INHIBITION OF JAPANESE ENCEPHALITIS VIRUS MATURATION AND TRANSPORT IN PS CELLS TO CELL SURFACE BY BREFELDIN A

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Summary. – A brefeldin A (BFA) treatment of porcine stable kidney (PS) cells resulted in inhibition of Japanese encephalitis virus (JEV) maturation and its transport to the cell surface. Interestingly, the antigenicity of the virus, in contrast, remained unaffected as no difference in epitope presentation/expression was observed in BFA-treated and control (untreated) infected cells even though in the former cells a loss of hemagglutinating (HA) activity was recorded. Thus it seems that the BFA treatment did not affect the glycoprotein E (gpE) synthesis and folding essentially required for the epitope presentation/expression in cells.

Key words: Japanese encephalitis virus; glycoprotein E; epitopes; PS cells; brefeldin A

Introduction

The N-linked glycosylation, a common modification of viral proteins plays an important role in their recognition and folding, thus has a strong influence not only on their functional activity but also on their antigenic reactivity (Basak and Compans, 1983; Alexander and Elder, 1984; Elbein, 1991; Fiedler and Simons, 1995). This process is highly conserved, involving the addition of a carbohydrate moiety and its sequential trimming and further processing in which the endoplasmic reticulum (ER) and Golgi complex play a major role (Kornfeld and Kornfeld, 1985; Moremen *et al.*, 1994; Roth, 1995).

The JEV gpE has one conserved N-glycosylation site at the amino acid 150 from the N'-terminus (McAda *et al.*, 1987; Sumiyoshi *et al.*, 1987; Nityaphan *et al.*, 1990). The glycans associated with the JEV gpE are converted into 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 NS1 and NS1' is completely abolished (Mason, 1989). The present study was undertaken to investigate the effect of BFA, a well known specific inhibitor of protein export from the ER to the Golgi complex (Fujiwara *et al.*, 1988; Lippincott-Schwartz *et al.*, 1989) on the JEV antigenicity and replication/maturation in PS cells.

Materials and Methods

Virus and cells. An Indian strain (733913) of JEV (Ghosh *et al.*, 1989), originally isolated from a human brain in Bankura, West Bengal, India in 1973 was passaged in 2-day-old infant Swiss mice inoculated by intracerebral (i.c.) route. This virus was also adapted to PS cells grown in the Earle's based Minimum Essential Medium (MEM), supplemented with 10% of goat serum. The virus passaging in PS cells was carried out in the MEM supplemented with 2% of goat serum as described earlier (Gupta *et al.*, 1991) and virus stocks were stored at -70°C.

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Abbreviations: AF = ascitic fluid; BFA = brefeldin A; ER = endoplasmic reticulum; GAM Ig-FITC = goat anti-mouse immunoglobulin fluorescein isothiocyanate; gpE = envelope glycoprotein; HA = hemagglutinin, hemagglutination; Hs = HAI-positive virus-specific; Hx = HAI-positive flavivirus cross-reactive; IF = immunofluorescence; i.c. = intracerebral; i.p. = intraperitoneal; JEV = Japanese encephalitis virus; MAb = monoclonal antibody; MEM = Minimum Essential Medium; MOI = multiplicity of infection; NHs = non-HI virus-specific; NHx = non-HI flavivirus cross-reactive; NPF = normal peritoneal fluid; NS = non-structural; PAb = polyclonal antibody; PS = porcine kidney stable cells; p.i. = post infection; TCF = tissue culture fluid; Tm = tunicamycin

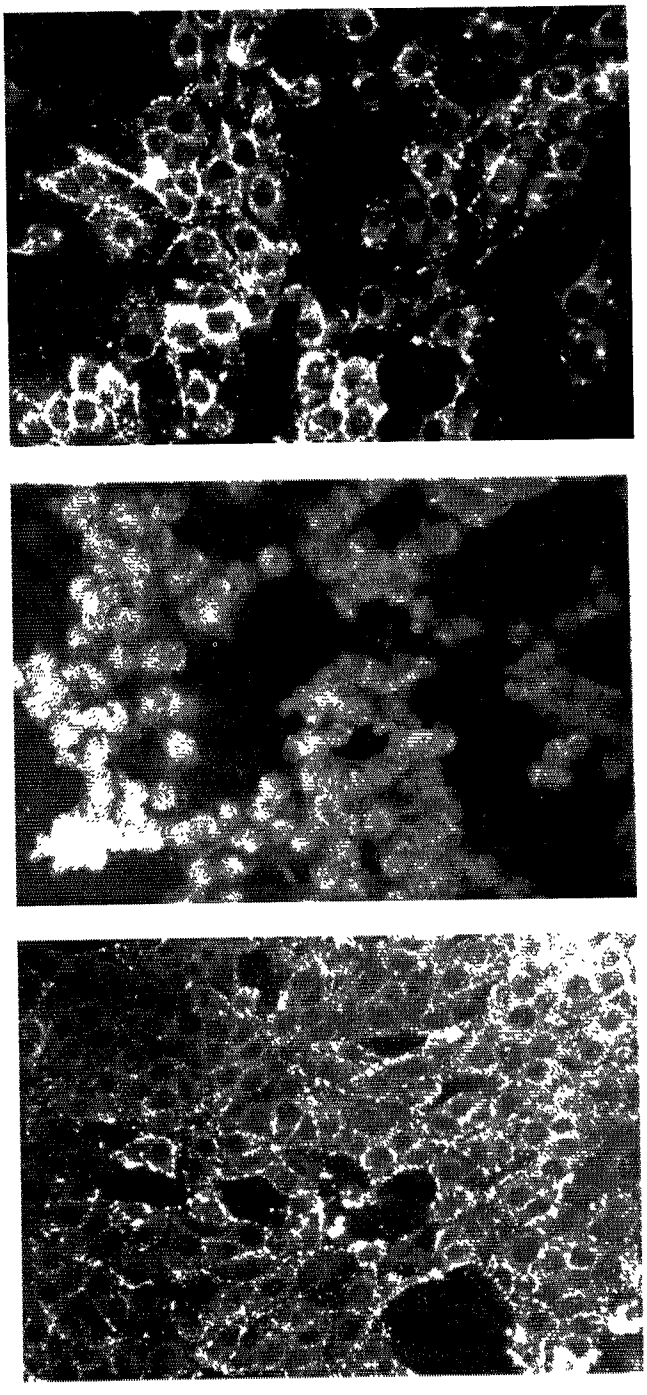

Fig. 1

Effects of BFA on the appearance of cytoplasmic and surface JEV gpE in PS cells as assessed by IF tests

(a) Virus-infected BFA-treated cells probed with MAb Hs-1 for cytoplasmic IF (positive result).
(b) Virus-infected BFA-treated cells probed with MAb Hs-1 for surface IF (negative result).
(c) Virus-infected BFA-untreated cells probed with MAb Hs-1 for surface IF (positive result).
Magnification x 400.

Monoclonal and polyclonal antibodies (MAbs, PAbs). The MAbs raised earlier in our laboratory against JEV gpE (Kedarnath *et al.*, 1986) were used. These MAbs have been grouped as Hs (hemagglutination-inhibition (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 viruses (Cecilia *et al.*, 1988). The respective hybrid cells were maintained in the Dulbecco's based MEM supplemented with 10% of fetal calf serum. The hybrid cells were inoculated intraperitoneally (i.p.) into Pristane-primed BALB/c mice and ascitic fluids (AFs) were collected by standard methods. The AF obtained by inoculating SP2/o cells served as the negative control. PAbs were raised in Swiss mice by immunization with JEV followed by production of ascites, employing Ehrlich's tumor cells by standard procedures. A normal peritoneal fluid (NPF), produced in non-immunized mice, served as the negative control.

JEV infection and BFA treatmen of PS cells. Confluent PS monolayers grown in 2.5" Petri dishes were infected with JEV at the multiplicity of infection (MOI) of 10. After the virus adsorption at 37°C for 1 hr, the virus inoculum was removed and the MEM supplemented with 2% of goat serum and 2 mg/ml BFA (Sigma) was added. The cell cultures grown on the medium without BFA served as controls. The cells were incubated for various times at 37°C prior to assaying the infectious virus. The cell monolayers grown on coverslips in Leighton tubes, infected with the virus and treated with BFA as mentioned above, were employed for immunofluorescence (IF) tests.

Indirect IF test. Coverslips with virus-infected cell monolayers either treated or untreated with BFA, were fixed at 24 or 48 hrs p.i. in chilled acetone (-20°C) for 20 mins, probed with different MAbs or PAbs diluted 1:100 in MEM containing sodium azide and stained with a goat anti-mouse immunoglobulin fluorescein isothiocyanate (GAM Ig FITC) conjugate (Sigma) as described earlier (Gupta *et al.*, 1991).

Surface IF test. Unfixed infected cells either treated or untreated with BFA were probed for the surface IF at 36 hrs p.i. with different MAbs and PAbs diluted 1:100 in MEM containing sodium azide as mentioned earlier (Gupta *et al.*, 1993). The cells were subsequently fixed and stained with GAM Ig-FITC conjugate (Sigma) as above.

Assay of infectious virus. Tissue culture supernates (TCFs) and cell lysates collected at 36 hrs p.i. from infected cultures treated or untreated with BFA were assayed for the extracellular infectious virus both *in vitro* (by a plaque titration in PS cells in 24-well-plates) and *in vivo* (by i.c. inoculation of infant mice) as described earlier (Lad *et al.*, 2000).

Hemagglutination (HA) assay. The effect of BFA on HA activity of the virus was studied by assaying HA titers of the TCFs and cell lysates collected from both BFA-treated and untreated virus-infected cells, employing goose erythrocytes as described earlier (Lad *et al.*, 2000).

Results and Discussion

Interestingly, even in the BFA presence, the intracellular epitope presentation appeared to be unaffected in the virus-

Table 1. Reactivities of MAbs and PAbs in JEV-infected PS cells in the absence and presence of BFA as assessed by IF tests

MAbs/PAbs	Indirect IF test 24 hrs/48 hrs p.i.		Surface IF test 36 hrs p.i.	
	Without BFA	With BFA	Without BFA	With BFA
HAI-positive MAbs				
(a) JEV-specific, Hs-1 to Hs-4	3+/4+	3+/4+	3+/4+	–
(b) Flavivirus-cross-reactive, Hx-1 to Hx-3, Hx-5	3+/4+	3+	3+/4+	–
HAI-negative MAbs				
(a) JEV-specific, NHs-1, NHs-2	3+/4+	3+/	3+	–
(b) Flavivirus-cross-reactive, NHx-1, NHx-3	3+	2+	2+	–
PAbs	4+	4+	4+	–

(2+) to (4+) = degree of IF.
(–) = no IF.

infected cells as characteristic cytoplasmic IF was observed with all the MAbs and PAbs in the indirect IF test (Fig. 1a). All the anti-JEV gpE MAbs such as Hs (Hs 1-4), Hx (Hx-1, 2, 3, and 5), NHs (NHs-1 and 2) and NHx (NHx-1 and -3) reacted similarly with both the BFA-treated and -untreated virus-infected cells at 24 or 48 hrs p.i. and showed characteristic bright apple-green cytoplasmic IF (Table 1). The PAbs also reacted similarly with the BFA-treated and untreated virus-infected cells. Uninfected cells did not show any IF by either test with the MAbs/PAbs tested. Also no IF was seen with SP2/o or NPF in BFA-treated or untreated virus-infected cells by either test. These findings are in contrast to those which we have recorded earlier with Tm-treatment (Lad *et al.*, 2000), wherein except one MAb (NHs-2) and the PAbs all the MAbs failed to detect the cytoplasmic as well as the cell surface IF in the indirect IF test. It indicated that in the BFA-treated cells the inhibition of terminal N-glycosylation, i.e. no addition of sialic acid due to the inavailability of sialyl transferases and its location in trans-Golgi-network (Chege and Pfeffer, 1990; Locker *et al.*, 1992), probably does not seem to affect the epitope expression/presentation on JEV gpE. Thus, it seems that the BFA treatment does not affect JEV gpE and its folding essentially required for the epitope expression/presentation.

Though the epitope presentation/expression within the cell was unaffected, the MAbs as well as PAbs failed to detect the same on the cell surface (Fig. 1b,c). Moreover, a drastic reduction by about 6 log units and more in infectious virus titers (Table 2) and undetectable HA titers were observed in the presence of BFA, while the BFA-untreated controls showed HA titers of 48 to 96.

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Table 2. Virus infectivity titers in TCFs and cell lysates collected from BFA-treated and untreated JEV infected PS cells

Virus-infected cells	Titers	
	In vitro (log PFU/ml)	*In vivo* (log LD_{50}/0.02 ml)
BFA-treated, TCF	2.0	<1.0
BFA-treated, cell lysate	1.3	ND
BFA-untreated, TCF	8.2	6.25
BFA-untreated, cell lysate	7.4	ND

ND = not done.

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