EFFECT OF TUNICAMYCIN ON EXPRESSION OF EPITOPES ON JAPANESE ENCEPHALITIS VIRUS GLYCOPROTEIN E IN PORCINE KIDNEY CELLS

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Summary. – The effect of tunicamycin (Tm), a glycosylation inhibitor, on the epitopes expressed on Japanese encephalitis virus (JEV) glycoprotein E (gpE) in porcine kidney stable (PS) cells was studied. At Tm concentration of 2 μg/ml, the virus-infected cells showed markedly reduced or no reactivity with any of the monoclonal antibodies (MAbs) directed against JEV gpE except NHs-2 and also with polyclonal antibodies (PAbs) directed against JEV. With the increase in Tm concentration to 3 μg/ml, a complete loss of the conventionally detected reactivity of the MAbs except NHs-2 was recorded, while the Pabs showed no decrease in their reactivity. However, the MAb NHs-2 and PAbs lost their reactivity when the cells treated with 3 μg/ml Tm were stained for epitopes expressed on their surface indicating that glycosylation plays a role in this phenomenon. Tissue culture fluid (TCF) displayed a low virus content in the presence of 3 μg/ml Tm, indicating probably a down-regulation of virus maturation inside the cells. Since preM and NS-1 proteins possess besides gpE conserved N-glycosylation sites and play a role in the maturation of JEV, their expression in nascent, i.e. non-glycosylated form might be responsible for the observed low virus content of TCF. Thus, the glycosylation of JEV gpE seems essential for the acquisition of native conformation of its epitopes and their expression in cells.

Key words: Japanese encephalitis virus; glycoprotein E; epitopes; PS cells; tunicamycin; monoclonal antibodies; polyclonal antibodies

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Abbreviations: AF = ascitic fluid; BABS = bovine albumin borate saline, HA = hemagglutination; HAI = hemagglutination-inhibition; Hs = HAI- positive, JEV-specific; Hx = HAI-positive, flavivirus cross-reactive; IF = immunofluorescence; i.c. = intracerebral; JE = Japanese encephalitis; JEV = JE virus; gpE = glycoprotein E; LCM = lymphocytic choriomeningitis; MAb = monoclonal antibody; MEM = Minimum (Eagle's) Essential Medium; ML = murine leukemia; NHs = HAI-negative, JEV-specific; NHx = HAI-negative, flavivirus cross-reactive; NS = non-structural, PS cells = porcine kidney stable cells; PAb = polyclonal antibody; p.i. = post infection; RBC = red blood cells; TBEV = tick-borne encephalitis virus; TCF = tissue culture fluid; Tm = tunicamycin; VGM = virus growth medium; WN = West Nile

Introduction

JE caused by a mosquito-borne flavivirus, JEV, represents a major public health problem as evident from small and major outbreaks/epidemics occurring from time to time in South-East Asian and Western Pacific regions, including India (Rodrigues, 1984; Vaughn and Hoke, 1992; Monath and Heinz, 1996). The virus replicates in a variety of cells of mammalian, avian and mosquito origin (Brinton, 1986; Hase *et al.*, 1989) and is released probably by a budding process (White and Fenner, 1986). The replication cycle of the virus is mostly confined to the cytoplasm of infected cells (Gould *et al.*, 1983; Mason, 1989). A small part (15–20%) of the infected cells shows intranuclear immunofluorescence (IF) as the sign of the presence of viral

antigen, in addition to cytoplasmic IF during the early phase of virus infection, which is not observed by or after 24 hrs post infection (p.i.) (Gould *et al.*, 1983; Gupta *et al.*, 1991). Enucleated cells are incapable of supporting the virus replication, indicating some role of cell nucleus in the JEV replication (Lad *et al.*, 1993a).

Most of the enveloped animal viruses utilize the host cell glycosylation machinery to synthesize and process oligosaccharides attached to viral gps (Neutra and Leblond, 1986). The processing probably involves a conversion of simple oligosaccharides acquired by nascent polypeptides during their assembly in endoplasmic reticulum into a complex form, which is characteristic of mature proteins. It seems that oligosaccharides on gps play an important role in the initiation and maintenance of folding of gps into biologically active conformation and protecting them from proteolytic digestion, thus influencing their immunogenicity (Elbein, 1987; Pfeffer and Rothman, 1987).

Both virus-specific and group-specific epitopes have been detected on JEV gpE (Kimura-Kuroda and Yasui, 1983; Kobayashi *et al.*, 1984; Cecilia *et al.*, 1988). From the sequence data it has been deduced that the viral gpE has a conserved N-glycosylation site at around the 150th amino acid from the N terminus (Sumiyoshi *et al.*, 1987; Nityaphan *et al.*, 1990).

Studies carried out by Mason (1989) indicate that the newly synthesized JEV gpE is released slowly into TCF and the glycans associated with the gpE are converted into complex forms prior to the virus release from infected cells.

The present study was carried out to analyze (1) the effect of Tm, a glycosylation inhibitor, on the epitopes on JEV gpE expressed in PS cells and (2) the down-regulation of the infectious virus production in the presence of Tm.

Materials and Methods

Virus and cells. An Indian strain of JEV (strain 733913, Ghosh *et al.*, 1989), since isolation maintained by intracerebral (i.c.) passaging in 2-day-old suckling Swiss mice, was used. For the experiments with Tm, the virus was adapted to PS cells. In these cells, it was grown in the Earle's solution-based Minimum Essential Medium (MEM) of Eagle supplemented with 10% goat serum (virus growth medium, VGM). PS cells were grown almost to confluency on coverslips kept in Leighton tubes.at the 10th–15th passage level. The virus passaging in PS cells was carried out in VGM as described earlier (Gupta *et al.*, 1991).

MAbs and PAbs. The MAbs against JEV gpE described earlier (Kedarnath *et al.*, 1986) have been used. The respective hybrid cells have been maintained in the Dulbecco's solution-based MEM supplemented with 10% fetal calf serum. The hybrid cells were inoculated into Pristane-primed BALB/c mice and the produced ascitic fluid (AF) was collected by standard methods. The AF obtained by using SP2/o cells served as negative control. The MAbs were grouped as Hs (HAI-positive, JEV-specific), NHs (HAI-negative, JEV-specific), Hx (HAI-positive, flavivirus cross-reactive), and NHx (HAI-negative, flavivirus cross-reactive), depending on their reactivity with JEV, West Nile, and Dengue (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988) viruses. PAbs were raised in Swiss mice by immunization with JEV followed by production of ascites employing Ehrlich's tumor cells by standard procedures.

Tm treatment of PS cells. Cell monolayers were infected with JEV and Tm (Sigma) was added to VGM up to the required concentration (Lad *et al.*, 1992).

Indirect IF test. After fixation in chilled acetone, cells were stained with the MAbs in the form of respective AFs diluted 1:50 (Gupta *et al.*, 1991). The PAbs (against JEV) in the form of AF diluted 1:100 served as a positive control, while "a normal" AF diluted 1:100 and the AF obtained from mice inoculated with SP2/o cells served as negative controls. Different intervals (15, 24 and 36 hrs p.i.) were used for the staining.

Surface IF test. Unfixed cells were treated at 36 hrs p.i. with the MAbs or PAbs in the presence of sodium azide, then fixed and stained as in the indirect IF test (Gupta *et al.*, 1993). Appropriate positive and negative controls were included.

Effect of Tm on virus infectivity. Cell monolayers in Petri dishes (2.5") were infected with the virus in the presence or absence of 3 mg/ml Tm (Gupta *et al.*, 1991; Lad *et al.*, 1992). The TCFs were collected after 36 hrs and their PFU titers were assayed in PS cells (Ghosh *et al.*, 1989) and their LD_{50} titers were estimated by intracerebral (i.c.) inoculation of 2-day-old suckling mice (Lad *et al.*, 1993b).

Effect of Tm on virus HA activity. The TCFs obtained in the experiments described above were assayed for HA activity employing goose red blood cells (RBC) as described earlier (Gupta *et al.*, 2000) with some modifications. Two-fold dilutions of the TCFs made in 0.4% bovine albumin borate saline (BABS) pH 9.0 were mixed with 0.4% goose RBCs and incubated at room temperature. The proper controls were included.

Results

Results of the indirect IF test

All the four Hs-MAbs (HAI-positive, JEV-specific MAbs) and two (Hx-1 and Hx-5) of the four Hx-MAbs (HAI-positive, flavivirus-cross-reactive MAbs) showed cytoplasmic IF in 2 μg/ml Tm-treated infected cells at 15 hrs p.i., but the IF intensity was markedly reduced as compared to that in the Tm-untreated, infected cells (Table 1). Even the extended incubation period from 24 to 36 hrs yielded similar results. However, with Tm concentration increasing to 3 μg/ml, a complete loss of the MAb reactivities was observed at 24 hrs p.i. In contrast, the remaining two Hx-MAbs (Hx-2 and Hx-3), one (NHs-1) of the two NHs-MAbs (HAI-negative, JEV-specific MAbs), and two NHx-MAbs (HAI flavivirus-non-cross-reactive MAbs) showed loss of IF at 15 hrs p.i. with 2 μg/ml Tm. The extended incubation to 36 hrs p.i. and/or Tm concentration increased to 3 μ/ml yielded negative results. Interestingly, another MAb belonging to the NHs-group (NHs-2) reacted similarly to

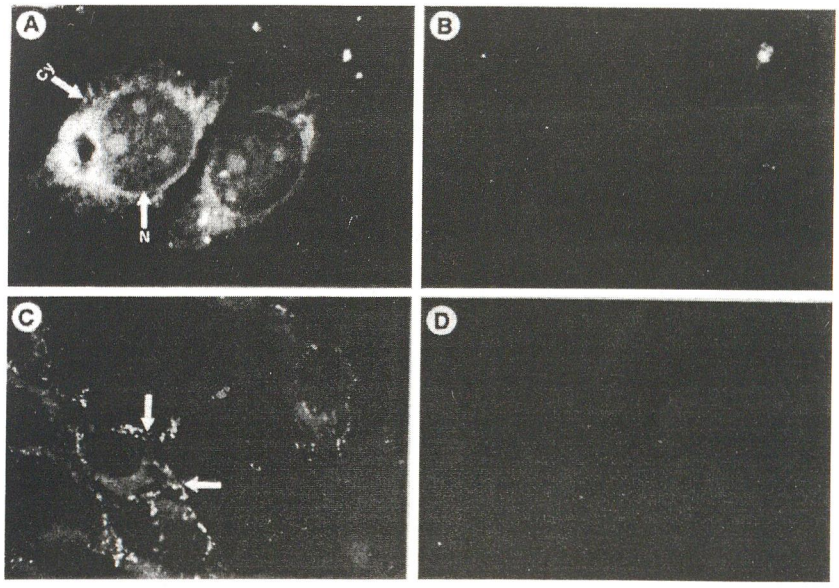

Fig. 1
Effect of Tm on IF of PS cells infected with JEV
The infected PS cells treated with 3 μg/ml Tm showing both nuclear and cytoplasmic IF (A, stained with MAb NHs-2 at 24 hrs p.i., magnification 1000x). The uninfected cells showing no IF with MAb NHs-2 (B). The Tm-untreated infected cells showing surface IF (C) with MAb NHs-2 which was lost after the Tm treatment (3 μg/ml) of cells (D, magnification 400x).

the Tm-untreated, infected cells and Tm-treated, infected cells at 15 to 36 hrs p.i. No loss in the MAb reactivity was observed even at a higher (3 μg/ml) Tm concentration (Fig. 1A). Also no difference in IF was recorded in the infected, Tm-treated cells (2 μg/ml and 3μg/ml) by the indirect FA test with the PAbs as compared to the MAbs.

Results of the surface IF test

The surface IF test on infected cells at 36 hrs p.i. revealed a complete loss of IF in the Tm-treated cells (3 μg/ml) with all the MAbs and PAbs as compared to the Tm-untreated cells (Table 1). The loss of IF with the MAb NHs-2 and Pabs is noteworthy (Fig. 1C and D). This is in contrast to the reactivity of MAb NHs-2 and PAbs in the Tm-treated cells (3 μg/ml), showing similar IF intensity to that observed in the Tm-untreated cells in the conventional indirect IF test.

Effect of Tm on infectivity and HA activity of the virus

Tm (3 μg/ml) caused a drop in the virus infectivity titer by 4.5 log units as determined *in vitro* by plaque assay and by more than 4.1 log units as determined *in vivo* by i.c. inoculation of mice (Table 2).

The TCF collected from the infected, Tm-untreated cells showed a HA titer of more than 32 HAU/ml, while that from infected, Tm-treated cells (as well as that from the control cells) did not show any HA titer.

Discussion

Various studies have been carried out earlier to elucidate the role of carbohydrates in epitope presentation by different viruses. The neutralizing epitopes on non-glycosylated viral gps were not detected when LCM and ML viruses were grown in the presence of Tm, thus indicating that glycosylation plays an important role in the epitope reactivity (Pleortti *et al.*, 1981; Wright *et al.*, 1989). However, a deglycosylation by enzymatic treatment of ML virus did not appear to alter its reactivity with MAbs raised against native viral gpE (Pleortti *et al.*, 1981). This suggests that a removal of sugar moieties after protein folding has a lesser effect than blocking glycosylation during protein synthesis prior to the protein folding.

In the present study, the loss of IF in JEV-infected cells in the presence of 3 mg/ml Tm was recorded with all the MAbs except one (Mab NHs-2). The fact that at 2 μg/ml Tm a few MAbs reacted weakly in the indirect IF test and the same MAbs did not react at all at 3 μg/ml Tm indicates that the inhibition of glycosylation by 2 μg/ml Tm could be incomplete and thus could produce some of the gpE (leaky phenotype). This clearly shows that either glycans are essential for the acquisition of correct conformation of epitopes recognized by the entire set of MAbs (except NHs-2) used and that NHs-2 is an high affinity MAb which reacted with gpE formed in the presence of 2–3 μg/ml Tm. This is well supported by the observation that the MAbs raised

Table 1. MAb and PAb reactivities with JEV-infected cells treated with Tm in indirect IF and surface IF tests

MAbs/PAbs	Indirect IF test					Surface IF test	
	Infected, Tm-untreated cell	Infected, Tm-treated cells				Infected, Tm-untreated cells	Infected, Tm-treated cells (3 μg/ml Tm)
		2 μg/ml Tm		3 μg/ml Tm			
		15 hrs	24 hrs	36 hrs	24 hrs		36 hrs
MAbs							
HAI-positive, JEV-specific							
Hs-1	3+/4+	1+	1+	1+	–	4+	–
Hs-2	3+	1+	1+	1+	–	3+	–
Hs-3	3+	1+	1+	1+	–	3+	–
Hs-4	3+/4+	1+	1+	1+	–	3+	–
HAI-positive, flavivirus cross-reactive							
Hx-1	2+	1+	1+	1+	–	2+	–
Hx-2	2+	–	–	–	–	2+	–
Hx-3	3+	–	–	–	–	3+	–
Hx-5	2+	1+	1+	1+	–	1+/2+	–
HAI-negative, JEV-specific							
NHs-1	3+	–	–	–	–	2+/3+	–
NHs-2	3+	2+	3+	3+	3+*	3+	–
HAI-negative, flavivirus cross-reactive							
NHx-1	3+	–	–	–	–	3+	–
NHx-2	2+	–	–	–	–	2+	–
PAbs	3+/4+	2+	3+	3±	3±	3±	–

(–) = no IF.
(1+) to (4+) = the degree of IF.
*(3+) IF at 36 hrs p.i.
Uninfected cells did not show any IF with the MAbs. "Normal" PAbs and AF from SP2/o cells included as negative controls in all the experiments with untreated infected cells.

against gpE of mature tick-borne encephalitis virus (TBEV, another flavivirus) do not recognize the immature TBEV gpE (Heinz, 1999).

Alexander and Elder (1984) have demonstrated that the reactivity of MAbs and immune serum raised against the influenza hemagglutinin and gp 70 of Rauscher Moloney leukemia virus dramatically decreased after deglycosylation. Also the immunological reactivity of avian myeloblastosis virus gp85 seems to depend on an intact carbohydrate side chain (van Eldik *et al.*, 1978). Carbohydrates play an important role in transport and expression of viral glycoproteins on the cell surface as detected in bovine herpes virus 1-infected cells (Van Drunen *et al.*, 1985). In the present study, the epitope recognized by MAb NHs-2 probably does not need glycosylation for its expression and is thus a glycosylation-independent epitope. However, the non-detection of the epitope by the MAb on the surface of infected cells (surface IF) treated with 3 μg/ml Tm, despite the detection of the epitope inside the cells (cytoplasmic IF) by the same MAb, indicates that glycosylation plays some role in the expression and recognition of this epitope on the cell surface. As a matter of fact, the non-reactivity of PAbs with infected cell surface in the presence of Tm may indicate that the nascent (unglycosylated) E protein itself was not expressed on the surface of the infected cells. This, in turn, indicates that the MAb NHs-2 non-reactivity was not due to the non-expression of NHs-2 epitope on the cell surface but was due to the non-expression of nascent E protein on the cell surface. It is possible that the increased Tm concentration beyond 3 μg/ml might also affect the expression of NHs-2 epitope inside the infected cells, as the recognition of this epitope along with other epitopes on the cell surface precedes their detection within the cells (Gupta *et al.*, 1993).

In the case of Sindbis virus grown in cells in the presence of Tm, unglycosylated E1 is inserted into the plasma membrane but the virion release is prevented (Ulug and Bose, 1985). In other studies with Tm, the absence of glycans in gps of herpes simplex virus 1 (Pizer *et al.*, 1983) and influenza viruses (Gandhi *et al.*, 1972) led either to dramatic reduction in virus production or complete inhibition of virus maturation. In the

case of JEV, the release of gpE into TCF was found to be impaired significantly in the presence of Tm whereas the release of NS-1 and NS-1' was completely abolished (Mason, 1989).

In the present study, a low virus content of TCF in the presence of Tm was found, which seems to be related to a down-regulation of virus maturation inside the cells. This assumption is based on the fact that markedly low intracellular virus titers were found in lysates of the cells treated with Tm (V.J. Lad, unpublished data). Since NS-1 has been implicated in the maturation of virions in infected cells (Lee *et al.*, 1989), such an inhibition of virus maturation might also indicate an inhibition of NS-1 and NS-1' glycosylation by Tm. In flaviviruses, the glycosylation site for NS-1 is conserved and all other NS proteins (NS2A, NS2B, NS3, NS4A, and NS4B) are non-glycosylated. In the presence of Tm, a strong selection pressure might result in JEV virions containing nascent (unglycosylated) proteins (Mason, 1989). It is possible that nascent E protein is incapable of expression on the surface of infected cells due to the absence of glycosylation of preM and NS-1 proteins as well as inefficient chaperoning of nascent E protein.

In the present study, the detection of IF by some MAbs in 2 μg/ml Tm-treated cells, despite the incubation extended to 36 hrs p. i., was lost with increasing the Tm concentration to 3 μg/ml. Despite the loss of virus recognition by MAbs in the two IF tests, the virus maturation yet continued in the cells at higher Tm concentration (3 μg/ml), indicating that the virus released into TCF probably contains proteins which are either unglycosylated or glycosylated incompletely. This is in contrast to E protein of WN (Wengler *et al.*, 1985) and Kunjin viruses (Coia *et al.*, 1988), which do not carry a N-glycosylation site and are thus non-glycosylated.

The role of carbohydrates in HA activity has been studied with deglycosylated rubella virions, resulting in significant HA titer decrease and suggesting that carbohydrates are functionally involved in HA. In contrast, the deglycosylation of TBEV impaired neither its HA activity nor its infec-tivity (Winkler *et al.*, 1987). In the present study, a complete loss of HA activity in the TCF collected from the Tm-treated infected cells was found, indicating that the absence of glycans affects the biological activity of the virus. However, a complete absence of carbohydrates in the WN virus E protein (Wengler *et al.*, 1985) supports the idea that the presence of carbohydrates may not be necessary for maintaining essential biological activities of some flaviviruses.

Our earlier studies have revealed the presence of conformational-dependent acetone-labile epitopes on gpE of some JEV strains (Gupta *et al.*, 1993, 1999). These epitopes of some Indian (755468, G9473, and 641686) Japanese (Yoken), and Sri Lankan (691004) strains were found to have lost reactivity in the HAI test employing sucrose-acetone extracted antigens and also in the conventional indirect IF test despite being recognized by the MAbs in a modified HAI test (employing a polyethylene glycol-precipitated antigen) and the surface IF test. It is possible that such acetone-labile epitopes, though recognized by the MAbs, might either be unglycosylated or glycosylated incompletely, thus leading to their denaturation by the acetone treatment. Our preliminary studies in mice indicated that such an epitopes are not involved in the protection against JEV infection (data not shown). Studies on the immune response to virions matured in the presence of Tm are to be undertaken to understand the role of unglycosylated epitopes either in protection against or immunopathogenesis of JE.

Table 2. Virus titers in TCFs collected from Tm-untreated and Tm-treated infected cells

Virus titer	Tm-untreated infected cells	Tm-treated infected cells (3 μg/ml Tm)
*In vitro** (log PFU/ml)	7.0	2.5
*In vivo*** (log LD_{50}/ml)	>7.0	2.9

*By the plaque assay in PS cells.
**By i. c. inoculation of 2-day-old suckling mice.

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