Infection of polarized epithelial cells with flavivirus West Nile: polarized entry and egress of virus occur through the apical surface

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Both polarized epithelial Vero (C1008) and non-polarized Vero (control) cells were grown on permeable cell culture inserts and infected either apically or basolaterally with West Nile (WN) or Kunjin (KUN) virus. KUN virus (closely related to WN virus) was used as a comparison. Using indirect immunofluorescence and plaque assays of productive virus titres, entry of WN and KUN viruses was confined to the apical surface of polarized epithelial cells. For the first time, these results provided evidence on the distribution of flavivirus-specific receptor(s) in polarized epithelial cells; that is to say that receptor expression was shown to be predominant at the apical surface. In addition, the release of these viruses from polarized Vero C1008 epithelial cells was also examined. Egress of WN virus strain Sarafend (S) was observed to occur predominantly at the apical surface of Vero C1008 cells. In contrast, the release of KUN virus was bi-directional from polarized Vero C1008 cells. Furthermore, disruption of the cellular microtubule network was shown to inhibit the apical release of WN (S) virus but had no effect on the release of KUN virus. Hence, the difference in the release of these closely related viruses suggested the involvement of a microtubule-dependent, polarized sorting mechanism for WN virus proteins but not for KUN virus proteins in polarized epithelial cells.

Introduction

The family Flaviviridae comprises more than 70 closely related RNA viruses and is one of the most medically important groups of emerging arthropod-borne viruses (Heinz et al., 2000). Flaviviruses are small, enveloped viruses consisting of three structural proteins [the envelope (E), capsid (C) and membrane] and a single-stranded RNA genome. Using cross-neutralization tests, De Madrid & Porterfield (1974) classified flaviviruses into seven subgroups. Both West Nile (WN) and Kunjin (KUN) viruses are members of the Japanese encephalitis virus serocomplex. WN virus is the aetiological agent of WN fever and severe human meningoencephalitis is a common complication of WN virus infection (George et al., 1984). The recent re-emergence of WN virus in the western hemisphere has resulted in several fatalities (Rappole et al., 2000). KUN virus causes Australian encephalitis but is probably less overt and is localized to Australia and Southeast Asia (Monath & Heinz, 1996). Previous studies have documented that WN and KUN viruses can infect a variety of cell cultures and continuous cell lines of human, primate, rodent and insect origin (Odelola & Fabiyi, 1977; Wengler et al., 1978; Peiris & Porterfield, 1979; Ng & Westaway, 1979; Brinton, 1982).

Polarized epithelial cells line the major cavities of the body and form a selective barrier against the invasion of many pathogens. The plasma membranes of these polarized cells are highly specialized and are divided into two discrete surfaces, the apical and the basolateral surface. The apical and basolateral domains of polarized cells each display a distinct set of specialized proteins, lipids and cellular receptors (Gumbiner & Simons, 1987; Madara, 1988; Simons & Wandinger-Ness, 1990). These discrete domains function in the selective absorption and release of many cellular proteins and pathogens. Polarized epithelial cells have been used extensively to study the sorting of cellular and viral proteins in polarized cells. Sorting cellular receptors and proteins to the distinct membrane domains of polarized cells has been reported to be mediated by the interaction of the cytoskeletal network (Wandinger-Ness & Simons, 1991). Several studies over the years have investigated polarized entry, the intracellular sorting mechanism of viral proteins and the release of virus progeny from distinct domains.
of polarized cells (Tucker & Compans, 1993; Compans, 1995). These studies have provided crucial information on the distribution of virus cellular receptors, the intracellular trafficking mechanism of viral proteins during morphogenesis and the domains of virus egress. The polarized entry and release of viruses might also be significant for virus pathogenicity (reviewed by Compans, 1995).

So far, the interaction of flaviviruses with polarized cells has not been documented. In this study, polarized entry and release of WN virus strain Sarafend (S) and KUN virus from specific domains of polarized epithelial cells (Vero C1008) were defined. Microtubule-dependent, polarized sorting of flavivirus E proteins in polarized Vero C1008 cells was also demonstrated.

Methods

- **Cells and viruses.** Non-polarized Vero cells and polarized Vero C1008 cells were grown on 0.4 μm porous support membrane inserts (Becton Dickson) immersed in medium 199 containing 10% inactivated foetal calf serum. A total of 1 x 10⁴ cells per insert was seeded and allowed to grow to confluency. The integrity of the cell monolayers was monitored by measuring the transepithelial electrical resistance with the Millicell-ERS apparatus (Millipore). The net resistance of the cell monolayers was calculated as described by Blau & Compans (1995).

- **Reagents and antibodies.** Propidium iodide and vinblastine sulphate were purchased from Sigma. The antibody for the WN virus E protein was a monospecific antibody raised in rabbits and was a kind gift from Vincent Deubel, Pasteur Institute, France. The anti-KUN virus E protein antibody, produced in our laboratory, was a polyclonal antibody derived from rabbits. Anti-tubulin and secondary antibodies conjugated to either FITC or Texas red (TR) were purchased from Amersham Pharmacia.

- **Indirect immunofluorescence microscopy.** For immunofluorescence microscopy, cell monolayers were grown on cell inserts and infected with WN (S) or KUN virus at an m.o.i. of 10. At 12 and 24 h p.i. for WN (S) and KUN virus, respectively, the cells were fixed with cold absolute methanol for 10 min, followed by a wash in cold PBS for 15 min. The cells were then washed three times for 10 min each in cold PBS containing 0.1% BSA to eliminate non-specific binding. Cells were incubated with the primary antibodies [1:30 dilution, anti-WN (S) virus E protein antibody; 1:10 dilution, anti-KUN virus E protein antibody; 1:50 dilution, anti-tubulin antibody] in a humidity chamber for 1 h at 37 °C. After three washes for 5 min each with PBS, cells were incubated further with FITC- or TR-conjugated secondary antibodies before washing again with PBS. Virus-infected cells were also incubated for 10 min with propidium iodide at a concentration of 0.1 μg/ml. The processed cell inserts were mounted onto ethanol-cleaned glass slides using Dabco mountant. Specimens were viewed under a laser scanning confocal inverted microscope (Leica TCS SP2) and an optical microscope (Olympus, BX60).

- **Ultrastructural analysis.** Vero and Vero C1008 cells were grown until confluent on cell inserts and were then infected apically with either WN (S) or KUN virus at an m.o.i. of 10. At the end of the infection period, 12 h for WN (S) virus and 24 h for KUN virus, the infected cells grown on 0.4 μm porous membranes were washed twice with cold PBS and prefixed with a 2.5% glutaraldehyde–2% paraformaldehyde mixture. This was followed by post-fixation for 90 min at 4 °C in 1% osmium tetroxide. The samples were then dehydrated through a series of ethanol solutions of increasing concentration and embedded in low-viscosity epoxy resin. Ultrathin sections (50–70 nm) were stained with 2% uranyl

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Fig. 1. Polarize entry of WN (S) and KUN viruses into Vero C1008 cells. At 12 [WN (S) virus] and 24 (KUN virus) h p.i., supernatants from both apical and basolateral chambers were pooled for plaque assays to assess total extracellular virus yield. Higher extracellular virus yields are observed upon apical inoculation of WN (S) virus (a) and KUN virus (b).
acetate and post-stained with 2% lead citrate before viewing under a Philips electron microscope, CM 120 Biotwin.

**Microtubule-disrupting drug treatment.** Polarized Vero C1008 cells grown on cell culture inserts were infected apically with WN (S) or KUN virus at an m.o.i. of 10. At 6 [WN (S) virus] and 12 (KUN virus) h p.i., culture media from both apical and basolateral chambers were supplemented with 10 µg/ml vinblastine sulphate (Sigma). The supernatants from both apical and basolateral chambers were then harvested at 12 h p.i. for WN (S) virus and 24 h.p.i. for KUN virus. Titres of productive virus were determined by plaque assays and the infected cells on the culture inserts were also fixed and processed for immunofluorescence assays.

**Results**

**WN and KUN virus entry into polarized Vero C1008 epithelial cells occurs preferentially at the apical surface**

Previous studies have shown that several viruses exhibit polarized entry into Vero C1008 cells. Simian virus 40, measles virus and Black Creek Canal virus each entered Vero C1008 cells at the apical surface, whereas entry of vesicular stomatitis virus was restricted to the basolateral surface of the cell’s plasma membrane and entry of poliovirus occurred bidirectionally (Clayson & Compans, 1988; Blau & Compans, 1995; Ravkov et al., 1997; Bergmann & Fusco, 1988; Tucker et al., 1993). Polarized entry of these viruses into Vero C1008 cells has indicated that there could be differential expression of the virus cellular receptors at different domains (apical or basolateral) of the polarized cells. To determine whether WN (S) and KUN viruses exhibit polarized entry into Vero C1008 cells, WN (S) and KUN viruses (m.o.i. of 10) were used to infect either the apical or the basolateral domains of confluent Vero C1008 cells grown on 0.4 µm porous cell culture inserts. Total extracellular virus production [at different times p.i. for WN (S) and KUN viruses] was determined by plaque assay. Higher yields of both WN (S) and KUN viruses were observed when the viruses were inoculated at the apical surface of Vero C1008 cells as compared to those when the viruses were inoculated at the basolateral surface (Fig. 1a, b).

The levels of WN (S) or KUN viral proteins and virus production after inoculation from either the apical or the basolateral surface of Vero C1008 cell monolayers were also assessed by immunofluorescence microscopy and immunoblotting detection. Both anti-KUN virus and anti-WN (S) virus E protein sera were used as markers to detect the presence of expressed viral proteins and virus particles. By using immunofluorescence staining, it was noted that Vero C1008 cells were more susceptible to both WN (S) and KUN virus infection when the viruses were inoculated at the apical surface. From three independent experiments it was observed that higher proportions of Vero C1008 cells were infected when the flaviviruses were inoculated at the apical surface (Fig. 2a, d). In

![Fig. 2. Indirect immunofluorescence detection of WN (S) and KUN virus antigen in infected Vero C1008 cells. Nuclei are stained with 0.1 µg/ml propidium iodide and the virus E protein is detected with anti-WN (S) or anti-KUN E-specific serum and FITC-conjugated secondary antibodies. Either WN (S) or KUN virus at an m.o.i of 10 was inoculated to either the apical (a, d) or the basolateral (b, e) chambers. High levels of viral antigen expression are observed when the viruses are inoculated to the apical surface of Vero C1008 cells. (c, f) Mock-infected Vero C1008 cells.](image-url)
Fig. 3. Release of WN (S) virus occurs predominantly at the apical domain, while KUN virus release occurs bi-directionally in Vero C1008 cells. Vero C1008 cells (a, b) and non-polarized Vero cells (d, e) are inoculated apically with either WN (S) or KUN virus. Productive virus titres from supernatants collected from the apical and basolateral chambers are determined by plaque assay at 12 and 24 h p.i. for WN (S) and KUN viruses, respectively. (a) Apical release of WN (S) virus from Vero C1008 cells. (b) Bi-directional release of KUN virus from Vero C1008 cells. (c) Bi-directional release of poliovirus from Vero C1008 cells (positive control). (d, e) Bi-directional release of WN (S) and KUN viruses from non-polarized Vero C1008 cells.

In a time-sequence study, a progressive increase in virus E protein synthesis was detected in Vero C1008 cells infected at the apical domain using immunoblotting (data not shown). In contrast, minimal virus E protein production was observed in Vero C1008 cells that were infected through the basolateral domain for both WN (S) and KUN virus infections. However, non-polarized Vero cells did not exhibit any preferential entry of either WN (S) or KUN virus into the cells.

contrast, a much lower number of cells was infected following basolateral inoculation (Fig. 2b, e). Mock-infected Vero C1008 cells were also labelled with anti-WN (S) virus E protein and anti-KUN virus E protein FITC-conjugated secondary antibodies, as shown in Fig. 2(c, f). Infections of non-polarized Vero cells were observed to be equally susceptible to WN (S) and KUN viruses from either apical or basolateral inoculation (data not shown).
Hence, these results have provided strong evidence that entry of both WN (S) and KUN viruses was restricted to the apical surface of polarized Vero C1008 cells. This could suggest that the receptor molecules for both WN and KUN viruses were highly expressed at the apical domain of polarized epithelial cells.

Release of WN virus is restricted to the apical domain of Vero C1008 cells while KUN virus is released bi-directionally

To determine the release site of the flaviviruses used in this study, WN (S) or KUN virus was inoculated as described in Methods. At different time-points, infected supernatants from either the apical or the basolateral chambers were assessed for progeny virus by plaque assays. WN (S) virus was released predominantly from the apical surface of Vero C1008 cells (Fig. 3a). At 12 h p.i., virus titres of WN (S) virus from the apical surface were more than 4 log units higher than those at the basolateral surface. In contrast, bi-directional release of KUN virus was observed (Fig. 3b). The titres of KUN virus released from the apical domain were approximately equivalent to those from the basolateral domain throughout the time-sequence study.

As a positive control, the release of poliovirus from Vero C1008 cells was determined. Fig. 3(c) shows the bi-directional release of poliovirus from Vero C1008 cells. This was consistent to the previous study conducted by Tucker et al. (1993). In comparison, non-polarized Vero cells were also infected with WN (S) or KUN virus. Not surprisingly, bi-directional release of WN (S) virus from non-polarized cells was observed (Fig. 3d). This was in contrast to that from polarized Vero C1008 cells. However, the release of KUN virus from non-polarized Vero cells remained bi-directional (Fig. 3e).

To confirm the above results further, localization of virus E proteins and virus particles at certain domains of Vero C1008 cells during virus release was visualized using immunofluorescence coupled with confocal microscopy and transmission electron microscopy. The WN (S) virus E protein and probably virus particles were observed as yellowish speckles of fluorescence localized exclusively at the apical surface of the infected cells (Fig. 4a). In contrast, KUN virus was localized at both domains of the infected cell monolayer (Fig. 4b).

At the ultrastructural level, cross-sections of the flavivirus-infected cell monolayers grown on porous membranes revealed budding of WN (S) virus (Fig. 5, arrows) at the apical surface of the plasma membrane (Fig. 5a, b). In contrast, exocytosis of intracellular mature KUN virus (Fig. 5, arrowheads) was noted at both apical and basolateral domains of polarized epithelial Vero C1008 cells (Fig. 5c-e). These results strongly indicated that the release of WN (S) virus is polarized and occurs predominantly at the apical domain of Vero C1008 cells, but this is not so for KUN virus.

Throughout this study, the integrity and polarity of flavivirus-infected Vero C1008 cells were also monitored by measuring the electrical resistance across the cell monolayers. The electrical resistance of the flavivirus-infected cell monolayers was 50–70 Ω cm². In addition, the integrity of the cell monolayers was also monitored by visualizing under the optical microscope. It was observed that flavivirus-infected Vero C1008 cells formed tight epithelial cell monolayers until late infection. Cell rounding was only observed after 14 and 24 h.p.i. for WN (S) virus and KUN virus, respectively (data not
Fig. 5. Ultrastructural studies of WN (S) and KUN virus release from Vero C1008 cells. WN (S) virus- and KUN virus-infected Vero C1008 cells are processed for transmission electron microscopy, as described in Methods. (a) Cross sections of the infected cells revealed distinct budding of WN (S) virus (arrows) from the apical surface of Vero C1008 cells. (b) Higher magnification of WN (S) virus budding from the apical surface. (c) Bi-directional release of KUN virus by exocytosis (arrowheads) was observed. (d, e) Higher magnification of (c) showing the apical and basolateral release of KUN virus, respectively. Bars, 300 (a), 500 (b), 300 (c), 300 (d) and 300 (e) nm. The apical (A) and basolateral (B) surfaces of Vero C1008 cells are indicated.

shown). Late in infection, the electrical resistance of flavivirus-infected cell monolayers was less than $20 \, \Omega / \text{cm}^2$.

**Polarized sorting of WN virus E protein to the apical domain of Vero C1008 cells is dependent on an intact microtubule network**

In a recent study involving non-polarized Vero cells, the E and C proteins of WN (S) virus were transported along cellular microtubules from the site of synthesis (i.e. endoplasmic reticulum and Golgi apparatus) to the cell periphery for assembly (Ng et al., 2001; Chu & Ng, 2002). However, it is not known whether microtubules are involved in the polarized sorting of WN (S) virus E protein in Vero C1008 cells; this could contribute to the apical release of WN (S) virus.

In these series of experiments, the possible involvement of microtubules in the polarized sorting of the E proteins of WN and KUN viruses was investigated. WN (S) or KUN virus-infected Vero C1008 cells were treated with a microtubule-disrupting drug, vinblastine sulphate, at a concentration of $10 \, \mu g/\text{ml}$. At this concentration, vinblastine sulphate induces rapid depolymerization of the cellular microtubules but has minimal cytotoxic effects on Vero C1008 cells. Despite the
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Fig. 6. Role of microtubules in the polarize release of WN (S) and KUN virus in polarized epithelial cells. The disruption of microtubules by vinblastine sulphate strongly inhibits the apical release of WN (S) virus but not KUN virus.

Fig. 7. Localization of WN (S) E protein in infected Vero C1008 cells treated with vinblastine sulphate. WN (S) virus E proteins (labelled with FITC, arrows) are observed as yellow speckles that were strongly associated with vinblastine sulphate-induced microtubulin paracrystals (labelled with TR, arrowheads).

formation of vinblastine sulphate-induced microtubulin paracrystals, minimal cell rounding and cell death was observed (data not shown).

As shown in Fig. 6, disruption of the microtubule network led to a drastic inhibition of the apical release of WN (S) virus but not KUN virus. However, the basolateral release of WN (S) virus was not affected when compared to that seen in Fig. 3(a). Furthermore, WN (S) and KUN virus-infected Vero cells treated with vinblastine sulphate were also processed for indirect immunofluorescence assay at 12 and 24 h p.i., respectively. In Fig. 7, entrapment of WN (S) virus E protein (Fig. 7, arrows) with the drug-induced microtubulin paracrystals (Fig. 7, arrowheads) was observed. Yellow staining indicated co-localization of the E protein (FITC) with the microtubulin paracrystals (TR). Staining of WN virus E proteins was not observed in the cytoplasm and cell plasma membrane. In contrast, KUN virus E proteins were localized to the cell cytoplasm and plasma membrane but not the drug-induced microtubulin paracrystals (data not shown). Together these results strongly suggested that the intracellular sorting of WN (S) virus E protein to the apical surface is dependent on the microtubule network, whereas KUN virus is not.

Discussion

This is the first study on the interaction of flaviviruses with polarized cells. In this study, it has been shown that flavivirus WN (S) and KUN viruses are capable of replication in polarized epithelial Vero C1008 cells with no drastic changes to the productive yield of the progeny viruses when compared to non-polarized Vero cells (Fig. 3). Although polarized epithelial cells might not be the prime targets of flavivirus infection, these cells were useful in providing a simple model for the study of cellular receptor distribution and intracellular trafficking of flaviviral proteins during replication.

Previous studies have shown that entry of WN (S) and KUN viruses was mediated by receptor-mediated endocytosis (Gollins & Porterfield, 1985; Kimura et al., 1986; Ng & Lau, 1988). However, the actual cellular receptors involved in the initial attachment and entry of these viruses have yet to be identified. In the present study, investigation revealed the polarized entry of WN (S) and KUN viruses into Vero C1008 cells. It was found that both WN (S) and KUN virus infections occurred with much higher efficiency when the viruses were inoculated at the apical surface of Vero C1008 cells (Fig. 1). This type of restriction in the entry of viruses has also been documented for simian virus 40, measles virus, Black Creek Canal virus, Epstein–Barr virus and hepatitis A virus (Clayson & Compans, 1988; Blau & Compans, 1995; Chodash et al., 2000; Blank et al., 2000). For these viruses, polarized entry was aided by the apical localization of their cellular receptors in Vero C1008 cells. Since polarized epithelial cells express distinct sets of receptors and cellular proteins at either the apical or the basolateral domain, the preferential entry of WN (S) and KUN viruses could imply the apical localization of their cellular receptors in Vero C1008 cells. With the receptor-rich region identified in this cell line, it could be a good cell system for the isolation of the receptor molecule(s) in future studies.

Polarized release of viruses from polarized epithelial cells was first observed in enveloped RNA viruses. Influenza virions were observed to bud from the apical membrane, while vesicular stomatitis virus was released basolaterally (Rodriguez-Boulan et al., 1983). The polarized fashion of virus release was subsequently found to be attributed to the polarized expression and intracellular sorting of the respective viral proteins to either the apical or the basolateral surface for...
intracellular maturation of flaviviruses, WN (S) virus was observed to mature by budding at the plasma membrane. In addition, our recent study (Chu & Ng, 2002) has demonstrated that the microtubule network is essential in the trafficking of WN (S) virus structural proteins to the plasma membrane for morphogenesis of the virus in non-polarized cells. Hence, the apical egress of WN (S) virus from polarized epithelial Vero C1008 cells was contributed to by the microtubule-dependent polarized sorting of the structural proteins to the apical membrane, while the intracellular delivery of mature KUN virus particles is independent of the microtubule network. Whether the differences in the polarized release of WN (S) and KUN viruses contribute to the pathogenesis of these viruses is currently under investigation.

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References


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