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Fusion of *Aedes Albopictus* Cells, Clone C6/36, by Japanese Encephalitis Virus is Triggered by Low pH

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Abstract: The fusion of *Aedes albopictus* cells, clone C6/36, by Japanese encephalitis virus (JE) has been investigated. Fusion from without is possible under condition of high multiplicities at a pH below 6.5. Fusion from within (FFWI) is discrete at 30 hours postinfection (hpi), however, it is rapid and complete at 48 hpi. The optimal pH range of the medium to elicit the FFWI is between 5.5 and 6.3. FFWI does not take place below 15℃. Exposition to low pH can be very short (10 sec).

Key words: Japanese encephalitis virus, C6/36 (mosquito cells), Fusion from within, Fusion from without

INTRODUCTION

Japanese encephalitis is an acute viral disease. Symptoms are those of an encephalitis. Lethality is high. Survivors often suffer from grave sequelae (Shope, 1980; Monath, 1985, 1986). The agent, JE virus, is a member of the St. Louis complex of the Flaviviridae (Westaway *et al.*, 1985); it used to be classified as a mosquito-borne group B arbovirus (Clarke and Casals, 1965). The virus is the most common cause of arthropod-borne human encephalitis in several Asian countries. Epidemic disease occurs throughout China, in Northern parts of Southeast Asia and in areas of India, Nepal and Sri Lanka. It has decreased in frequency in Japan and Korea, however, it is spreading in Southeast Asia, Northeast India and Nepal (Johnson, 1987; Umenai *et al.*, 1985). JE accounts for 20,000 acute illnesses per year (Johnson, 1987). The major vectors are rice field breeding culicine mosquitoes, and pigs are important mammalian amplifier hosts.

The flaviviruses are small enveloped viruses which contain a single-stranded positive sense RNA genome. The mature virus particle is composed of three structural proteins: the nucleocapsid protein C (M_r approximately 14,000), the envelope glycoprotein E (M_r 50,000−60,000) and a membrane protein M (M_r ca. 8,000) (for reviews: Brinton, 1986; Rice *et al.*, 1986). The E protein is the hemagglutinating protein containing the neutralization epitopes

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(Mathews and Roehrig, 1984; Srivastava and Igarashi, 1987; Srivastava *et al.*, 1987).

The genomic RNA of the wildtype strain of JEV (JaOArS982) has been sequenced. It contains 10,976 nucleotides with a long open reading frame of 10,296 nucleotides corresponding to 3,432 amino acid residues. In contrast to Alphatogaviruses (Garoff *et al.*, 1982) the structural proteins are encoded by the 5'-region of the genome (Sumiyoshi *et al.*, 1987) in the gene order C [viral protein 2], pre M [viral protein 1] and E [viral protein 3]; then follow the genes for the nonstructural proteins as reviewed by Chambers and Rice (1987). From analysis of the nucleotide sequence the primordial C protein is composed of 127 amino acid residues with a M_r of 13,859; the pre M contains 167 and the M protein 75 amino acid residues (M_r 8,329). The M protein is cleaved in the infected cell from the precursor pre M during release of virus (Shapiro *et al.*, 1973a, b; Wengler and Wengler, 1989) which exhibits one potential glycosylation site in the part not expressed in the virion. M protein which is the carboxyterminal segment of pre M is quite hydrophobic and may be embedded in the lipid bilayer of the virion envelope thus being the link between the envelope and the nucleocapsid. The E protein finally with 500 residues has a M_r of 53,334. It shows several hydrophobic domains, mainly in the C-terminal transmembrane region. This protein possesses one putative N-glycosylation site. The hydrophobic domains may lead to oligomerization into spikes, e.g. E_3, as noted in case of West Nile virus (Wengler *et al.*, 1987; Wengler and Wengler, 1989); they may also favour the interaction with the M protein. Probably the intracellular cleavages resulting in the mature structural proteins follow the scheme as outlined for West Nile virus (Nowak *et al.*, 1989) and yellow fever virus (Ruiz-Linares *et al.*, 1989): signalase(s) cleaves between C and pre M, between pre M and E and, between E and the ensuing nonstructural protein NS1. Cleavage of pre M occurs presumably by a cellular enzyme located in the Golgi stacks between -arg-arg and ser-val-ser-val.

The mode of entry of flaviviruses into the cell is an important question. In principle, enveloped virions can either enter by fusion with the plasma membrane at pH 7 or by fusion with the endosomal membrane at mildly acidic pH after endocytosis. The correlate is that the former viruses provoke in the late phases of infection a spontaneous cytopathic effect (CPE) in form of syncytia (cell-cell fusion) at a pH of 7 of the cell culture medium; the latter viruses do not form syncytia spontaneously. However, in this case, syncytium formation can be enforced by intentionally lowering the pH of the medium. By definition, a "fusion from without" (FFWO) occurs when parental virions adsorbed to the cell surface fuse with the plasma membrane and then act as bridges to fuse neighbouring plasma membranes; a "fusion from within" (FFWI) takes place when during virus maturation in later phases of infection progeny viral proteins in the plasma membranes link the cells. In all cases fusogenic viral proteins are implicated as mediators of fusion (Bratt and Gallaher, 1969; for reviews: White *et al.*, 1983; Spear, 1986; Koblet *et al.*, 1987). To obtain further insight into the entry mechanism of Japanese encephalitis virus and into the systematics of viral fusion reactions we have examined FFWO and FFWI in *Aedes albopictus* cells. This cell type is especially suited for such studies (Koblet, 1990).

Materials and Methods

Cells

Aedes albopictus cells, clone C6/36 (Igarashi, 1978), were grown at 28℃ in 25 or 75 cm^2 Roux bottles or in 12-well semimicroplates (Linbro, Flow, USA). Eagle's minimal essential medium supplemented with 0.2 mM each of 7 nonessential amino acids and 9% heat-inactivated (56℃, 30 min) fetal bovine serum (FBS) (Sanko Pure Chemicals, Japan) was used throughout.

Vero cells in 12-well semimicroplates were grown in the medium as above at 37℃ in a humidified 5% CO_2 atmosphere.

Virus

For stock production the wildtype strain of JE, JaOArS982 (Hori *et al.*, 1986), was grown in C6/36 cells. After 2−3 days of growth a titer of maximally 2×10^7 plaque forming units (PFU) per ml was reached. This seed virus was stored at −70℃.

Titration of PFU

Titration was performed with Vero cells essentially according to the protocol of Shameem *et al.* (1989). Virus in 0.2ml Eagle's medium containing 5% FBS was adsorbed for 2 h at 37℃ in a CO_2 atmosphere. The overlay contained 2% FBS and 1.5% methyl cellulose. Cells were then incubated for 5 days at 37℃.

Fusion Assay

Subconfluent C6/36 cells were infected with a multiplicity of infection (MOI) of 1−10 PFU/cell. They were then incubated with Eagle's medium containing 2% FBS at 28℃. After the appropriate times of incubation the medium was replaced by a medium with a preset pH (either Eagle's medium containing 2−10% FBS or phosphate buffered saline [PBS±25mM glucose]). Fusion was evaluated by light microscopy.

Results

Viral Growth and CPE

In order to find the optimal period of time after infection for assaying for a FFWI, subconfluent C6/36 cells were infected with a MOI of 1−10. Cells were then inspected for CPE. Virus shed into the medium was titrated on Vero cells.

Development of a CPE was slow and appeared after one week. This CPE corresponded to a starlike appearance of the cells which contacted each other; syncytia were never observed at pH 7. The virus titers grew slowly. After 12 and 24 hpi no extracellular virus could be detected. At 48 hpi the titer was 5×10^6. at 72 hpi 5×10^7 and at 96 hpi 10^8 PFU/ml.

Infected Monolayers of C6/36 Fuse at pH 5.6 within 30 min

Monolayers were infected at pH 7 with a MOI of 1−10. At different times after infection the medium was replaced by Eagle's medium containing 10% FBS at a pH of 5.6. Discrete foci of fusion involving not more than 2−3 cells could be visualized 30 hpi. At 48 hpi fusion was rapid and complete. Whole monolayers formed huge syncytia within 30 min after lowering the pH and more than 90% of the cells fused. At that time fusion was independent of the composition of the pH 5.6 medium (Eagle containing 2%, 5% or 10% FBS, PBS containing glucose or no glucose), or of the multiplicity of infection. Figure 1 shows an example of syncytia several hours after lowering the pH.

Infected Monolayers Can Be Kept at pH 6 and Fuse at a Given Time

It has been noticed before that C6/36 cells can be grown at pH 6 whether not infected or infected with Semliki Forest virus (SFV) (Koblet *et al.*, 1987). If cells infected with SFV were kept at pH 6 virus production was drastically reduced; probably this was due to the fact that p62, the precursor of SFV envelope proteins E_3 and E_2, was only partially cleaved under these conditions, or that virions shed were rapidly inactivated. However, the envelope proteins appeared in due time in the plasma membrane and were fusion competent (Omar *et al.*, 1986; Koblet *et al.*, 1985; unpublished observation). Correspondingly, monolayers were infected with JE virus at pH 7 with a MOI of 1−10 PFU/cell. At 24 hpi, when no fusions occurred, the medium was exchanged by a medium of pH 5.6 and the cells were incubated at 28℃. The fused areas increased in a regular fashion and 48 hpi more than 90% of the cells

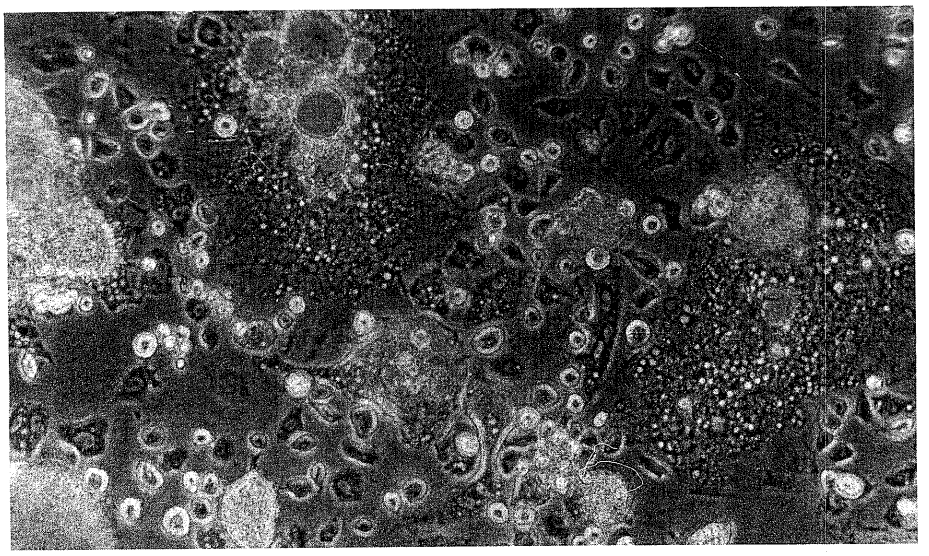

Fig. 1. Syncytium formation of C6/36 cells. A subconfluent monolayer was infected with 10 PFU/cell. After 48 h the pH of the medium was lowered to 5.6. The picture was taken 6 h after lowering the pH. Some non-fused cells are visible. The syncytia contain already many large vacuoles.

had fused. However, if the pH was increased above 6.5 at any time between 24 and 48 hpi, then the fusion was arrested at a given level.

These experiments so far show that (i) at pH 7 expression of a fusogenic factor requires 36−48 h and develops in a nonsynchronized fasion (ii) there is a certain correlation between viral titers and extent of cell-cell fusion (iii) the time for completion of fusion as visualized in the light microscope takes 30 min after lowering the pH (iv) the critical pH triggering fusion is between 5.6 and 6.5 (v) the time course favours the assumption of a late fusion (FFWI).

The Critical pH of the Medium

Monolayers were infected at pH 7 with a MOI of 1−10 PFU/cell. At 55 hpi the media were exchanged by media of preset pH from 5.5 to 7. At pH 6.5 and 6.3 foci of fusions in form of cell clusters were discernible, however, if the pH of the medium was dropped to 6.2 or below the entire monolayer fused. The same phenomenon could be elicited with PBS with or without 25 mM glucose. Thus, the critical pH of the medium is around 6.2. This situation is similar to that in case of *Aedes albopictus C7* cells infected with Dengue virus type 2 or St. Louis encephalitis virus (LAV 5−156) (Randolph and Stollar, 1990), where the pH range over which maximal fusion occurred was broad. In contrast, in case of C6/36 cells infected with SFV the pH of the medium triggering FFWI is very critical, being at pH 6.1.

Exposition to Low pH Can Be Very Short

For FFWI to occur in Alphavirus infected mammalians cells, cultures must be exposed to a low pH for a short time only and then returned to neutral pH (Edwards and Brown, 1984; Kempf *et al.*, 1988). In contrast, SFV infected C6/36 cells will fuse under all conditions, whether left at pH 6 or returned to neutral pH. The reasons for this discrepancy between mammalian and mosquito cells have been reviewed (Koblet, 1990). The shorter the time needed at low pH to induce fusion after reestablishing the neutral pH, the more persuasive is such a result for a conformational change of a fusogenic protein. There are several examples of conformational changes of viral fusogenic proteins as a prerequisite for fusion to occur (White *et al.*, 1983; Spear, 1986; Koblet *et al.*, 1985; Koblet *et al.*, 1987; Koblet, 1990).

Therefore, monolayers were infected as above. Three days later they were exposed to PBS without glucose of pH 6 for various times from 10 sec to 2 min. Thereafter, they were immediately brought to pH 7 with Eagle's medium containing 2% FBS and examined for fusion in the light microscope 1 h later. All manipulations were performed at room temperature. Monolayers fused under all conditions. Uninfected monolayers never fused. Exposition to low pH was mandatory. Thus an exposition of 10 sec to low pH was amply sufficient to trigger the syncytium formation. A conformational change of a (viral) protein is therefore most probable. Kimura and Ohyama (1988) using a FFWO assay with West Nile virus came to a similar conclusion calculating the half-maximal conversion of the conformation to take place within 15 sec after acidification.

FFWI Does Not Take Place Below 15℃

To find the limiting temperature of fusion, C6/36 monolayers were infected with JE virus for 48 h at 28℃. The medium was then replaced with Eagle's medium containing 10% FBS of pH 5.8 at various temperatures and kept at these temperatures. Patchwise fusion was seen at 15℃; at 17℃ complete syncytia formed. Below 15℃ fusion was never observed.

However, a period of 15 sec at pH 5.8 and 4℃ was sufficient to elicit the fusion at pH 5.8 or 7 and 28℃ within 30 min. Thus, fusion is initiated by a triggering event which is rapid, pH-dependent and temperature-independent. It probably occurs at the outer surface of the plasma membrane. Below 15℃ fusion is blocked at an early step after the triggering event; this cannot be overcome by prolonged incubation at this temperature. This second step is temperature-dependent and pH-independent and seems to be slow.

The same situation prevails in C6/36 cells infected with SFV (Kolbet *et al.*, 1987; Koblet, 1990). It is tempting to assume, therefore, that the first temperature-independent step is due to the conformational change of the viral fusogenic protein, while the second temperature-dependet step concerns an inherent characteristic of the mosquito cell.

Assay for FFWO

At least with high multiplicities it should be possible to enforce a FFWO at low pH. Monolayers were incubated with 3×10^6 PFU [MPO=1] or 10^7 PFU for 1 h at pH 7 and 4℃ to absorb the virions on the cell surfaces. Then the inoculum was replaced with medium of pH 5.8 and 28℃. Only 5 days later fusions could be observed. This does not reflect a FFWO; it corresponds to an infection and FFWI which is possible under these conditions in case of C6/36 cells (see above). However, when a MOI of 200 was adsorbed the above protocol yielded strong fusion reactions englobing the whole monolayer within 60 min. Again the most intense reactions could be visualized at pH 6−6.2 and above 17℃.

Discussion

This paper presents data giving firm evidence that JE virus belongs into the category of the viruses provoking fusion at low pH. It confirms observations with other flaviviruses. Randolph and Stollar (1990) reported FFWI 36−48 hpi in case of Dengue infected mosquito cells in a pH range of 5−6.5 and in case of St. Louis encephalitis virus infected mosquito cells in a pH range of 5−5.5. Fusion could be prevented by monoclonal antibodies to the E protein. Lysosomotropic amines inhibited replication consistent with the idea that low pH induced fusion is necessary for virus entry by endocytosis. However, cells had to be exposed to an acidic pH for as long as 15−30 min to induc fusion. This may have been due to a low level of expression of the envelope proteins on the cell surface. This level of expression may in turn be influenced by the strains of virus and cells used (see below). Summers *et al.* (1989) demonstrated FFWO with C6/36 cells with a high MOI of Dengue types 1, 2, 3, 4, JE and yellow fever viruses at pH 6 and 36℃. Dengue 2 monoclonal antibodies against the E protein inhibited fusion, whereas monoclonal antibodies to pre M and NS1 proteins did not inhibit fu

sion. Kimura and Ohyama (1988) showed that acidic pretreatment of West Nile virus rendered the E protein trypsin-resistant in a pH-dependent manner. This conversion was irreversible and was complete at pH 6.4. It is a clear indication of an acid-induced conformational change.

Thus several observations locate the fusion activity into the E protein which seems to be a further example of a viral multifunctional protein (hemagglutination, hemolysis, neutralization, attachment to the cell surface). The fusogenic sequence is unknown. Hydrophobicity plots indicated several regions of hydrophobicity in the E protein of yellow fever and other flaviviruses (Dalgarno *et al.,* 1986). Whether any of these hydrophobic areas are involved with the fusion process is unknown.

FFWI seems to be a symptom of viral envelope proteins residing in the plasma membrane and budding at the plasma membrane. Rapid fusion of C6/36 cells in case of Alphaviruses 16 hpi can be explained by the fact that there is not only intracellular maturation but also budding at the plasma membrane (Koblet *et al.,* 1987; Simizu and Maeda, 1981). However, in case of flaviviruses the question is equivocal. For example, Hase *et al.* (1987) examined the morphogenesis of JE and Dengue 2 viruses under comparable conditions in C6/36 cells. Dengue virus matured in cisternae of the rough endoplasmic reticulum (RER) as well as at the plasma-membrane. In contrast, JE virions were found exclusively within the cisternae of the RER and cytoplasmic vesicles. Therefore, assembled virions seem to pass through the host secretory pathway including the Golgi apparatus and be shed through a secretory type of exocytosis. Targeting to the plasma membrane might then involve binding of the virions to membrane "receptors" in the inner leaflet of the membrane. Kunjin virions were observed scattered in the cytoplasm of *Aedes albopictus* (Singh) cells between 24 to 30 hpi. However, 48 hpi large numbers of morphologically mature particles were visible in association with the RER (Ng, 1987). However, one type of maturation or the other does not seem to be specifically related to a given virus taxon (Hase *et al.,* 1987). JE virus rather budded at the plasma membrane in porcine kidney cells (Ota, 1965). A virus may exhibit one or the other type of maturation based on passage history and adaptation to a host cell system (Hase *et al.,* 1987). This question deserves more investigation.

Despite the fact that the replication strategies and the gene order are different in Alpha- and flaviviruses (Chambers and Rice, 1987) there are some fascinating analogies between these viruses. In both cases it is not the fusogenic protein which is terminally cleaved to be activated (as for example in influenza virus); rather it is the nonfusogenic envelope precursor which is cleaved (pre M in case of Flavivirus, p62 in case of Alphavirus). In both cases, p62 and E_1 (Alpha) (Ziemiecki *et al.,* 1980; Naim and Koblet, 1980) and pre M and E (Flavi) (Wengler and Wengler, 1989) form intracellular heterodimes, which are restructured later into trimers ($[E_1\ E_2\ E_3]_3$, Alpha; $(E)_3$, Flavi; Wengler *et al.,* 1987). Therefore, p62 and Pre M may stabilize the protein complexes and cover up the fusion potency of E_1 and E respectively (Wahlberg *et al.,* 1989; Randolph and Stollar, 1990). In both cases the unknown fusogenic sequence is buried in the fusogenic protein; it is not a newly formed N-terminal after a cleavage event as for example in myxo- or paramyxoviruses. Therefore, there is a

similar situation in Alpha-, Flavi- and Rhabdoviruses. All these genera can replicate in mosquito cells. Vesicular stomatitis virus grows to high yields in the C−7 clone of *A. albopictus* cells (Gillies and Stollar, 1980). Whether there is a deeper meaning hidden in these relations remains to be seen.

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