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Analysis of the endocytic pathway mediating the infectious entry of mosquito-borne flavivirus West Nile into *Aedes albopictus* mosquito (C6/36) cells

J.J.H. Chu, P.W.H. Leong, M.L. Ng *

Flavivirology Laboratory, Department of Microbiology, 5 Science Drive 2, National University of Singapore, Singapore 117597, Singapore

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Abstract

The initial interaction between mosquito-borne flavivirus West Nile and mosquito cells is poorly characterized. This study analyzed the endocytic and the associated signaling pathway that mediate the infectious entry of West Nile virus (WNV) into mosquito cell line (C6/36). Pretreatment of C6/36 cells with pharmacological drugs that blocks clathrin-mediated endocytosis significantly inhibited virus entry. Furthermore, the transfection of functional blocking antibody against clathrin molecules and the overexpression of dominant-negative mutants of Eps15 in C6/36 cells caused a marked reduction in WNV internalization. WNV was shown to activate focal adhesion kinase (FAK) to facilitate the endocytosis of virus but not the mitogen-activated protein kinases (ERK1 and ERK2). Subsequent to the internalization of WNV, the virus particles are translocated along the endosomal pathway as revealed by double-immunofluorescence assays with anti-WNV envelope protein and cellular markers for early and late endosomes. Specific inhibitor for protein kinase C (PKC) was shown to be highly effective in blocking WNV entry by inhibiting endosomal sorting event. The disruption of the microtubule network using nocodazole also drastically affects the entry process of WNV but not the disruption of actin filaments by cytochalasin D. Finally, a low-pH-dependent step is required for WNV infection as revealed by the resistance of C6/36 cells to WNV infection in the presence of lysosomotropic agents.

Keywords: Flavivirus; Virus entry; Clathrin-mediated endocytosis; Endosome; Cytoskeleton

Introduction

The entry process of virus into cells is often a complicated process that involves virus binding to the surface of cells, entry into the cytosol and transport of viral genome to appropriate site for replication. The close association of virus with the host cellular component is essential for the establishment of a successful infection. Binding of virus to specific cellular receptor(s) on the surface of cells can be triggered at least two pathways that facilitate virus entry into cells. Some of the viruses utilize receptor-mediated endocytosis and subsequent acidification along the endo-lysosomal, which will trigger the release of viral genome for replication (Sieczkarski and Whittaker, 2005). This type of entry process has been shown in both enveloped (influenza) and non-enveloped (adenovirus) viruses (Lakadamyali et al., 2004; Meier and Greber, 2004). Alternatively, other enveloped viruses are documented to fuse with the host cellular membrane to gain access into the cells by releasing the viral core into cytosol (Sieczkarski and Whittaker, 2005). Once the virus binds to the cellular receptor, it often triggers a cascade of downstream signaling events that will facilitate its entry process into cells and creating a suitable environment for its subsequent replication (Pelkmans, 2005). Therefore, understanding the fundamentals of the virus entry process into host cells often provides opportunities for intervention to help combat viral infections.

West Nile virus (WNV) is a medically important mosquito-borne flavivirus that causes West Nile fever and meningo-encephalitis (Hayes, 2005). It is a small-enveloped virus classified as a member of the Japanese encephalitis virus serocomplex of the genus *Flavivirus* in the family Flaviviridae (Rice, 1996). The emergence of WNV in several parts of the world has posed significant global health problem. WNV is

* Corresponding author. Fax: +65 7766872.
E-mail address: micngml@nus.edu.sg (M.L. Ng).

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usually maintained in a mosquito–bird–mosquito transmission cycle while mammals and human are often considered as incidental or dead-end hosts (Campbell and Dreher, 2002). It was speculated that the movement of WNV to the Western Hemisphere was caused by migratory birds that acted as introductory hosts and perhaps by infecting ornithophilic mosquitoes (Rappole et al., 2000). WNV is transmitted to vertebrate hosts mainly from the inoculation of virus harboring mosquitoes of the *Culex*, *Aedes*, *Anopheles*, *Minomyia* and *Mansonia* species in Africa, Asia and the United States (Burke and Monath, 2001; Ilkal et al., 1997).

In our recent study, we have identified two putative receptor molecules that are involved in the binding and entry process of WNV (Chu et al., 2005) into susceptible C6/36 (*Aedes albopictus*) cell line. However, little is currently known about the entry process and pathway of WNV into mosquito cells. Therefore, this is the first study that focuses on deciphering WNV entry process into C6/36 cells. Understanding the infection dynamics of WNV in mosquito cells can provide an alternative means of anti-viral strategies that can act against the infectious entry of WNV in mosquitoes.

Results

Infectious entry of WNV is inhibited by the disruption of clathrin-mediated endocytosis

To investigate whether WNV entered mosquito cells by a clathrin or caveola-dependent endocytosis pathway, the C6/36 mosquito cells were treated with a panel of compounds that are known to selectively inhibit each of the specific pathways. C6/36 cells were treated with monodanslycadervine (selectively inhibit receptor-mediated endocytosis), chlorpromazine and sucrose (inhibits clathrin-dependent endocytosis) and filipin (inhibits caveola-dependent endocytosis by disrupting the cholesterol-rich caveola-containing membrane microdomain) and subjected to WNV infection (M.O.I. of 10). The pretreatment as well as the administration of monodanslycadervine (at 15 min p.i.) on C6/36 cells significantly inhibited WNV infection by more than 75% (Fig. 1a). This result implied that cellular receptor molecule(s) was involved in mediating the internalization of WNV into C6/36 cells. In addition, the pretreatment and the early administration (with 10 min p.i.) of C6/36 cells with chlorpromazine (Fig. 1b) and sucrose (Fig. 1c) also strongly inhibited WNV infection. It was noted that the treatment of C6/36 cells with monodanslycadervine, chlorpromazine and sucrose had minimal inhibitory effect on WNV infection when these drugs were added after 1 h p.i. Therefore, this may suggest that these drugs selectively exert their effects in an early step of the virus entry pathway. In contrast, treatment of C6/36 cells with filipin had no significant inhibitory effect on WNV infection regardless of the timing of the administration of this drug (Fig. 1d).

To affirm the involvement of clathrin-dependent endocytosis pathway in mediating the entry of WNV into C6/36 cells, molecular inhibitors in the form of dominant-negative mutants of Eps-15 and arrestment of clathrin activities by specific functional blocking antibodies were carried out. As an

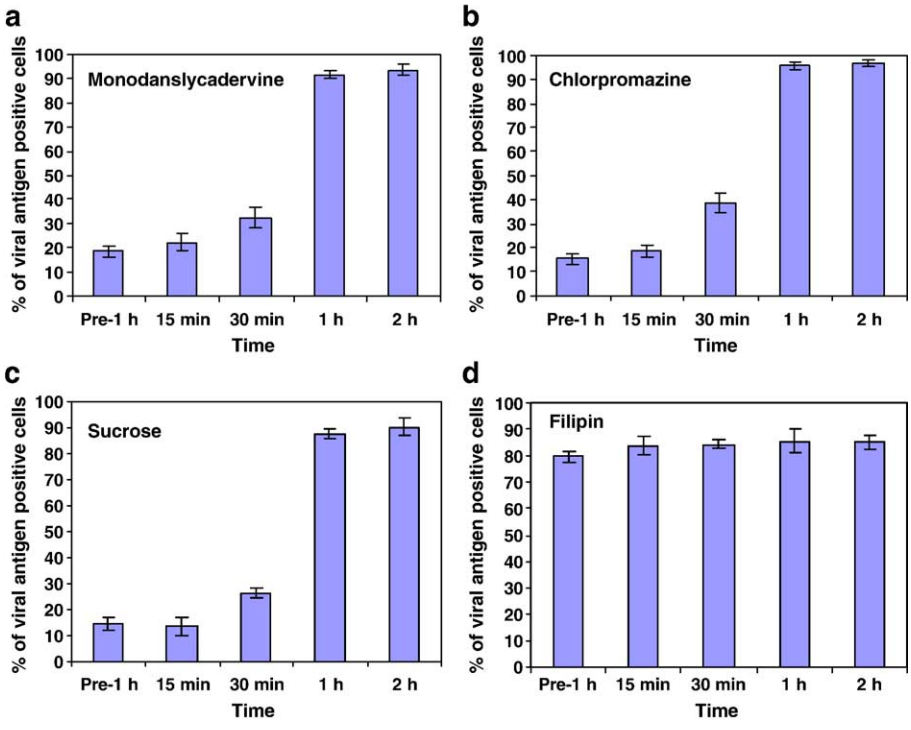

Fig. 1. Effects of receptor-mediated endocytosis disrupting drugs on WNV entry into C6/36 cells. The percentage of viral antigen positive cells was plotted against treatment time. C6/36 cells were treated with (a) monodanslycadervine, (b) chlorpromazine, (c) sucrose and (d) filipin. Endocytosis of WNV into C6/36 cells was significantly inhibited by monodanslycadervine, chlorpromazine and sucrose between 15 and 30 min p.i. whereas filipin has minimal effect on virus entry. Pretreatment for 1 h was equally effective except for filipin. The average of 3 independent experiments is shown.