

Early Inhibition of Glycosylation with Deoxynojirimycin Has No Effect on the Replication of Flavivirus West Nile

Mah Lee NG and Lay Tin AW

Department of Microbiology, National University of Singapore, Kent Ridge 0511, Singapore

Abstract: Deoxynojirimycin (dNM) is a specific and reversible inhibitor of the trimming actions of glucosidase I and II in the rough endoplasmic reticulum. The importance of the effects of dNM on the replication of West Nile (sarafend – glycosylated) and West Nile (Wengler – nonglycosylated) viruses were studied. It was found that dNM treatment increase the molecular weights of the E and prM proteins in both virus strains without affecting the yields of the virus. The intracellular transport of the E protein from the rough endoplasmic reticulum to Golgi apparatus was not affected despite the change in molecular weights. It would appear that the prevention of removal of glucose molecules by dNM did not affected the maturation of E protein or the virus.

Key words: Glucosidase inhibitor, Deoxynojirimycin, West Nile virus

INTRODUCTION

The envelope (E) protein molecules of flavivirus have a role in the attachment and entry of the virus (Gollins and Porterfield, 1985). Despite the role of the E protein, evidence have shown that some mature viruses have glycosylated envelope (E) proteins but are absent in other viruses (Wengler *et al.*, 1985; Wright, 1982). Little is known about the significance of glycosylation in the process of maturation of viruses. Over the past decade, there has been wide-spread use of inhibitors of N-linked oligosaccharide processing to study their effects on viral assembly, maturation and infectivity (Gruters *et al.*, 1987; Kang and Elbein, 1983; Pal *et al.*, 1989; Pan *et al.*, 1983; Romero *et al.*, 1983; Schwarz *et al.*, 1976).

Deoxynojirimycin (dNM) is an antibiotic which inhibits early glycosylation process. It is specific and reversible and act on the trimming actions of glucosidase I and II in the rough endoplasmic reticulum (RER). The two naturally occurring forms of West Nile (WN) virus i.e. the glycosylated Sarafend strain and the unglycosylated Wengler strain, provided a very good model to study the significance of the glycosylation process since both can be cultivated in similar host cell system. Any discrepancies due to differences in host system which might give rise to variation in the ability to produce mature glycoprotein could be eliminated.

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Correspondence: M.L.Ng, Department of Microbiology, National University of Singapore, Kent Ridge 0511, Singapore.

The WN (Sarafend) and WN (Wengler) virus-infected cells were exposed to dNM at appropriate concentrations under optimum conditions throughout the post-infection period. The aspects investigated were the yields of the virus, production of virus-specified glycoproteins and the intracellular transportation of the E protein.

MATERIALS AND METHODS

Cells and viruses: Vero cells is a continuous cell line first isolated by Dr Y Yasumura at Chiba University, Japan from the kidney cells of African green monkey. The cells were grown to confluency in Medium 199 containing 5% foetal calf serum and maintained after infection in Eagle's minimum essential medium (MEM) containing 0.1% bovine serum albumin. The two strains of WN virus used were WN (Sarafend) and the WN (Wengler). The WN (Sarafend) was from Professor E G Westaway, Australia and the WN (Wengler) was from professor G Wengler, Germany.

Drug treatment during cell infection: The drug, deoxynojirimycin. HCL (dNM, Sigma, USA) was in powder form packed in 10mg quantity. Deoxynojirimycin was easily dissolved in PBS to make up a working solution of 10mM.

For continuous drug treatment on cell monolayer, drug-supplemented MEM was added after infection and incubated at 37°C. The infection procedure was done on a confluent monolayer grown in 25cm² flask (Falcon, USA).

Harvesting of intracellular and extracellular virus: The extracellular virus was harvested by transferring the culture medium to sterile centrifuge tubes and centrifuged at 1,000 rpm for 1 minute to remove the cell debris. The supernatant was then dispensed into sterile glass ampoules. The glass ampoules were frozen in a mixture of absolute ethanol and dry ice and stored at -70°C until assayed.

After the removal of the culture medium for the extracellular virus, 2ml of MEM was added. The flask was left horizontally in -70°C freezer for 15 minutes. The frozen cells were quickly thawed under tap water. The freezing and thawing steps were repeated for another two more times in order to detach and break up the cells, to release the intracellular virus. Cell debris was spun down and the supernatant aliquoted into glass ampoules as mentioned above. The virus titers were obtained by plaque assay.

Radioactive labelling with ³H-mannose: Confluent monolayers of Vero cells grown in 25 cm² tissue culture flasks were infected. Drug supplemented MEM was added to the infected cells after the 1 hour adsorption period.

To label the virus-specified glycoproteins with ³H-mannose, the infected monolayer was exposed to medium deficient of glucose for 2 hours and subsequently replaced with labeling medium containing 40μCi/ml of ³H-mannose for 6 hours. All the media above contained the dNM to the final concentration of 2.5mM and 5mM. A mock-infected control and an infected control without drug treatment were incubated similarly as above.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The 12% polyacrylamide gels used were the Laemmli gel system (1970). Twenty-five μl of each labelled sample were used for each lane. Prior to loading, the sample was mixed with 5 μl of cocktail containing dithioerythritol and bromophenol blue and boiled for 1 minute.

Indirect immunofluorescence microscopy: The cells were grown on coverslips in 24-well plates (Falcon, USA). The cell monolayers were infected with WN (Sarafend) and WN (Wengler) when they reached 75% confluency. The cells were fixed with cold methanol for 10 minutes before rinsing in cold PBS. The processing for immunofluorescence staining was as described previously by Westaway and Goodman (1987).

Indirect immunofluorescence with single labelling using anti-envelope serum was carried out for WN (Sarafend) virus at a series of time intervals (4 hour-intervals upto 20 hours post infection). As for WN (Wengler) virus the timings were 32, 48 and 64 hours post infection. Double labelling were done at 20 hours post infection and 48 hours post infection for WN (Sarafend) and WN (Wengler) viruses respectively.

The antisera used were anti-virus envelope protein (Dr Vincent Duebel, Pasteur Institute, France), anti-Golgi complex (Dr Paul Gleeson, Monash University, Australia) and the concanavalin A (Vector Laboratory, USA). The concanavalin A was used as a tag for endoplasmic reticulum membrane and was a gift from Dr Hong Wan Jin, IMCB, Singapore.

RESULTS

Extracellular and intracellular virus yields: The results are tabulated in Tables 1 and 2. Only small differences in virus titres were discernible for WN (Sarafend) virus (Table 1) and WN (Wengler) virus (Table 2). From the results, it could be seen that generally, in the WN (Sarafend) virus infections, the intracellular virus titers were higher than the extracellular virus titers. However for the WN (Wengler) virus, the reverse was true. A closer examination of the data showed that virus titers varied by approximately 1.5 to 5 times between the drug-treated and untreated samples for the WN (Sarafend) virus infections and a difference of 1.8 times for WN (Wengler) virus infections. These small difference were insignificant and

Table 1. Virus titres from drug-treated and untreated West Nile (Sarafend) virus-infected cells.

Experimental Samples	Controls (PFU/ml)	Drug treatment (PFU/ml)	
		2.5mM	5mM
Intracellular	2.6×10^8	1.69×10^8	
	1.05×10^9		2.6×10^8
Extracellular	1.3×10^7	4.5×10^7	
	1.8×10^8		1.4×10^8

Table 2. Virus titres from drug-treated and untreated West Nile (Wengler) virus-infected cells.

Experimental Samples	Controls (PFU/ml)	Drug treatment (PFU/ml)	
		2.5mM	5mM
Intracellular	1.18×10^6	2×10^6	
	2.0×10^6		3.8×10^5
Extracellular	3.4×10^7	1.88×10^7	
	2.7×10^7		2.0×10^8

hence showed that the drug had no effect on the infectivity and final maturation of the viruses.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of virus-specified glycoproteins labelled with ^3H -mannose: Since dNM inhibits the early step in the glycosylation pathway, further experiments were carried out to examine the effects of the drug on the virus-specified glycoproteins. The experiments used ^3H -mannose as it is more specific since only the glycoprotein side chains were labeled.

From the autoradiograph (Fig. 1), the E and the precursor membrane (prM) proteins were heavily glycosylated and appeared as a broad smear. It was observed that the E and the prM glycoprotein bands in the drug-treated samples showed higher molecular weights (MW) compared with the untreated samples. This effect of the drug was observed for both WN (Sarafend) and WN (Wengler) viruses infections.

For the WN (Sarafend) virus, the MW of the E protein band from the drug-treated samples in lanes 2 and 3 (Fig. 1) were found to be approximately 60.95 kd to 61.66 kd respectively. The E protein of the untreated infected control had a MW of 58.20 kd (Lane 4). This showed that dNM increased the MW of E protein by about 2 to 3 kd. The prM protein bands from the WN (Sarafend) virus infections were also affected by the drug. The MW of the drug-treated samples was increased from 21.38 kd (untreated—Lane 4) to 21.63 kd (Lane 2) and 22.13 kd (Lane 3). This slight differences in MW of PrM (about 1 kd) is quite significant since the prM is a small protein.

It was observed that the intensities of the E and prM glycoprotein bands in the WN (Wengler) virus samples decrease due to the drug treatment. This effects being more severe in the sample treated with dNM at the concentration of 2.5 mM (Fig. 1, Lane 5). Similar increase in MWs of E and prM glycoproteins were observed in the drug-treated samples as with the WN (Sarafend) infection. There was about an increase of about 1 kd for both the E and prM proteins.

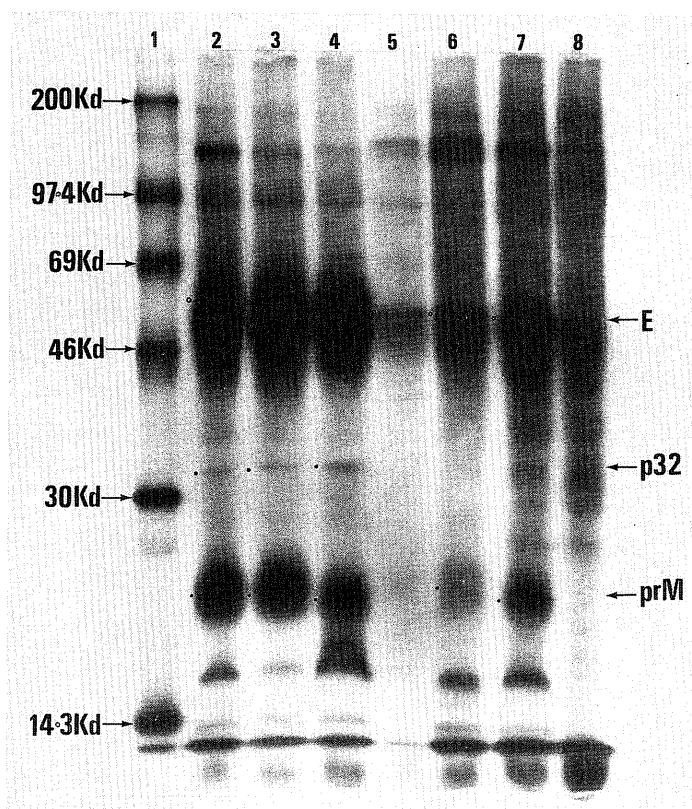


Fig. 1 Autoradiography of SDS-Page of cell lysates from West Nile virus-infected Vero cells labeled with ^3H -mannose.

Lane 1—the molecular weight markers were a [^{14}C] methylated mixture consisting of myosin (200 kd), phosphorylase b (97.4 kd), bovine serum albumin (69 kd), ovalbumine (46 kd), carbonic anhydrase (30 kd) and lysozyme (14.3 kd).

Lane 2—the West Nile [(WN) (Sarafend)] virus-infected sample treated with 2.5mM of dNM.

Lane 3—the WN (Sarafend) virus-infected sample treated with 5mM of dNM.

Lane 4—the untreated WN (Sarafend) virus-infected control sample.

Lane 5—the WN (Wengler) virus-infected sample treated with 2.5mM of dNM.

Lane 6—the WN (Wengler) virus-infected sample treated with 5mM of dNM.

Lane 7—the untreated WN (Wengler) virus-infected control sample.

Lane 8—the untreated and uninfected control sample.

In the autoradiograph, only the structural proteins such as the envelope (E) proteins, precursor membrane (prM) proteins and p32 proteins are seen. The p32 protein bands are only found in the WN (Sarafend) virus-infected samples. Close circles denote the viral proteins excluding the E proteins (indicated by open circle).

Indirect immunofluorescence: The study on the effects of dNM on intracellular transport of E protein in WN virus replication was carried out using indirect immunofluorescence. The subcellular localisation of E protein was first examined using anti-envelope serum. To visualise the distribution of the E protein in the RER and the Golgi complex, rhodamine-conjugated concanavalin A (Con A) and anti-Golgi serum were used as markers for the respective compartments.

The location of E protein was similar to the other flaviviruses as described by Ng and group (1983) and Westaway and Goodman (1987). There was a fluorescent rim at the perinuclear region which became more intense as the infection progressed (not shown). The fluorescence radiated from the boundary of the nucleus and spread in a polar fashion into the cytoplasm. With dNM treatment, there was no change in the pattern and intensity of fluorescence (not shown). These observations were consistent for both strains of WN virus and agreed with the reported works.

Double labelling with anti-envelope serum and concanavalin A: In order to investigate if dNM affect the glycosylation pathway or intracellular transport of the E protein in WN (Sarafend) and WN (Wengler) virus-infected cells, the E proteins were first co-localised with the RER via double labelling immunofluorescence. In the double-labelling immunofluorescence, the anti-E serum was conjugated with fluorescein isothiocyanate (FITC) while the Con A was conjugated with rhodamine.

The cells were infected with West Nile (Sarafend) virus for 20 hours and reacted with anti-envelope serum and Con A (Fig. 2). The fluorescence staining with anti-envelope serum was most intense at the perinuclear region (Fig. 2a) and radiated out into the cytoplasm in a reticular pattern. With the rhodamine-Con A staining (Fig. 2b), the fluorescence pattern was very similar to the anti-envelope reaction. When the infected cells were treated with either 2.5 mM or 5 mM of dNM, the fluorescence patterns did not show any significant changes (Fig. 2c and d).

Similarly, when WN (Wengler) virus was used in the infection, the fluorescence patterns after 48 hours of infection was comparable with the WN (Sarafend) infection (Fig. 3a and b). The drug also did not alter the localisation of the envelope protein in the endoplasmic reticulum (Fig. 3c and d).

Double labelling with anti-envelope and anti-Golgi complex sera: Since the dNM affects the early glycosidases actions, it was of interest to observe if these unprocessed E proteins would be transported from the endoplasmic reticulum to the Golgi complex. Double labelling was performed using anti-envelope and anti-Golgi complex sera under the same conditions as the above. The results showed that for both WN viruses infections (without dNM treatment), the E protein was transported to the Golgi complex (see Fig. 4a, b and 5a, b). There were coincidence in the fluorescence staining patterns between the anti-envelope and the anti-Golgi complex reactions. Once again the dNM did not showed any drastic changes in the fluorescence for both virus infections (Fig. 4c, d and 5c, d).

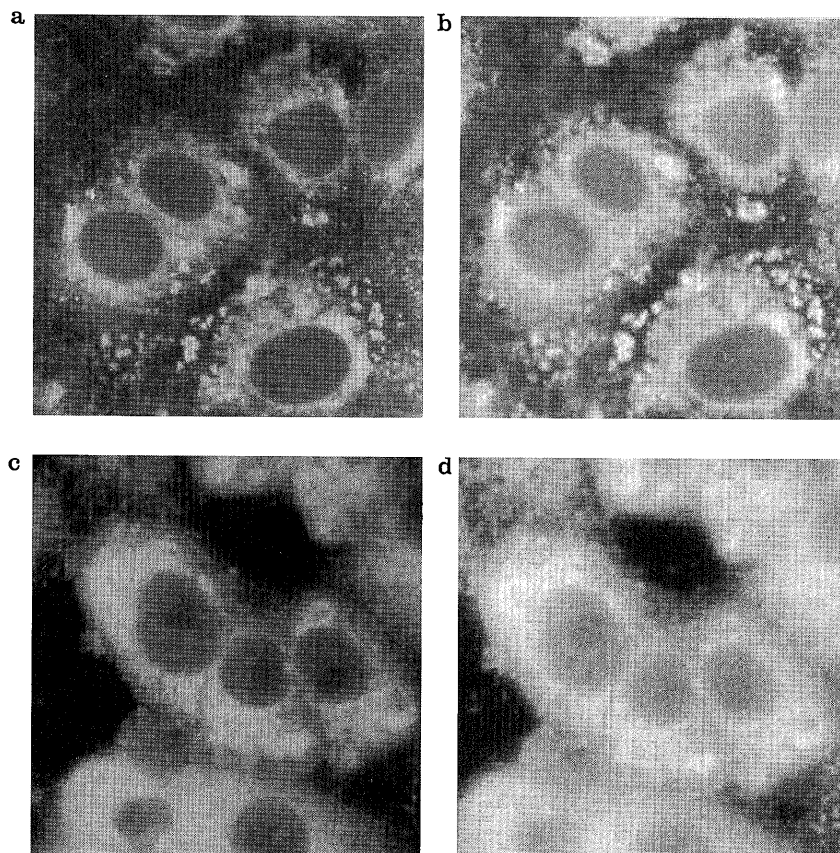


Fig. 2 Double indirect immunofluorescence of West Nile (Sarafend) Virus-infected Vero cells reacted with anti-envelope serum and concanavalin A.

The cells were infected for 20 hours and treated with anti-envelope (α -E) serum and concanavalin A (Con A). For the drug treatment, 2.5 mM or 5 mM of dNM were added to the media immediately after infection and incubated at 37°C until 20 hours post infection. Magnification of all the figures is 1000 times.

Infected cells not treated with dNM: (a) shows the fluorescence pattern of the α -E which was conjugated to fluorescein isothiocyanate (FITC). A diffuse reticular pattern radiates from the perinuclear region and encompasses the cytoplasm of the cell. The fluorescence is most intense at the perinuclear region, showing distinct nuclei that are unstained. 'speckled' fluorescence pattern is observed near the peripheral regions of the cells. (b) Con A is conjugated with rhodamine and binds at the membrane of the endoplasmic reticulum (ER). It could be observed that the fluorescence pattern is similar to that observed in (a). The coincidence of the fluorescence pattern shows that the E proteins in the WN (Sarafend) virus-infected cells are found in the ER.

Infected cells treated with dNM: There was no difference in the fluorescence intensities in cells treated either with 2.5 mM or 5 mM dNM. (c) An intense perinuclear fluorescence pattern extends into the cytoplasm in a slightly polar fashion, with decreasing intensity towards the perinuclear region. Very fine punctuated dots are seen near the boundary of the cell. (d) Bright and 'speckled' fluorescence pattern in the perinuclear region as well as encompassing the cytoplasm is observed. The strong fluorescence pattern of anti-envelope serum (FITC) co-localised with that for rhodamine Con A. This shows that the E proteins accumulate in the endoplasmic reticulum (RE) even in the presence of dNM. The drug did not seem to affect the subcellular distribution of the E protein in the ER as compared to the untreated samples (a & b).

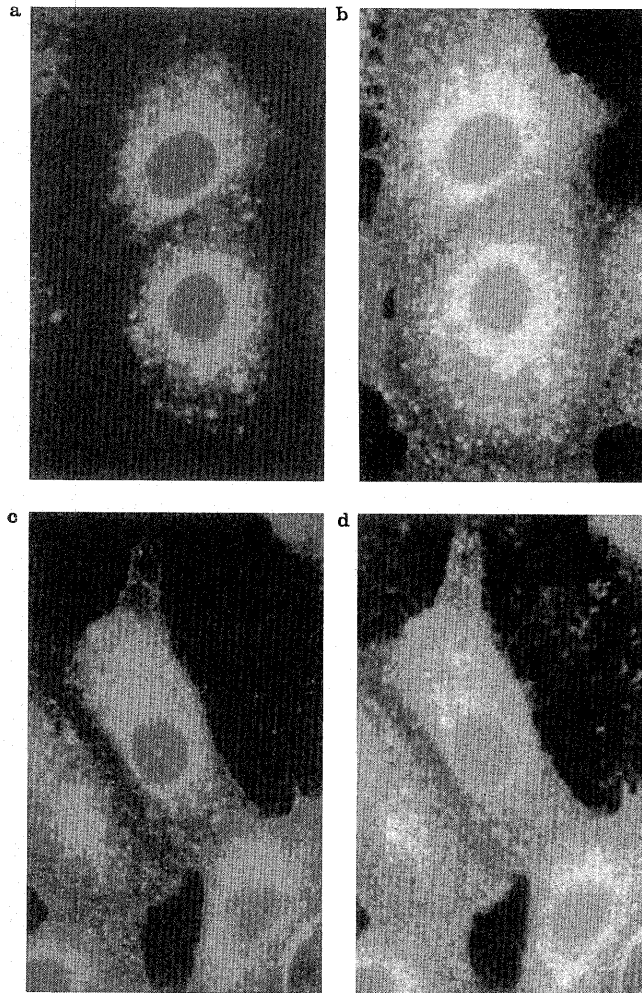


Fig. 3 Double indirect immunofluorescence of West Nile (Wengler) virus-infected Vero cells reacted with anti-envelope serum and concanavalin A.

The cells were infected for 48 hours and stained with anti-envelope serum and Con A. The drug-treated infected cultures were exposed to the dNM at either 2.5 mM or 5 mM immediately after infection for 48 hours. Magnification of all figures is 1000 times.

Infected cells not treated with dNM: (a) Very intense network of fluorescence is seen around the perinuclear region. More 'speckled' fluorescence pattern is seen in the cytoplasm. The intensity of the fluorescence decreases from the perinuclear area to the peripheral region. (b) the intense fluorescence from labelling with rhodamine conjugated Con A is shown. Intense fluorescence is observed over the entire cytoplasm with the intensity higher at juxtannucleus position. The coincidence of the fluorescence pattern from (a) and (b) indicated that the E proteins in the West Nile (Wengler) virus-infected cells are also localised in the ER.

Infected cells treated with dNM: Again there was no difference in the intensities of the fluorescence after treatment with either 2.5 mM or 5 mM of dNM. (c) The fluorescence pattern obtained is similar to (a) except that the cells have more cytoplasmic extensions (filopodia). The intense particulate fluorescence encompasses the cells in a polar fashion with higher intensities around the perinuclear regions. (d) The bright fluorescence pattern resulting from staining with rhodamine Con A appears similar to (c). Thus dNM also does not affect the distribution of the E proteins in the ER.

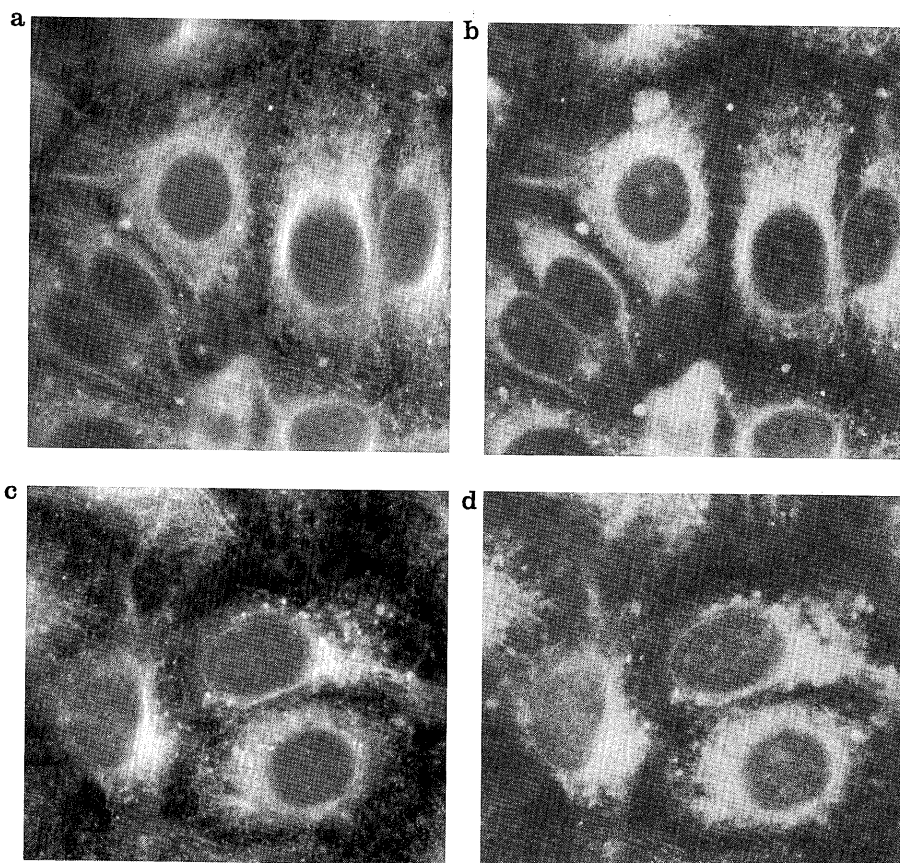


Fig. 4 Double indirect immunofluorescence of West Nile (Sarafend) virus-infected vero cells reacted with anti-envelope and anti-Golgi sera.

The treatment of the infected cells were similar to Fig. 2 except that the cells were stained with anti-envelope and anti-Golgi sera. The conjugate used were FITC and Texas red respectively. Magnification of all figures is 1000 times.

Infected cells not treated with dNM: (a) A reticular network of fluorescence spread over the entire cytoplasm, with higher intensity at the perinuclear region. (b) The staining using anti-Golgi serum exhibit less bright fluorescence pattern when compared to (a). The fine filaments of fluorescence encompass the unclear, with some filaments extending into the cytoplasm.

Infected cells treated with dNM: The staining patterns and intensities of the fluorescence were similar for both treatment at 2.5 mM and 5 mM. (c) A reticulate network radiating from an intense point fluorescence at one side of the nucleus is observed. Some speckles are observed near the peripheral region. (b) The polar extension of the filamentous and 'speckled' fluorescence patterns are also observed with staining of the Golgi apparatus with Texas red.

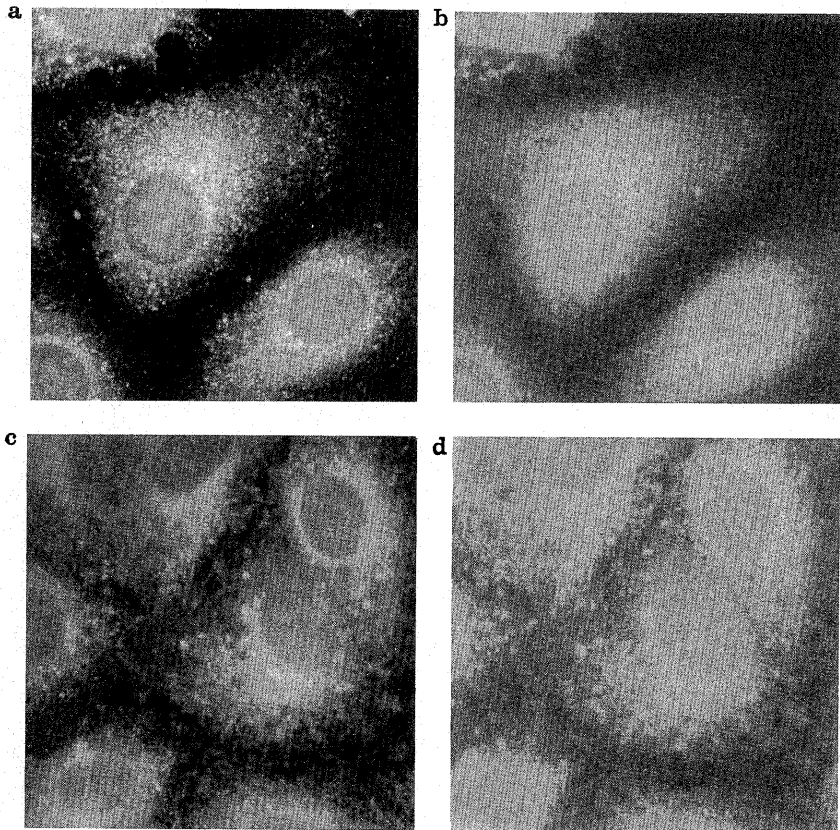


Fig. 5 Double indirect immunofluorescence of West Nile (Wengler) virus-infected Vero cells reacted with anti-envelope and anti-Golgi sera.

The infected cells were treated similarly as in figure 3 except that staining were done using anti-envelope and anti-Golgi sera. Magnification of all figure is 1000 times.

Infected cells not treated with dNM: The particulate fluorescence pattern is distributed throughout the cytoplasm. (b) The dim 'speckled' pattern of the fluorescence extends from the perinuclear region to the cytoplasm. The fluorescence seem to be slightly polar. The patterns of (a) and (b) appear to be similar. *Infected cells treated with dNM:* There was again no difference in the staining intensities between the two dNM concentrations. (c) Intense filamentous network of fluorescence is seen concentrated around the perinuclear region and extends in a polar fashion into the cytoplasm. The intensity of fluorescence decreases at the cell periphery. Some granular specks of fluorescence are seen at the peripheral region. (d) The perinuclear reticular fluorescence pattern of the Golgi complex is observed with Texas red staining. The intensity of the fluorescence remains greatest at the boundary of the nucleus.

The patterns from (a) to (d) are similar, thus dNM treatment does not affect the distribution of E in the Golgi complex nor the intracellular transport of E proteins from the endoplasmic reticulum to the Golgi complex.

DISCUSSION

The significance of glycosylation in the infectivity and maturation process of viruses is not known. In an attempt to study the importance of glycosylation in flavivirus, two WN viruses known to have the glycosylated and nonglycosylated E proteins in mature virions were chosen. The two viruses were the WN (Sarafend) and WN (Wengler). West Nile (Wengler) virus was established by Wengler and colleagues (1985) to have nonglycosylated E proteins in the extracellular virus from nucleotide sequence analysis. It was found that the membrane-associated protein did not contain an Asn-X-Ser/Thr sequence which would allow addition of N-linked carbohydrate chains in this protein. The intracellular E protein is however glycosylated (V. Sreenivasan, personal communication).

Deoxynojirimycin (dNM) and antibiotic known to inhibit at the very early stage of glycosylation was used in this study. The dNM prevents trimming actions of the glycosidases in the RER (Fuhrmann *et al.*, 1985). In addition, dNM showed little toxicity *in vitro* and *in vivo* (Gruters *et al.*, 1987). The effects of inhibiting protein glycosylation have been reported to either lead to an increase in susceptibility of the nonglycosylated protein to proteolytic cleavage, inefficient intracellular transport of the protein to the cell surface or inhibition of virus assembly and infectivity (Sim, 1990). This approach of using inhibitor of glycosylation to study the replication process of viruses has the advantage of allowing conclusions to be made about the roles of sugars during synthesis and maturation of glycoproteins.

Analysis of the experimental results showed that dNM does not affect the infectivity and final maturation of both the WN viruses (Tables 1 and 2). The titers obtained from the drug-treated WN (Sarafend) and WN (Wengler) virus-infected cells were similar to the titers for their respective untreated infected cells. The presence of the dNM did not prevent virus release. This result is consistent with that obtained in the infection with vesicular stomatitis virus, influenza virus and fowl plaque virus in the presence of dNM (Burke *et al.*, 1984; Romero *et al.*, 1983).

The dNM treatment did increase the molecular weights of the E and prM proteins as seen in the ³H-mannose labelling (Fig. 1). However, this increase in molecular weights did not affect the maturation process of these viruses as shown in Table 1. The E proteins of the viruses have been reported to be important in attachment and recognition of host cell receptors (Gollins and Porterfield, 1985). The non-removal of glucose residues from the E proteins of WN viruses after dNM treatment, did not result in any impairment in the functions such as attachment and recognition of host cell receptors (as seen in the plaque assay — Tables 1 and 2). It could be postulated then that the change in the E proteins were so slight that the proteins configurations were not affected to an extent which might affect recognition of cellular receptors.

On the other hand, the maturation process of the viruses might not be a tightly controlled system and would allow slight variability in the carbohydrate side chains on the viral glycoproteins. The sugar moieties on these structural proteins (E and prM) did not appear to play essential roles. In the formation of the mature virion of WN (Wengler) virus, the

carbohydrate moiety of the precursor glycoproteins could be trimmed off before or during the final maturation of virions. Such a cleavage probably would not depend on the presence of unique oligosaccharides structure, as inhibition of glucose trimming by dNM does not affect final maturation of the WN (Wengler) virus. Similar explanation was given by Wright (1982) who worked on Kunjin virus (member of the West Nile subgroup) which showed that Kunjin virus have the glycosylated E proteins only in the intracellular virus but not the extracellular particles.

The results obtained from the SDS-PAGE and plaque assays prompted further analysis of the effect of dNM on the subcellular localisation of the E protein and the intracellular transport of this protein. This was carried out with immunofluorescent technique using anti-envelope serum, Con A and anti-Golgi serum. It was observed that dNM did not affect the intracellular transport of the E proteins from the RER to the Golgi complex (Fig. 2 to 5). Thus, prevention of trimming of the outermost glucose moieties on the core oligosaccharid $\text{Glc}_3 \text{Man}_9 \text{GlcNAc}_2$ by dNM did not inhibit the transport of the envelope glycoproteins of WN viruses.

This result is in contrast to that obtained for vesicular stomatitis virus (Schlesinger *et al.*, 1984) but similar to that for haemagglutinin (HA) of influenza virus (Romero *et al.*, 1983; Burke *et al.*, 1984), as well as for envelope glycoprotein of Rous sarcoma virus. This again showed that the sugar moieties did not have a direct role in the recognition process by the receptor proteins in Golgi complex during the trafficking of the E protein precursors. It seems that both the WN (Sarafend) and the WN (Wengler) viruses follow the same glycosylation pathway from the RER to the Golgi complex in the infected Vero cells.

Another interesting observation from the autoradiograph was that the drug concentration at 2.5 mM appeared to have a greater effect on the WN (Wengler) virus infection compared with 5 mM (Fig. 1, Lanes 5 and 6). This phenomenon was not observed with the WN (Sarafend) virus. One possibility might be that 5 mM of the drug exceeded the k_i (inhibition constant) by two to three order of magnitude. This according to Fuhrmann and colleague (1985) could lead to unreliable results.

From this study the conclusions are that both WN (Sarafend) and WN (Wengler) viruses possess similar glycosylation pathway whereby the E proteins are transported from the RER to the Golgi complex. The deoxynojirimycin does not affect the intracellular transport of the E proteins. Therefore, prevention of removal of glucose molecules does not affect the intracellular maturation of the E protein, showing that this process may not be tightly controlled in the WN viruses. The presence of glucose residues in the high-mannose oligosaccharide precursors of the E protein does not interfere with the maturation of the progeny viruses.

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