Effect of catanospermine, 1-deoxynojirimycin or 1-deoxymannojirimycin on biological and functional activities of Japanese encephalitis virus in porcine stable kidney cells

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Abstract

In the present study, effect of catanospermine (CST), 1-deoxynojirimycin (DNJ) or 1-deoxymannojirimycin (DMJ) was studied on porcine stable kidney (PS) cells infected with Japanese encephalitis virus (JEV). As both CST and DNJ are potent inhibitors of ER alpha-glucosidases 1 and II, while DMJ is an inhibitor of Golgi mannosidase which removes alpha (1, 2) Man residues from the N-glycan precursor. Treatment of infected cells with CST (200 uM/mL), DNJ (100 uM/mL) or DMJ (200 uM/mL) did not produce much effect on viral gpE epitope presentation within the cells as well as on the cell surface as detected in the immunofluorescence employing monoclonal (MAbs) and polyclonal (PAbs) antibodies. As well the treated (infected) cells showed only a marginal decrease in infectious virus yield along with a slight decrease in haemagglutination activity of the virus that was recorded in comparison to the untreated infected (control) cells and the cells infected with Dengue virus. Immuno-blotting of the separated proteins from infected lysed cells and probed with anti-gpE MAbs also revealed a band corresponding to JEV gpE (MW 53kDa) both with inhibitor treated and the untreated cells; the reactivity with the former however, was somewhat less intense and prominent in comparison to latter (control untreated) indicating some effect on JEV. The present results indicate that these inhibitors by in large, do not affect maturation and the release of infective JE virions in PS cells.

Introduction

Japanese encephalitis virus (JEV) is one of the most dreaded mosquito-borne flaviviruses (genus: *Flavivirus,* family: *Flaviviridae*) that causes frequent epidemics of acute encephalitis throughout South-east Asia and Western Pacific regions.[1-3] The disease has also emerged in the non-Asian region such as Northern Australia as reported in recent past.[4] Initially both adults and children are affected, while lately it is mostly children who suffer from JE in affected areas during subsequent attacks. The mortality as high as 40% was recorded in some of the JE affected areas. Moreover, many survivors face some neurological problems and complications.[2,3] Even though there are vaccines available, no any specific antiviral therapeutics are yet available for the treatment.[5,6]

JEV as like many other flaviviruses are known to replicate exclusively in the cytoplasm and mature on the intracellular membranes of infected cells.[7,8] Employing the intrinsic secreting pathway of infected cells, flaviviruses bud from the membranes of endoplasmic reticulum (ER) and the Golgi apparatus to release mature virions.[9] It has earlier been shown with some enveloped viruses viz., human immunodeficiency virus, hepatitis B and C viruses, dengue viruses that are greatly affected in cells when alpha-glucosidase mediated N-linked oligosaccharide trimming is inhibited.[10-16] The initial steps of N-linked oligosaccharide processing on the glycoprotein in the ER involve the sequential trimming of the glucose residues on oligosaccharide precursor Glc3Man9GlcNAc2 after it is transferred from the dolichol diphosphate to the growing polypeptide backbone.[17] ER alpha-glucosidases I and II are involved initially in the trimming pathway; alpha-glucosidase I removes the terminal alpha (1,2)-linked glucose from Glc3Man9GlcNAc2, whereas alpha-glucosidase II removes the second and possibly the third alpha(1,3)-linked glucose residues.[18] As castanospermine (CST) and deoxynojirimycin (DNJ) are well-known ER alpha-glucosidase inhibitors both inhibiting the early stages of glycoprotein processing whereas 1-deoxymannojirimycin (DMJ) is an inhibitor of Golgi mannosidase which removes alpha (1, 2) Man residues from the N-glycan precursor. In the present study, we therefore investigated whether these inhibitors are effective in inhibiting JEV production in a model using PS cells.

Materials and Methods

Virus and cells

The details of JEV and PS cells used during the study are given elsewhere.[19,20] In brief, JEV strain 733913, originally isolated from a fatal case of JE was adapted to PS cells grown in Earle's based minimum essential medium (MEM, Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% of goat serum. Also strain P23085 of DENV-2 adapted to PS cells was included. The virus passaging in PS cells was carried out in MEM supplemented with 2% goat serum and the virus stocks were stored at –70°C.

Monoclonal and polyclonal antibodies

Monoclonal antibodies (MAbs) were raised earlier in our laboratory against JEV gpE and were grouped as HAI-positive, HAI-negative virus-specific, HAI-positive, HAI-negative flavivirus cross-reactive and HAI-negative autoreactive MAbs depending on their reactivity with JEV, West Nile and Dengue (DEN) viruses.[21,22] The respective hybrid cells were maintained in the Dulbecco's based MEM supplemented with 10% fetal calf serum (both from Gibco) and were inoculated intra-peritoneally into pristane-primed BALB/c mice, ascitic fluids (AFs) were collected by standard methods. Control AF was also obtained by inoculating SP2/0 cells or a MAb prepared against Chikungunya virus (CHIKV-an *Alphavirus*, the family: *Togaviridiae*) which did not cross-react with JEV, served as negative control. Polyclonal antibodies (PAbs) to JEV and a control AF were raised in Swiss mice by immunization with

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JEV or control (uninfected) antigen respectively, followed by ascites production employing Ehrlich's tumor cells by standard procedures.

Japanese encephalitis virus and Dengue virus-2 infections and catanospermine, 1-deoxynojirimycin or 1-deoxymannojirimycin treatment of PS cells

Confluent PS monolayers grown in 2.5" petri dishes and on coverslips in Leighton tubes were infected with JEV as mentioned elsewhere.[19,20] After virus adsorption at 37°C for 1 hr, the virus inoculum was removed and MEM supplemented with 2% goat serum and CST (200 uM/mL), DNJ (100 uM/mL) or DMJ (200 uM/mL) (all three from Sigma-Aldrich Corp., St. Louis, MO, USA) was added. The cells grown on medium but without CST, DNJ or DMJ served as controls. As these inhibitors have shown to inhibit DEN virus replication, therefore cells infected with DENV 2, both treated and untreated cells were included in the experiments. The concentrations of CST, DNJ or DMJ have been worked out prior to the experimental work and at the given concentrations no any morphological changes indicative of cell toxicity were observed. The cells were incubated for various times at 37°C prior to assaying the infectious virus and for antigenicity by IF. Also tunicamycin (Tm-3 ug/mL) (Sigma) treated cells were additionally included as positive controls.[19]

Immunofluorescence studies

Coverslips with virus-infected cell monolayers either treated or untreated with CST, DNJ, DMJ or Tm were fixed at 24 h and 48 h post-infection (p.i.) in chilled acetone (–20°C) for 20 min, probed with anti-gpE JEV MAbs or PAbs diluted 1:100 and stained with a goat anti-mouse immunoglobulin fluorescein isothiocyanate (GAM Ig-FITC) conjugate (Sigma).[23] The cells were also probed for surface immunofluorescence (IF) at 36 h p.i. by treating the unfixed cells with different MAbs or PAbs diluted 1:100 in MEM containing sodium azide as mentioned earlier.[24] Subsequently, the antibody-treated cells were fixed in chilled acetone (–20°C) for 20 min and stained with a GAM Ig-FITC conjugate as above.

Assaying of infectious virus

Tissue culture supernates (TCFs) and cell lysates collected from the treated cells and the untreated (control) cells at 48 h p.i. were assayed for the infectious virus in vitro (by plaque titration in PS cells using 24-well plate) as described earlier.[19,20] The results were expressed as log PFU/mL. Further the TCFs were titrated for the infectious virus yield by intracerebral (i.c.) inoculation in 2-3 days old infant Swiss mice as detailed elsewhere.[19]

Hemagglutination assay

The effect of CST, DNJ, DMJ or Tm on HA activity of the virus was additionally studied by assaying HA titres of the TCFs and cell lysates collected from both the treated and the untreated virus-infected cells employing goose erythrocytes as described earlier.[19,20]

Western blotting

Treated and untreated cells were lysed with Laemmmli buffer were electrophoresed using 10% SDS-polyacrylamide (SDS-PAGE) as described by Laemmli.[25] This was followed by transfer onto nitrocellulose membrane (Schleicher and Schuell, West Germany). The membranes were blocked and probed with JEV anti-gpE MAbs and detected with an alkaline phosphatase-conjugated goat anti-mouse antibody (GAM Ig-AP, Bio Rad).

Results

Incubation of JEV-infected cells in presence of CST, DNJ or DMJ added immediately after virus adsorption resulted in reduction by about 0.6-1.0 log PFU/mL both in the intracellular (*i.e.* cell lysates) and extracellular (*i.e.* TCFs) infectious virus yield at 48 h p.i. (Figure 1). Similarly, HA activity of the virus was only marginally affected as the treated cells showed a little lower HA titres (a titre of 1:48 to 1:64) in comparison to the untreated cells (a titre of 1:96). In contrast, DENV-2 infected cells showed a drastic reduction (yielding about 1.0 log PFU/mL of the virus) in presence of the inhibitors in comparison to the untreated controls yielding approx. 7.0 log PFU/mL of the virus. Also Tm treated cells showed marked reduction in the intracellular and extra-cellular infectious virus yield (untreated controls yielding 7.0-8.0 log PFU/mL of the virus as against 2.0-3.0 log PFU/mL with the Tm treated cells). Similarly, the i.c. titration of the TCFs from Tm treated infected cells yielded a virus titre of 2.9 log LD_{50}/0.02 mL as against 7.2 log LD_{50}/0.02 mL of the virus obtained with untreated controls.

The results thus indicated that these glucosidase inhibitors did not affect much the JEV production in comparison to DENV-2 grown under similar conditions. Although there were no direct indications of the inhibitors absorption, the DENV-2 inhibition in present study suggested that the inhibitors were invariably absorbed to PS cells. Further, the treatment of cells with the inhibitors produced a dose dependent response as observed with DENV-2 (data not shown). Also more than 95% of the cells incubated for 24 h with CST (200 uM/mL), DNJ (100 uM/mL) or DMJ (200 uM/mL) still excluded trypan blue and no obvious morphological changes such as cell rounding, shrinking or swelling and detachment of cells from solid surfaces were observed between treated and untreated cells.

The intracellular presentation of the epitopes on JEV gpE appeared to be unaffected by the CST, DNJ or DMJ treatment of infected cells which showed characteristic apple- green IF at 24 h and 48 h p.i. with the MAbs and PAbs to JEV. Also the treated cells produced IF of similar magnitude on the cell surface at 36 h p.i., whereas no IF (both cytoplasmic and surface IF) was detected at all with the Tm cells. No IF with SP2/0 or CHIKV MAb and control AF was seen with treated or untreated infected cells by either method. Immuno-blotting of the separated proteins probed with anti-gpE MAbs also revealed a band corresponding to JEV gpE (MW 53kDa) both with inhibitor treated (CST, DNJ or DMJ) and the untreated cells; the reactivity with the former cells was somewhat less intense and prominent in comparison to latter

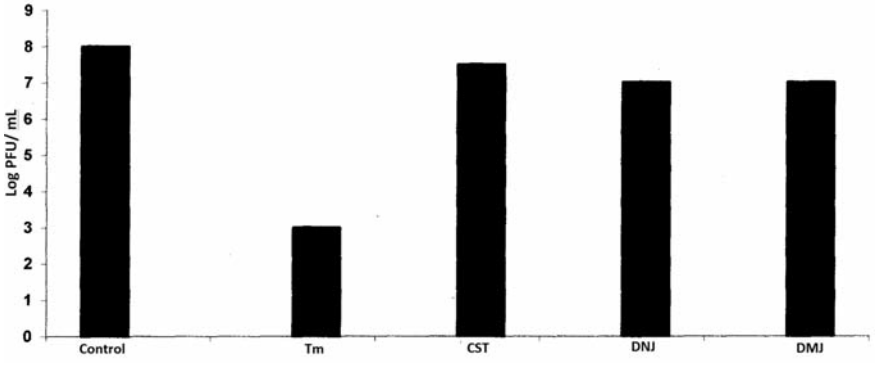

Figure 1. Virus infectivity in tissue culture supernates collected from the untreated and the treated cells at 48 h p.i.

(control untreated) indicating some effect on JEV (Figure 2). In contrast, no band corresponding to JEV gpE (MW 53kDa) was however, revealed with Tm treated cells.

Discussion

The entry of flavivirus in host cell usually occurs via receptor mediated endocytosis and heparan sulfate present on mammalian cells that commonly act as receptors in the virus host-cell interactions.[26-28] Following endocytosis, acidic pH of the endosome causes conformational changes in E protein exposing the hydrophobic domain required for membrane fusion that releases nucleocapsid into cytoplasm of the infected cell.[29,30] Most of the gps undergo folding which attain native conformation being chaperoned through the ER resident molecular chaperons *i.e.*, membrane-bound calnexin and its soluble homologue calreticulin.[31-33] As Tm is known to block the attainment of native structure by E protein that in turn, affects its expression on the surface of infected PS cells.[19,34] On other hand, in Tm absence co-translationally added N-linked glycan precursor (glyococalices) on newly synthesized protein is processed initially by removing terminal glucose residues by the ER alpha-glucosidases 1 and II. Both CST and DNJ are well known inhibitors of ER alpha-glucosidases 1 and II.[14,35,36] and the prevention of Glc residues trimming by ER alpha-glucosidases 1 and II inhibits the activity of ER resident molecular chaperons which specifically binds to monoglucosylated gps till the proteins attain native conformation.[32,33,36] After removal of three glucose residues in the ER, one mannose (alpha1, 2) is additionally cleaved (cleaved from the Man alpha 1, 6 branch) by ER mannosidase as a result high mannose type glycans ($GlcNAc_2Man_8$) are formed. In order to further processing, the gps are transported through vesicles to the Golgi alpha (1, 2) mannosidase which specifically cleaves alpha 1, 2 mannose residues and convert the glycan to $GlcNAc_2Man_5$ type. In contrast, DMJ is the inhibitor of Golgi alpha (1, 2) mannosidase 1 that prevents the formation of hybrid/complex type of oligossaharides; thus the glycans in its presence are rather of high mannose type ($GlcNAc_2Man_8$).[36, 37]

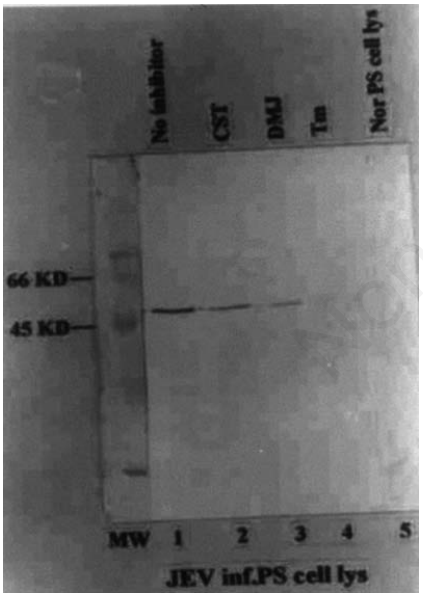

Figure 2. Reactivity of cell lysates of the untreated and treated Japanese encephalitis virus infected cells with anti-gpE MAb in Western blot showing single band at around 53 kDa in lanes Nos. 1, 2 and 3. MW, molecular markers; 1, control (untreated infected) cells; 2, CST treated cells; 3, DMJ treated cells; 4, Tm treated cells; 5, uninfected PS cells; CST, catanospermine; DMJ, 1-deoxymannojirimycin; Tm, tunicamycin.

Conclusions

The present results indicate that glucosylated or high mannosylated gpE of JEV has no effect on the attainment of proper gpE conformation as evident by the reactivity of CST, DNJ or DMJ treated cells with the MAbs or PAbs in IF. Further, such a treatment of the cells has by in large, did not much affect the virus assembly as indicated by the infectious virus contents retaining biological (HA) activity of the virus. In studies by others, the cell type has however been shown to affect the susceptibility of vesicular stomatitis virus to some of glucosidases inhibitors which has been attributed to a Golgi apparatus-resident endomannosidase.[38] Although there were no direct indications of these inhibitors absorption, the inhibition of DENV-2 grown under similar conditions in present study suggested that the inhibitors were invariably absorbed to PS cells. Also some variations in the susceptibility to glucosidases amongst flaviviruses may be related to a differential requirement for associating with the chaperones calnexin and calreticulin.[15] Therefore, at least one of the glycans on the prM or E of DENV, but not in case of WNV or JEV as indicative from the present results that may essentially be required for calnexin-mediated protein folding, oligomerization, and virion assembly.[39] There are however some contra-indications to this as well as DENV structural proteins have also been reported not to be associated with chaperones in infected cells.[40]

Earlier, it was reported that both DENV-2 and JEV were inhibited by N-nonyl(NN)-DNJ,[41] indicating some role of ER glucosidases in E protein processing and thus of molecular chaperons in proper folding/conformation of the viral gps. Further studies in this aspect however, has indicated that long alkyl chain present in iminosugar of N-nonyl(NN)-DNJ itself produced anti-viral effect by interfering in glycolipid synthesis rather than inhibition of ER glucosidases.[42] Also a differential effect of DNJ and NN-DNJ on Kunjin virus (KUNV) has been reported as the treatment of KUNV-infected cells with the latter *i.e.*, NN-DNJ resulted in reduced infectious virus yield but none with the former treatment.[43] Furthermore, the flaviviruses which are strongly retained in the ER would probably be more susceptible as they do not appreciably traffic through the Golgi network and encounter the resident escape mannosidase that allows carbohydrate processing and proper viral protein folding.[15] Alternatively, amongst flaviviruses even a difference in transit time through the Golgi network could affect exposure to escape mannosidases that however, remains to be determined.[20]

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