Effect of brefeldin A and monensin on Japanese encephalitis virus maturation and virus release from cells

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Abstract

Japanese encephalitis virus (JEV) replicates in a variety of cells, the exact intracellular site of virus assembly is somewhat obscure. The aims of this study were to investigate the role of Golgi apparatus in JEV maturation by utilizing two Golgi-disrupting agents- brefeldin A (BFA) and monensin (MN) that inhibit virus assembly at specific cellular sites. JEV-infected porcine kidney stable (PS) cells were treated with BFA (2 µg/mL) or MN (10 µM/mL) at different h post-infection (p. i.) and the virus contents were assayed after 48 h p. i. The treated cells were further subjected to immuno-fluorescence (IF) using antibodies directed against JEV envelope glycoprotein (gpE) for localization of intracellular viral antigen as well as the antigen expression on the cell surface. Addition of BFA or MN to cells immediately after virus adsorption or at 4 h and 12 h post-infection (p. i.), resulted in 4- or 8- fold reduction in infectious virus contents along with inhibition of its transport to the cell surface, indicating an essential role of the Golgi-associated membranes in JEV replication. Interestingly, the antigenicity of the virus, in contrast, remained unaffected as no difference in epitope presentation/ expression was observed in BFA/MN-treated and control (untreated) infected cells even though in the former cells a loss of hemagglutinating (HA) activity was observed. Further, BFA addition at 18 h or 24 h p. i. showed only a negligible effect on virus suggesting that once the viral-associated membranes are formed, these membranes appear to be stable. In contrast, the inhibition with MN persisted even after its addition to cells at 18 h and 24 h p. i., indicating its sustained effect on JEV. Although BFA inhibits protein transport from endoplasmic reticulum (ER) to the Golgi complex while MN inhibits transport from medial to trans cisternae of the Golgi complex, none of the two agents however affected the gpE synthesis and folding essentially required for the epitope presentation/ expression within the cells. As flaviviruses are known to encode three glycoproteins (gps) within their genomes i. e., prM, E, and NS, it will be worthwhile in future to

determine whether vesicular transport occurs within or between the virus-induced membranes and how the individual JEV-encoded proteins are transported to discrete compartments further remain to be seen.

Introduction

Japanese encephalitis virus (JEV) is one of the most dreaded mosquito-borne flaviviruses (the genus- *Flavivirus* and 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 The virus replicates in a variety of cells- of mammalian, avian and mosquito origin and is probably released by a budding process.⁵ Using Kunjin virus (KUNV) as a model to study the events of flavivirus replication, it appears that virus particles are closely associated with the KUN factories in which compartmentalization within the induced membranes occurs to ensure efficient replication; the exact intracellular site of flavivirus assembly somewhat remains obscure.^{6,7} Ultrastructural studies of flavivirus-infected cells have consistently revealed apparent mature virions within distended endoplasmic reticulum (ER), large cytoplasmic vesicles, and vacuoles.⁸ In addition, occasional sections have shown individual particles within Golgi cisternae, indicating that flavivirus maturation might be proceeding through the Golgi apparatus.^{9,10} However, this process may not be a generalized phenomenon for all the flaviviruses as the viral E protein which is the binding site of neutralizing antibodies, is glycosylated in some flaviviruses while in others it is unglycosylated.^{6,11} The JEV gpE has one conserved N-glycosylation site at the amino acid 150 from the N-terminus.¹²⁻¹⁴ The glycans associated with the JEV gpE are converted in to complex forms prior to the virus release from cells and in the presence of tunicamycin (Tm), a potent N-glycosylation inhibitor, the secretion of gpE is significantly impaired while that of NS 1 and NS 1' is completely abolished.¹⁵ In contrast, Brefeldin A (BFA) is a fungal metabolite that disrupts intracellular membrane traffic at the ER-Golgi junction while monensin (MN) is a well-characterized metabolite of *Streptomyces cinnamomensis* that binds Na⁺, K⁺ and proteins, and inhibits trans-Golgi function by preventing the protein transport from medial to trans-cisternae of Golgi compartment.^{16,17} Both BFA and MN have effectively been used to inhibit the transport and secretion of a number of cellular and viral proteins in a variety of cells.^{6,7} Our preliminary studies with JEV

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revealed that treatment of PS cells with BFA added immediately after virus adsorption resulted in inhibition of virus maturation and its transport to the cell surface.¹⁸ The present study was undertaken utilizing both BFA and MN in order to attempt inhibition of virus assembly at specific cellular sites in PS cells and assessing their effect by determining the virus yield along with localization of intracellular viral antigen as well as the antigen expression on the cell surface using antibodies directed against JEV gpE by immuno-fluorescence (IF).

Materials and Methods

Virus and cells

The details of JEV and PS cells used during the study are given elsewhere.^{18,19} 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. 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 auto-reactive MABs depending on their reactivity with JEV, West Nile and Dengue viruses.^{20,21} 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-*Togaviridae*) which did not cross-react with JEV, served as negative control.²² Polyclonal antibodies (PABs) to JEV and a control AF were raised in Swiss mice by immunization with JEV or control (uninfected) antigen respectively, followed by ascites production employing Ehrlich's tumor cells by standard procedures.

Japanese encephalitis virus infection and Brefeldin A/Monensin 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.^{18,19} After virus adsorption at 37°C for 1 hr, the virus inoculum was removed and MEM supplemented with 2% goat serum and BFA (2 µg/mL) or MN (10 µM/mL) (both from Sigma-Aldrich Corp., St. Louis, MO, USA) was added. The cells grown on medium but without BFA or MN served as controls. The cells were incubated for various times at 37°C prior to assaying the infectious virus and for antigenicity by IF.

Immunofluorescence studies

Coverslips with virus-infected cell monolayers either treated or untreated with BFA/ MN were fixed at 24 h and 48 h post-infection (p. i.) in chilled acetone (-20°C) for 20 min, probed with different MABs or JEV antiserum 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 JEV antiserum 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.^{18,19} The results were expressed as log PFU/mL.

Hemagglutination assay

The effect of BFA and MN on Hemagglutination (HA) activity of the virus was studied by assaying HA titres of the TCFs and cell lysates collected from both treated and untreated virus-infected cells employing goose erythrocytes as described earlier.¹⁹

Treatment of Japanese encephalitis virus -infected PS cells with Brefeldin/Monensin for different times

BFA and MN were similarly added to the infected cells at various times viz. 4, 12, 18 and 24 h p. i. rather than immediately after virus adsorption. In some experiments, BFA and MN were added immediately after virus adsorption; these were removed from the treated cells at various times viz. 4, 12, 18 and 24 h p. i. and the cells were incubated further without BFA or MN. TCFs and cell lysates collected from the cells were assayed at 48 h p. i. for the infectious virus as above.

Results

Incubation of JEV-infected cells in presence of BFA or MN added immediately after virus adsorption drastically reduced the intracellular and extracellular infectious virus yield at 48 h p. i. by about 4-fold and 8-fold, respectively

(Figure 1). This was further accompanied with undetectable HA activity of the virus with the treated cells (HA titres of 1:48 to 96 with untreated cells yielding 7.5-8.0 logs of the virus as against 1.0-2.0 logs only with the treated cells). Whereas, the intracellular presentation of the epitopes on JEV gpE however, appeared to be unaffected either by the BFA or MN 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. In contrast, the MABs and PABs failed to produce any IF on the surface of BFA/ MN treated cells at 36 h. p. i., indicating a defective virus transport and/or virus assembly as reported earlier with BFA.¹⁸ No IF with SP2/0 or CHIKV MAB and control AF was seen with treated or untreated infected cells by either method.

Further, BFA or MN addition to the cells even at 4 h or 12 h p. i. resulted in a drastically reduced infectious virus yield (Figure 2). In contrast, the inhibitory effect of BFA was negligible at 18 h or 24 h p. i. as only a marginal decrease in virus yield was recorded in comparison to the MN. Even removal of MN (at 4, 12, 18 and 24 h p. i.) after its addition to the cells immediately after virus adsorption produced only a little change, indicating a sustained effect produced by MN in comparison to BFA (Figure 3). As the characteristic virus-induced membranes are not clearly formed until about the end of the latent period i. e. about 12 h-15 h, the present results with BFA therefore, indicate an essential involvement of some of the Golgi apparatus-associated membranes in JEV replication.^{6,7} Once these viral-associated membranes are formed as observed on BFA addition just after the end of latent period i. e., 18 h and later on, the previously induced membranes appear to be stable/ resistant. In contrast, the inhibition with MN

Figure 1. Virus infectivity titres in TCFs and cell lysates collected at 48 h p. i. from BFA or MN – treated and untreated infected PS cells. BFA tr. = BFA treated, MN tr. = MN treated.

Figure 2. Addition of BFA / MN at different time hours p.i. Effect of BFA (2 µg/mL) or MN (10 µM) added at different time interval on JEV replication in PS cells. BFA tr. = BFA treated, MN tr. = MN treated.

persisted even after the end of latent period, suggesting its sustained effect on JEV.

Discussion

Flaviviruses contain an electron dense icosahedral nucleocapsid core which is surrounded by a lipid bilayer containing envelope protein and the genomic RNA serves itself as mRNA that gets translated into a long polyprotein encoding three structural proteins (C, M and E) and seven NS proteins.²⁵ The polyprotein is processed, co- and post-translationally into individual proteins by the host and viral proteases.²⁵⁻²⁷ The M protein represents C' terminal cleavage product of the precursor protein i. e., prM undergoes delayed cleavage that is taking place just prior to or even during virions' release from cells.²⁸⁻³¹ The cleavage has shown to be catalyzed by the host protease-furin located specifically in trans cisternae of the Golgi complex.³² As the prM and not M protein which is glycosylated and is associated with E protein in a hetero-dimeric form namely, prM-E.³³ This interaction between prM and E protein, namely hetero-dimeric prM is necessary to protect E protein from irreversible conformational changes during maturation in the acidic vesicles of the exocytic pathway.^{29,31} Also the prM facilitates correct folding of E protein or alternatively masks a retention signal within it, thus allowing its exit from the ER.³⁴⁻³⁷

Conclusions

In the present study utilizing the metabolic inhibitors that inhibit protein and/or membrane traffic throughout the cell, the secretion of JEV particles late in infection was significantly affected/ impaired in the BFA or MN presence along with undetectable antigen on the cell surface. Thus, it seems that though the virus yield is affected, BFA or MN treatment does not affect JEV gpE and its folding essentially required for the epitope expression/presentation as the inhibition of terminal N-glycosylation, i. e. no addition of sialic acid due to non-availability of sialyl transferase and its location in trans-Golgi-network.^{38,39} As BFA treatment of cells restricts anterograde protein transport between the ER and Golgi apparatus leading to recycling of viral proteins between the ER and IC, the present results therefore suggest the movement of cellular or viral proteins from the ER to the Golgi apparatus that might essentially be required for membrane induction, rather than just accumulation of viral proteins within the ER and IC.⁴⁰ In contrast, the movement of virus particles within

Figure 3. Removal of BFA / MN at different time hours p.i. Effect of BFA (2 µg/mL) or MN (10 µM) added at different time interval on JEV replication in PS cells. BFA tr. = BFA treated, MN tr. = MN treated.

the medial Golgi compartment during the MN treatment may not be arrested but subsequent furin cleavage of prM was either not occurring or was very inefficient, thus not allowing the conformational change in the E protein (of immature particles containing prM) that is essentially required for un-coating and fusion with endosomal membranes after infection.¹⁵ In addition to the obvious inhibition of cleavage of prM, MN also appears in some unknown way to inhibit the cleavage.²⁹ Therefore, it will be worthwhile in future to determine whether vesicular transport occurs within or between the virus-induced membranes and how the individual JEV-encoded proteins are transported to discrete compartments further remains to be seen.

References

- Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Koipe DM, Howley PM, eds. *Field's Fundamental Virology*. 3rd Ed. Philadelphia: Lippincott- Raven; 1996. pp. 961-1034.
- Solomon T, Ni H, Beasley DW, et al. Origin and evolution of Japanese encephalitis virus in Southeast Asia. *J Virol* 2003; 77:3091-8.
- Vaughn DW, Hoke CH. The epidemiology of Japanese encephalitis: prospects for prevention. *Epidemiol Rev* 1992;14:197-221.
- Hanna JN, Ritchie SA, Philips DA, et al. Japanese encephalitis in North Queensland, Australia, 1998. *Med J Aust* 1999;170:533-66.
- Brinton MA. Replication of flaviviruses. In: Schlesinger S, Schlesinger MJ, eds. *The Togaviridae and Flaviviridae*. New York-London: Plenum Press; 1986. pp. 327-74.
- Mackenzie JM, Jones MK, Westaway EG. Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. *J Virol* 1999;73:9555-67.
- Mackenzie JM, Westaway EG. Assembly and maturation of the flavivirus Kunjin Virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J Virol* 2001;75: 10787-99.
- Murphy FA. St. Louis encephalitis. In: Monath TP, ed. *Morphology and Morphogenesis*. Washington, D.C.: American Public Health Association Inc.; 1980. pp. 65-103.
- Barth, OM. Ultrastructural aspects of the dengue virus (flavivirus) particle morphogenesis. *J Submicrosc Cytol Pathol* 1999;31:407-12.
- Hase T, Summers PL, Eckels KH, Baze WB. Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo. *Arch Virol* 1987;96:135-51.
- Roehring JT. The use of monoclonal antibodies in studies of the structural proteins of togaviruses and flaviviruses. 111. Flaviviruses. In: Schlesinger S, Schlesinger MJ. *The Togaviridae and Flaviviridae*. New York-London: Plenum Press; 1986. pp. 265-72.
- McAda PL, Mason PW, Schmaljohn CS, et al. Partial nucleotide sequence of the Japanese encephalitis virus genome. *Virology* 1987;158:348-60.
- Sumiyoshi H, Mori C, Fuke I, et al. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* 1987;161:497-510.
- Nityaphan S, Grant JA, Trent DW. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. *Virology* 1990;177:541-52.
- Mason PW. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 1989;169:354-64.
- Fujiwara T, Oda K, Yokoto S, et al. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J Biol Chem* 1988;263:18545-52.
- Misumi Y, Misumi Y, Miki K, Takatsuki A, Tamura G, Ikehara Y. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J Biol Chem* 1986;261:11398-403.
- Lad VJ, Gupta AK. Inhibition of Japanese

- encephalitis virus maturation and transport in PS cells to cell surface by brefeldin A. *Acta Virol* 2002;46:187-90.
19. Lad VJ, Shende VR, Gupta AK, et al. Effect of tunicamycin on expression of epitopes on Japanese encephalitis virus glycoprotein E in porcine kidney cells. *Acta Virol* 2000;44:359-64.
 20. Cecilia D, Gadkari D, Kedarnath N, Ghosh SN. Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. *J Gen Virol* 1988;69:2741-7.
 21. Kedarnath N, Dayaraj C, Sathe PS, et al. Monoclonal antibodies against Japanese encephalitis virus. *Indian J Med Res* 1986;84:125-33.
 22. Lad VJ, Gupta AK, Ghosh SN, Banerjee K. Immunofluorescent studies on the replication of some arboviruses in nucleated and enucleated cells. *Acta Virol* 1993;37:79-82.
 23. Gupta AK, Gore MM, Lad VJ, Ghosh SN. Nuclear immunofluorescence in porcine kidney cells infected with Japanese encephalitis virus. *Acta Virol* 1991;35:282-6.
 24. Gupta AK, Lad VJ, Ghosh SN. Detection of viral antigens on the surface of the cells infected with Japanese encephalitis virus by modified immuno-fluorescent technique. *Acta Virol* 1993;37:93-6.
 25. Rice CM. Flaviviridae: the viruses and their replication. In: Fields BN, Knipe DN, Howley PM, et al., eds. *Virology*. 3rd ed. Philadelphia, PA: Lippincott-Raven; 1996. pp. 931-59.
 26. Westaway EG, Mackenzie JM, Kenney MT, Jones MK, Khromykh AA. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA and of NS2B with NS3 in virus-induced membrane structures. *J. Virol.* 1987; 71:6650-61.
 27. Chambers TJ, Grakoui A, Rice CM. Processing of the yellow fever virus non-structural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. *J. Virol.* 1991; 65: 6042-50.
 28. Shapiro D, Brandt WE, Cardiff RD, Russell PK. The proteins of Japanese encephalitis virus. *Virology* 1971; 44:108-24.
 29. Randolph VB, Winkler G, Stollar V. Acidotropic amines inhibit proteolytic processing of flavivirus prM protein. *Virology* 1990;174:450-8.
 30. Guirakhoo F, Bolin RA, Roehrig JT. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. *Virology* 1992;191:921-31.
 31. Guirakhoo F, Heinz FX, Mandl CW, et al. Fusion activity of flaviviruses: comparison of mature and immature (prM containing) tick-borne encephalitis virions. *J Gen Virol* 1992;72:1323-9.
 32. Stadler K, Allison SL, Schalich J, Heinz FX. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol* 1997; 71:8475-81.
 33. Wengler G, Castle E, Leider U, et al. Sequence analysis of the membrane protein V3 of the flavivirus West Nile and of its gene. *Virology* 1985;147:264-74.
 34. Konishi E, Mason PW. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires co-synthesis with the pre-membrane protein. *J Virol* 1993;67:1672-75.
 35. Heinz FX, Stiasny K, Puschner-Auer G, et al. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology* 1994 198:109-17.
 36. Allison SL, Schalich J, Stiasny K, et al. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* 1995;69:695-700.
 37. Schalich J, Allison SL, Stiasny K, et al. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. *J Virol* 1996;70:4549-57.
 38. Chege NW, Pfeffer SR. Compartmentation of the Golgi complex, brefeldin A distinguishes trans Golgi cisternae from trans Golgi cisternae from the trans-Golgi network. *J Cell Biol* 1990;111:893-900.
 39. Locker JK, Griffiths G, Horzinek MC, Rother PJ. O-glycosylation of the Coronavirus M protein. Differential localization of sialyltransferases in N- and O-linked glycosylation. *J Biol Chem* 1992; 267:14094-101.
 40. Lippincott-Schwartz J, Donaldson JG, Schweizer A, et al. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* 1990;60:821-36.