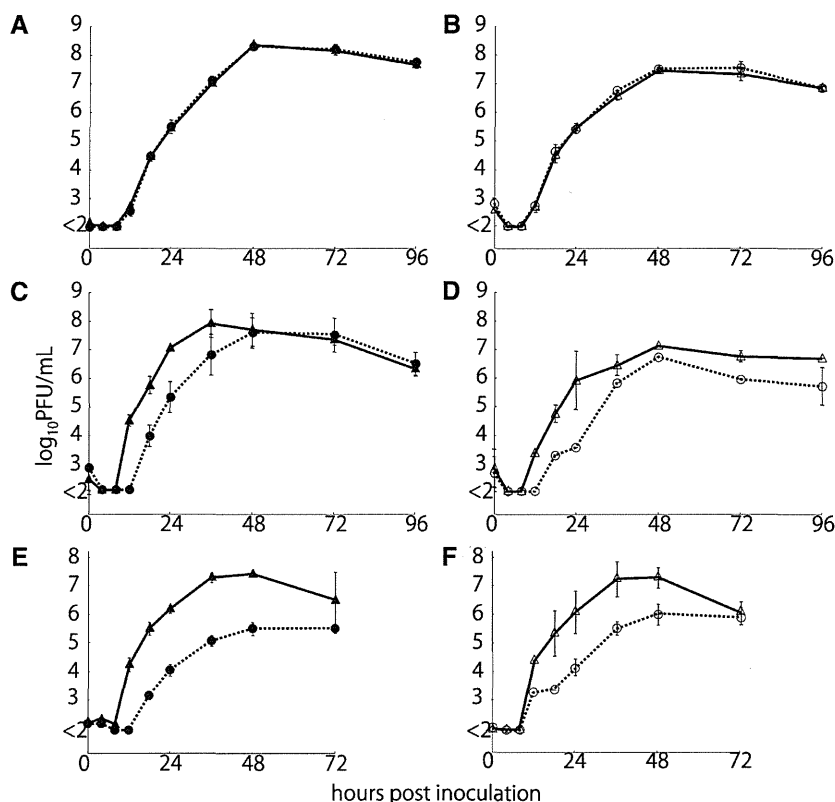


FIGURE 5. *In vitro* replication of WN virus variants in quail (QT6) cells. QT6 cells were inoculated with variants 6-LP (▲), 6-SP (●), B-LP (△), or B-SP (○) at an MOI of 0.01. QT6 cells were incubated at 37°C (A and B), 40°C (C and D), or 42°C (E and F). The viral titers were measured in the culture fluids using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (\pm SD) titers are from triplicate cultures.

the efficient replication of WN virus in QT6 cells at the higher temperature.

Comparisons of the intracellular and extracellular expression levels of the LP and SP variant viral proteins. At 42°C, viral titers of the SP variants of WN virus were lower than those of the LP variants in mammalian BHK cells and avian QT6 cells. To examine which step of the viral replication cycle is involved in the reduced multiplication of the SP variants, which

have non-glycosylated E proteins, at higher temperatures, intracellular and extracellular levels of viral proteins of the LP and SP variants were compared.

QT6 cells were infected with 6-LP or 6-SP variants and incubated at 42°C and 37°C, respectively. For comparison, C6/36 cells were also infected with 6-LP or 6-SP variants and incubated at 28°C. The cell lysates and culture fluids were harvested sequentially and subjected to antigen-detection ELISA. The levels of E protein of 6-SP in QT-6 cells and culture fluid were lower than those of 6-LP at 42°C (Figure 8A and B), but there was no difference between those of 6-SP and 6-LP at 37°C (Figure 8C and D). In contrast, there was no difference in the levels of E protein in either the cell lysates or the culture fluids of C6/36 cells infected with 6-SP or 6-LP and incubated at 28°C (Figure 8E and F). These results suggested that the

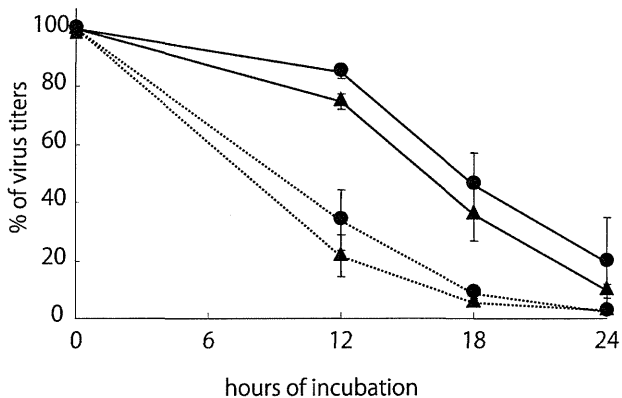


FIGURE 6. Thermostability of WN virus variants. Two variants, 6-LP (▲) and 6-SP (●), were incubated at 37°C (—) and 42°C (···) in culture medium without cells, and viral titers in the culture medium were measured using the plaque assay on BHK cells. The percentage of viral titers were (titers at each time point)/(titers at 0 hour of incubation). Mean (\pm SD) percent are from triplicate cultures.

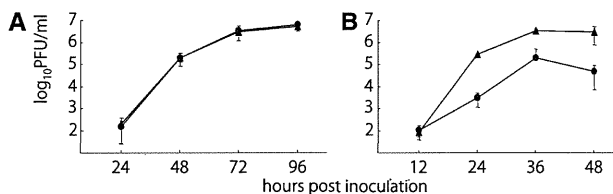


FIGURE 7. *In vitro* replication of WN virus strains recNY/Gly+ (▲) and recNY/Gly- (●). C6/36 (A) and QT6 (B) cells were infected with each strain at an MOI of 0.01. C6/36 cells were incubated at 28°C, and QT6 cells were incubated at 40°C. The viral titers in culture fluids were measured using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (\pm SD) titers are from triplicate cultures.

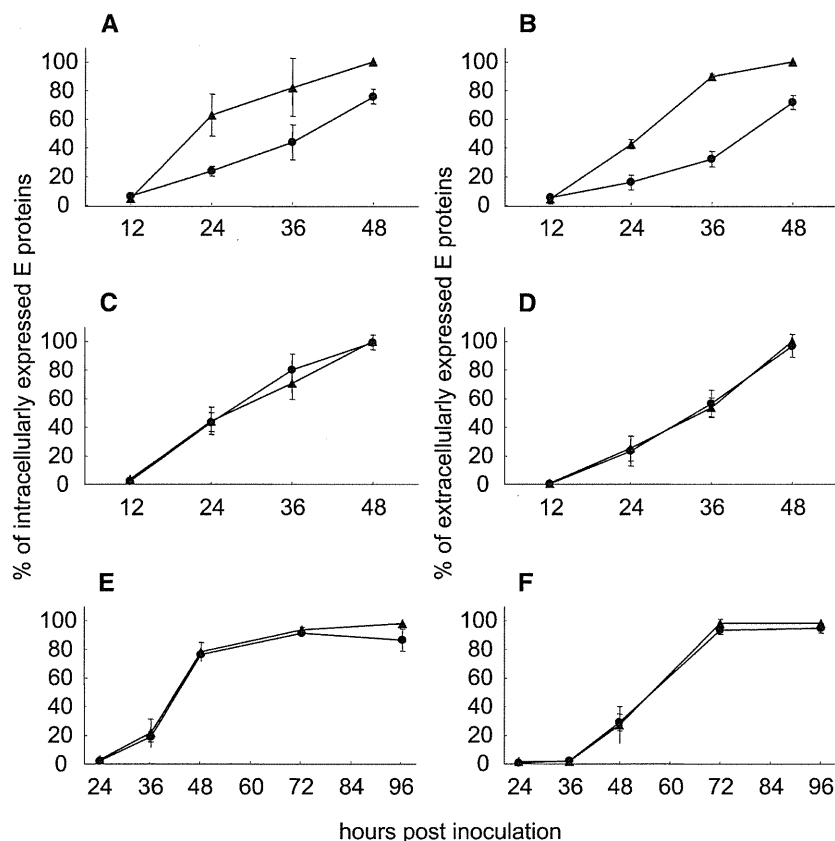


FIGURE 8. The effects of glycosylation of the E protein on intracellular expression and extracellular secretion of the E protein in QT6 (A–D) and C6/36 (E and F) cells. QT6 cells were incubated at 42°C (A and B) and 37°C (C and D), and C6/36 cells were incubated at 28°C. The amounts of intracellular and extracellular E protein of 6-LP (▲) and 6-SP (●) variants were measured by antigen-detection ELISA. Culture cell lysates (A, C, and E) and supernatants (B, D, and F) were harvested sequentially from QT6 and C6/36 cells. The percentage of expressed proteins were (OD values at each time point)/(OD values at 48 hours of LP in A–D or those at 96 hours of LP in E and F). Mean (\pm SD) titers are from triplicate cultures.

lack of glycosylation of the E protein affects viral replication at steps before the viral-release process.

DISCUSSION

Previously, we plaque purified two NY strains of the WN virus and isolated four variants that had different amino acid sequences at the N-linked glycosylation site in the E-protein sequence. The E protein was glycosylated in two of these strain variants. The glycosylated variants produce LPs, and the non-glycosylated variants produce SPs in BHK cells; the LP variants are more pathogenic in mice than are the SP variants. The gene sequences of WN viruses from various locations around the world were investigated and compared. We found that most of the strains occurring before the 1990s and some of the low pathogenic strains do not have the N-glycosylation site, whereas many of the highly pathogenic strains that emerged recently have the N-glycosylation site.¹² A rare WN virus isolated in Mexico lacks the glycosylation site on the E protein, and it was shown to have reduced pathogenicity in mice.¹⁸

N-linked glycosylation of the WN virus E protein was previously shown to be responsible for enhanced neuroinvasiveness of the virus in a mouse model.^{12,14,19} However, few studies²⁰ have been conducted to determine the role of glycosylation of the E protein in WN virus dynamics in birds and mosquitoes,

both natural hosts of the virus. We selected young chicks as a model to examine WN virus dynamics, and we found that young chicks can serve as a model to study the pathogenicity of WN virus in avian hosts. Subcutaneously injected LP variants resulted in a much higher mortality rate ($LD_{50} < 0.1$ PFU) than SP variants, suggesting that glycosylation of the E protein of WN virus is a determinant of pathogenicity in chicks that have been peripherally inoculated. Histopathological findings in dead chicks included necrosis in hepatocytes and necrotic myocarditis, and cardiovascular failure was the suspected cause of death in these birds. These histopathological changes were also seen in birds that had been naturally infected with WN virus.²¹

Efficient viral propagation both in avian and mosquito hosts is an important determinant of active viral circulation in the natural transmission cycle. We tested the viremic levels of LP and SP variants in chicks to reveal the effect of glycosylation of the WN virus E protein on the magnitude of viremic levels. The viremic levels of chicks inoculated with LP variants were higher than those inoculated with SP variants. The viremic titers of chicks inoculated with LP variants exceeded 10^5 PFU/mL blood during 2–4 dpi. Previous studies showed that avian viremic levels higher than 10^5 PFU/mL are crucial for the efficient infection of vector *Culex tritaeniorhynchus* mosquitoes.⁹ These results showed that N-linked glycosylation of

WN virus E protein is a determinant of high viremic levels in young chicks and suggest that glycosylated WN-virus variants may be more efficiently transmitted to vector mosquitoes than non-glycosylated variants because of higher viremia in infected birds.

To explain the differences in viremic titers of chicks inoculated with the two variants, growth characteristics of the LP variant, which is glycosylated, and the SP variant, which is not glycosylated, were examined in tissue culture cells at different temperatures. The results suggest that glycosylation of the E protein imparted heat stability to WN virus during propagation in cells at high temperature. We tested three kinds of cultured cells, namely BHK cells from a mammalian host, QT6 cells from an avian host, and C6/36 cells from mosquitoes, each representing an important host in the natural transmission cycle of WN virus. Viral growth characteristics were examined by culturing the cells at different temperatures. Compared with LP variants, SP variants showed a remarkable reduction in viral growth in BHK cells at 37°C and 40°C and in QT6 cells at 40°C and 42°C. Reduction rates of viral titers in the culture media without cells were not significantly different between SP and LP variants. Collectively, differences in the heat-stable characteristics of the LP variants and the heat-labile characteristics of the SP variants in BHK cells and QT-6 cells at high temperatures depended on the glycosylation status of the E protein of the variants, which affected the viral-replication steps within the cells.

In contrast, we did not detect a significant difference in viral titers between the LP and SP variants when *Culex pipiens* mosquitoes were inoculated intrathoracically with each variant, and the amount of virus was measured in the mosquitoes. The disseminated infection rates of mosquitoes orally infected with the variants did not show any difference between the LP and SP variants. Moreover, there were no differences in the propagation of the two variants in C6/36 cells at various temperatures. The results suggest that the glycosylation status of the E protein may not affect viral propagation and dissemination in mosquitoes. Recently, Moudy and others²⁰ reported that WN virus E-protein glycosylation is required for efficient viral transmission by *Culex* mosquitoes.²⁰ This contradiction might have been caused by the difference of the mosquito strains or method of viral measurement assays. In our experiment, a relatively small number of mosquitoes were examined compared with that of their experiment, which might also cause this contradiction. In addition, they mutated the E-protein glycosylation site from NYS to IYS in a full-length clone of the NY99 strain, which resulted in a virus that lacked the glycan at aa154. In our current study, we generated non-glycosylated variants by mutating aa156; the NYS glycosylation site was changed to NYP for 6-SP and to NYE for B-SP. It is interesting to establish how these differences in amino acid sequence among the variants affect viral propagation and dissemination in vector mosquitoes.

Furthermore, we conducted the same multiplication experiment using variants of the NY strain of the WN virus generated by infectious cDNA clone technology.¹⁵ This experiment was performed to exclude the possibility that some silent mutations in the coding regions other than the N-glycosylation site and the 3'NCR contribute to the differences between the SP and LP variants. The recNY/Gly+ and the recNY/Gly- strains only differed in the N-linked glycosylation site (S156P), and the recNY/Gly+ strain, with a glycosylated E protein, showed stable multiplication in QT6 cells at higher temperature. We

previously examined the role of the N-linked glycans of E protein in tick-borne encephalitis-virus particle secretion using subviral particles.¹³ Secretion of virus particles was greatly reduced in culture cells transfected with mutant vectors that have an amino acid substitution of T156A in the E protein, and the study also suggested that the reduced particle secretion is caused by glycan loss rather than to the amino acid substitution per se. The amino acid substitution of T156A is similar to that of S156P in our current study in terms of amino acid characteristics, and both mutations altered the protein such that it would not be recognized by oligosaccharyl-transferase.²² Collectively, the observed differences between LP and SP variants are most likely caused by glycan loss on the E protein rather than to the amino acid substitutions.

When the E proteins in samples from QT6 cells were quantified by ELISA, the E-protein levels were found to be lower both in the cell lysates and the supernatants of cells infected with the 6-SP variant than in those infected with 6-LP. This result suggests that glycosylation participates in the multiplication of WN virus at an earlier stage than the viral-release process.

There were several reports of temperature sensitive, attenuated WN virus variants, and those temperature sensitive phenotypes were caused by the amino acid substitutions at NS genes, which might reduce the efficiency of RNA replication.²³⁻²⁵ But there was no report studying the relationship among temperature sensitivity of WN virus, the glycosylation of envelope protein, and the intracellular viral protein maturation and trafficking. Our previous study¹³ using a subviral system of tick-borne encephalitis virus showed that a mutant lacking E-protein glycosylation has a large reduction in the level of secretion of the E protein; the E protein is retained at the endoplasmic reticulum and is rarely present in the Golgi complex. In the dengue virus, this glycosylation at aa154 occurs in E-protein domain I, close to the center of the fusion peptide of E-protein domain II, and glycosylation of the E protein is considered to increase the stability of the protein.^{26,27} Glycosylation of the E protein of WN virus may also be important for the folding and stability of the viral protein at high temperatures.

Mutations of NS3 or NS4B of the NY strain of WN virus were reported to be responsible for the increased pathogenicity and viremic level in avian or mammalian hosts.^{8,23} Importantly, the introduction of a T249P amino acid in NS3 helicase was shown to be crucial for the above-mentioned viral characteristics. We showed that N-glycosylation of the E protein facilitated efficient multiplication of the NY strain of WN virus at high temperatures in an avian cell culture, and it was responsible for the higher viremic level in an avian host. The observation that most recent isolates of lineage I WN virus carry the N-glycosylation site on the E protein¹² suggests that glycosylation of the E protein is a pre-requisite for the stable circulation of WN virus in the avian-mosquito transmission cycle, and it may be one of the multiple determinants for efficient transmission.

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