EFFECT OF MONENSIN ON MAYARO VIRUS REPLICATION IN MONKEY KIDNEY AND *AEDES ALBOPICTUS* CELLS

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Summary. – The effect of a cationic ionophore, monensin, on the replication of Mayaro virus in monkey kidney TC7 and *Aedes albopictus* cells has been studied. Treatment of these cells with 1 μmol/l monensin during infection did not affect the virus protein synthesis but inhibited severely the virus replication. Electron microscopy of the cells infected with Mayaro virus and treated with monensin revealed that the morphogenesis of Mayaro virus was impaired in TC7 but not in *A. albopictus* cells.

Key words: Mayaro virus; monensin; TC7 cells; Aedes albopictus cells; protein synthesis; virus morphogenesis

Introduction

Mayaro virus (the *Mayaro virus* species, the *Alphavirus* genus, the *Togaviridae* family) is an arbovirus, perpetuated in nature by its ability to infect and replicate in vertebrate and invertebrate cells (Casals and Whitman, 1957). Since the first isolation in Trinidad in 1954, this virus has been further isolated in Brazil, Bolivia and other countries, from human and mammalian species, especially at the frontiers of colonization in the Amazon region (Causey and Maroja, 1957; Talarmin *et al*., 1998). Clinical manifestations of human Mayaro virus infection have been described as a feverish illness accompanied by headache, chills, nausea, photophobia, myalgia and arthralgia. In some cases there is true arthritis and joint symptoms that persist up to months (Pinheiro *et al*., 1981).

The alphavirus genome consists of a plus-sense single-stranded RNA molecule, capped at the 5'-terminus and polyadenylated at the 3'-terminus. The 3' third of the genome codes for a polyprotein that is processed and gives rise to structural proteins consisting of a capsid protein (34 K), a precursor (62 K), later cleaved to two glycoproteins (E2 and E3), and another glycoprotein (E1). For most alphaviruses, only glycoproteins E1 and E2 (50 K each) are structural components of mature virions (Strauss and Strauss, 1994).

In vertebrate cells, the process of alphavirus morphogenesis has been analyzed by electron microscopy. Usually the assembly of virus nucleocapsids occurring in the cell cytoplasm involves association of capsid proteins with genomic RNA. The nucleocapsids acquire the envelope by budding trough the plasma membrane where virus-specific glycoproteins have been previously inserted (Strauss and Strauss, 1994; Mezencio *et al*., 1990; Rebello *et al*., 1993). However, virus maturation in mosquito cells is not well understood. In studying morphogenesis of Sindbis virus (SINV) in *A. albopictus* cells (clones C6/36 and C7-10) Miller and Brown (1992) have observed that virus buds at the cell surface or membranes of internal vesicles and is released by exocytosis. In Mayaro virus infected *A.*

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Abbreviations: DMEM = Dulbecco's Modified Eagle's medium; FCS = fetal calf serum; HA = hemagglutinin; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; p.i. = post infection; SDS = sodium dodecyl sulfate; SDS-PAGE = PAGE in the presence of SDS; SFV = Semliki Forest virus; SINV = Sindbis virus; TGN = trans-Golgi network; VSV = Vesicular stomatitis virus

albopictus cells (clone C6/36) Mezencio *et al*. (1989) have observed the presence of cytoplasmic vesicles containing viral nucleocapsids and mature virions. However, at no time during the viral replicative cycle, budding of the virus into such vacuoles has been observed.

Monensin is an ionophore that catalyzes the exchange of sodium ions for protons across biological membranes, disrupting in this way the gradient of ions. Ultrastructural examination of cells treated with this drug has shown morphological changes in the Golgi apparatus with accumulation of dilated cytoplasmic vacuoles. Monensin also affects intracellular transport of cell and virus proteins to the extracellular space (Mollenhauer *et al*., 1990). The effect of this drug on the replication of Semliki Forest and Sindbis viruses has been investigated more intensively in vertebrate compared to mosquito cells (Kääriäinen *et al*., 1980; Jain *et al*., 1991).

In this study we demonstrated that the effect of monensin on morphogenesis of Mayaro virus differs considerably in monkey kidney (TC7) and *A. albopictus* cells.

Materials and Methods

Virus. Mayaro virus was obtained from the American Type Culture Collection, Rockville, MD, USA. The virus stock was prepared in BHK-21 cells and stored at -70°C.

Cell cultures. TC7 cells, a clone of CV-1 cell line derived from kidneys of a male adult African green monkey were grown at 37°C in Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, USA) supplemented with 2% of fetal calf serum (FCS) and 8% of bovine serum. *A. albopictus* cells, clone C6/36, were grown at 28 °C in DMEM supplemented with 0.2 mmol/l no-essential amino acids, 2% of fetal calf serum, penicillin (500 U/ml), streptomycin (100 µg/ml) and amphotericin B (fungizone, 2.5 µmg/ml).

Monensin treatment. Stock solution of monensin (1 mg/ml, Sigma Chemical Co.) was made in ethanol and was stored at -20°C. The stock solution was diluted in growth medium to proper concentration, added to the cultures, and kept there throughout the whole experiment inclusive of labeling.

Virus infectivity titration. After 20 hrs post infection (p.i.) the cell culture supernatant was recovered and used for titration of extracellular infective virus. For the titration of intracellular infective virus, the monolayer was washed 3 times with phosphate-buffered saline (PBS) and treated with 0.25% trypsin and 1 mmol/l EDTA for 2 mins at room temperature in order to remove the virus particles attached to the cell surface. The cells were then centrifuged at 3,000 x g, washed with the growth medium and disrupted in distilled water with the help of a syringe. The preparation was then centrifuged at 10,000 x g for 10 mins and the supernatant was used for titration. The latter was performed by plaque assay in Vero cells that had just reached confluence in a standard way.

Polyacrylamide gel electrophoresis (*PAGE*). Confluent monolayers of TC7 or *A. albopictus* cells were labeled with [^{35}S] methionine (30 µCi/ml) in a methionine-free medium for 1 hr at 37°C or 28°C, respectively. The medium was then removed and the monolayer was dissolved in 70 µl of a loading buffer (63 mmol/l Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.001% Bromophenol Blue). The sample was then heated for 5 mins at 95°C and electrophoresed in 12.5% SDS-PAGE as described by Laemmli (1970). The dried gel was exposed to Kodak X Omat (YAR-S) film. Relative molecular mass (M_r) of proteins was determined protein standards (Life Technologies).

Electron microscopy. Confluent monolayers of TC7 or *A. albopictus* cells were infected with Mayaro virus at a multiplicity of infection of 5 PFU/cell. After 1 hr of incubation at 37°C for TC7 cells or 28°C for *A. albopictus* cells, the inoculum was replaced by the growth medium containing 1 µmol/l monensin. After 20 hrs the cells were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer pH 7.3, containing 0.2 mol/l sucrose and 5 mmol/l $CaCl_2$ in the same buffer for 2 hrs at room temperature. After a 3-fold rinsing in 0.1 mol/l sodium cacodylate buffer pH 7.3 and 0.2 mol/l sucrose, the cells were post-fixed with 1% OsO_4 (w/v) in the same buffer for 1 hr at room temperature, dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate for 20 mins followed by lead citrate for 5 mins, and observed in a Philips EM301 electron microscope operated at 80 kV.

Results

Effect of monensin on virus yield

We first examined the effect of monensin in various concentrations on Mayaro virus replication in TC7 and *A. albopictus* cells. The results of these experiments (Table 1) revealed that monensin decreased virus yields in both cell lines when added after the inoculum removal. At the concentration of 1 µmol/l the virus yields were inhibited by 75% in TC7 cells and by 29% in *A. albopictus* cells. The virus yields in both cell lines decreased progressively with increasing concentration of the drug, resulting to inhibition by 88% at 10 µmol/l.

Effect of monensin on protein synthesis

In order to determine whether monensin could act by specifically affecting the synthesis of Mayaro virus proteins,

Table 1. Effect of monensin on the production of infectious Mayaro virus in TC7 and *A. albopictus* cells

Monensin (µmol/l)	TC7 cells (PFU/ml)	Inhibition (%)	*A. albopictus* cells (PFU/ml)	Inhibition (%)
0	3.3×10^6	–	1.7×10^7	–
1	8.2×10^5	75	1.2×10^7	29
5	5.9×10^5	82	5.1×10^6	70
10	4.1×10^5	87	1.9×10^6	88

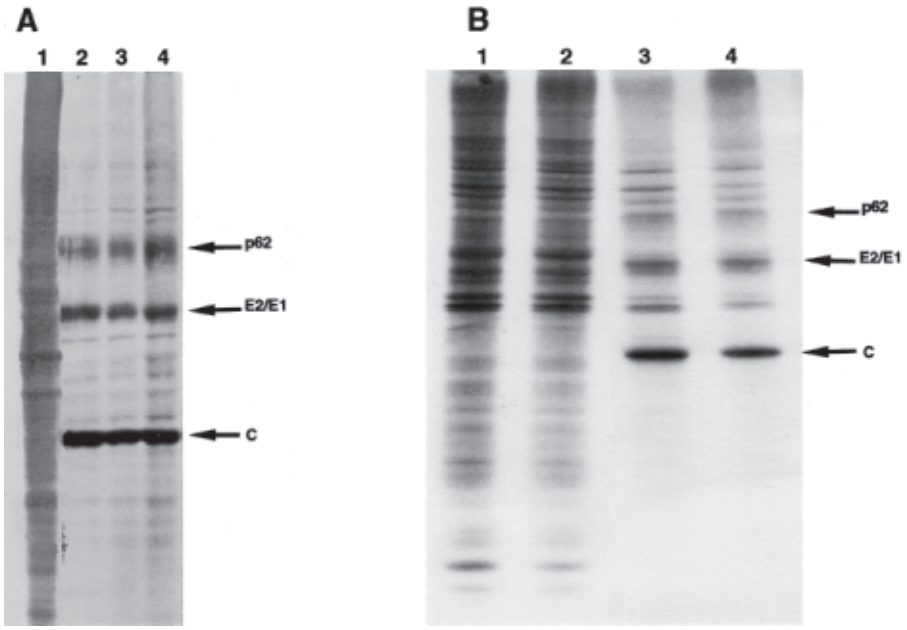

Fig. 1
Effect of monensin on Mayaro virus protein synthesis

(A) TC7 cells. Mock-infected cells (lane 1); virus-infected cells; virus-infected cells treated with 1 µmol/l (lane 3) and 10 µmol/l (lane 4) monensin; (B) *A. albopictus* cells. Mock infected cells (lane 1); mock-infected cells treated with 1 mmol/l monensin (lane 2); virus-infected cells (lane 3); virus-infected cells treated with 1 µmol/l monensin (lane 4).

the cells were infected and treated with 1 µmol/l monensin right after removal of the inoculum. After 20 hrs the cells were labeled with [^{35}S]methionine and cell extracts were prepared and subjected to PAGE and autoradiography.

Fig. 1 shows that the treatment with 1 µmol/l monensin did not inhibit protein synthesis in mock-infected or Mayaro virus-infected TC7 cells and *A. albopictus* cells. In all experiments described bellow 1 µmol/l monensin was used, which was not toxic to the cells tested, as revealed by the trypan blue exclusion test. Moreover, *A. albopictus* cells proved to be more sensitive to monensin. In experiments with monensin concentration over 5 µmol/l we observed an inhibition of cell protein synthesis and loss of cellular adherence to culture flasks (data not shown).

Effect of monensin on morphogenesis

To study the effect of monensin on Mayaro virus morphogenesis, TC7 or *A. albopictus* cells infected with the virus were treated with 1 µmol/l monensin as described above and at 20 hrs p.i., before any cytophatic effect (CPE) could be seen, the cells were harvested and processed for electron microscopy.

Figs. 2A and 3A show micrographs of uninfected cells. In monensin-treated cells (Figs. 2B and 3B) dilatated vacuoles in the cytoplasm of both cell lines were observed. In the virus-infected TC7 cells (Figs. 2C and 2D) budding (arrow) can be observed at the plasma membrane level. When these cells were infected and treated with monensin (Figs. 2E and 2F), we observed dilated vesicles surrounded by nucleocapsids, and mature virions inside these vesicles. Some of these vacuoles were near the cell surface and contained mature virus particles organized in a paracrystalline array (Fig. 2F). In the monensin-treated cells budding through the plasma membrane was rarely seen, indicating a shift in maturation at the plasma membrane level to internal membranes. This data suggests that the virus yield inhibition seen in the supernatant of infected TC7 cells (Table 1) could be rather due to inhibition of virus release than to a reduced infectivity of virions.

Monolayers of the virus-infected TC7 cells treated with 1 µmol/l monensin were examined for intracellular infectious virus. The experiments showed that for this concentration of monensin the intracellular virus titer increased to an average of 112%. In conclusion, the effect of monensin on replication of Mayaro virus in TC7 cells seems to be primarily caused by blocking the release of the virions to the extracellular medium.

Figs. 3C and 3D show the virus-infected *A. albopictus* cells. Numerous virions budding through the plasma

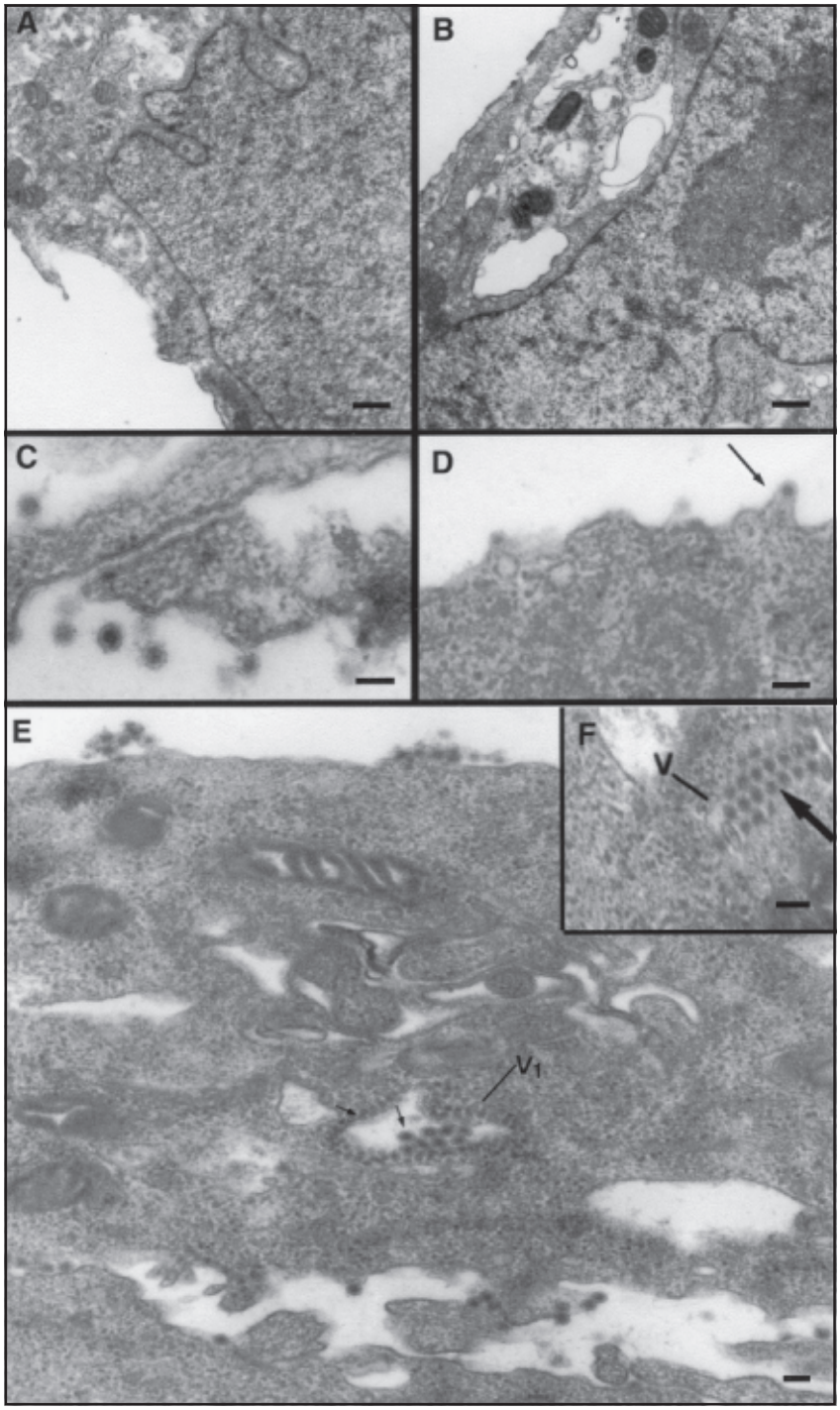

Fig. 2
Electron microscopy of TC7 cells

(A), Mock-infected cells, bar 300 nm; (B), mock-infected cells treated with monensin, bar 300 nm; (C, D), virus-infected cells, bar 100 nm; (E), virus-infected cells treated with monensin showing a cytoplasmic vacuole (V_1) with mature virus particles inside and nucleocapsids around the vacuole membrane (arrows), bar 100 nm; (F), the insert showing a vacuole (V) with nature virions (arrow) in a paracrystalline array, bar 100 nm.

membrane can be seen. When these cells were treated with monensin (Fig. 3D) no differences in virus morphogenesis could be seen. However, a reduction in the number of virions released and no retention of virions were observed.

Discussion

In this work we demonstrated that the effect of monensin on Mayaro virus replication differed in vertebrate (TC7) and invertebrate (*A. albopictus*) cells. The effect of monensin

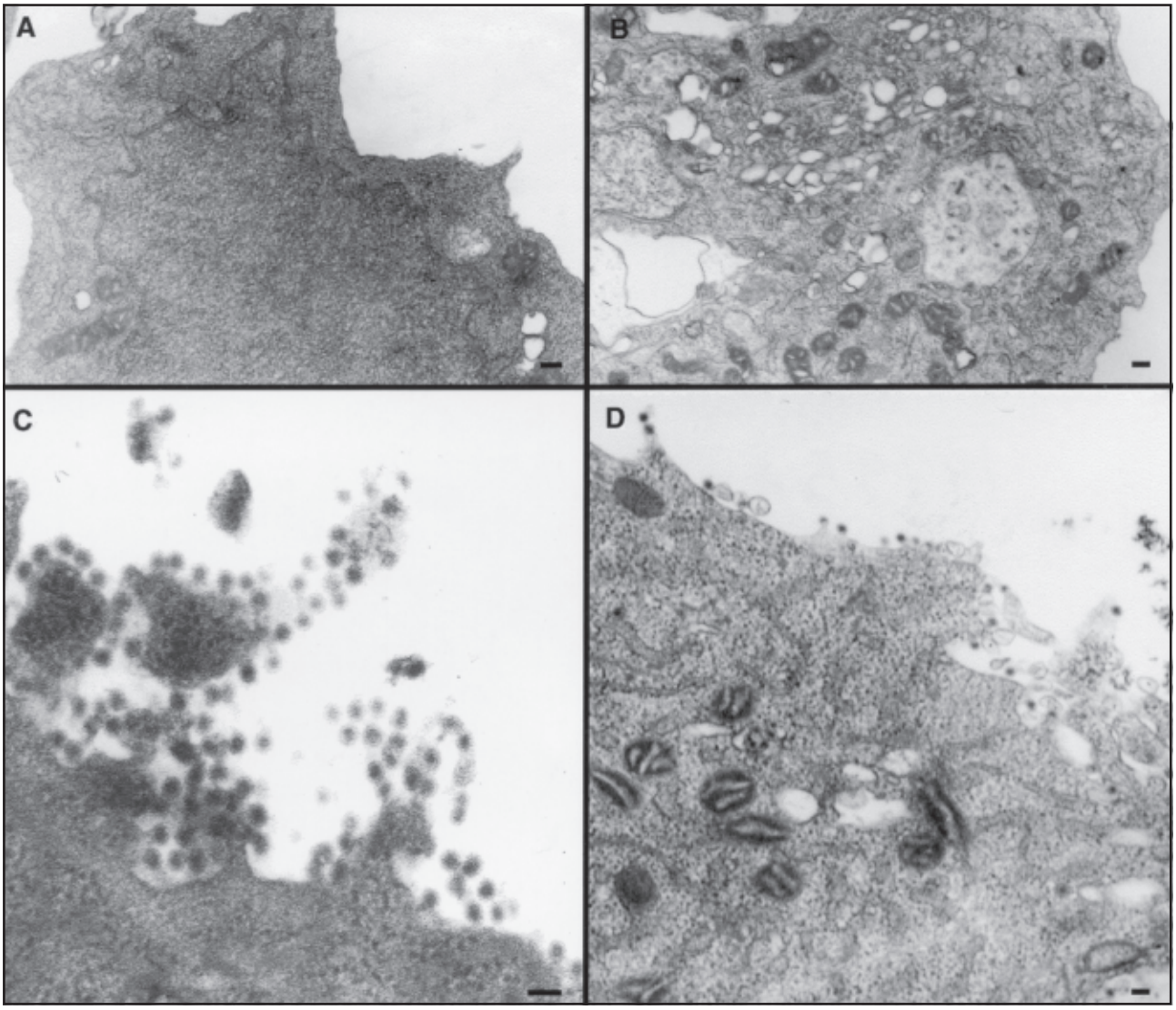

Fig. 3
Electron microscopy of *A. albopictus* cells

(A), mock infected cells, bar 200 nm. (B), cells treated with monensin, bar 200 nm. (C), infected cells, bar 100 nm. (D), cells infected and treated with monensin, bar 100 nm.

on the virus replication differs apparently in dependence on the cells tested. Johnson and Schlesinger (1980) have shown that 1 μmol/l concentration of monensin was enough to inhibit SINV or VSV replication in chicken embryo fibroblasts and in BHK cells. In both cases the yields of these viruses were reduced by 90%. Johnson and Spear (1982) have observed that 0.2 μmol/l monensin reduced yields of herpes simplex virus 1 in Hep2 and Vero cells by 15–30%. Toxic effects of monensin on cell cultures are not usually observed, although *A. albopictus* cells (clone C6/36) seem to be more sensitive to this drug and doses over 5 μmol/l proved to be toxic to these cells. Similar effects have been described for other cells such as HEP G2 (Hahn *et al*., 1992) and tumor Krebs II ascities cells (Contreras and Carrasco, 1979) showing that toxic effects of the drug vary in dependence on the cells.

In both cell lines studied here monensin reduced the yields of Mayaro virus. This inhibition was accentuated in TC7 cells, where monensin affected the virus morphogenesis. This finding is not surprising since the same phenomenon has been described for many other viruses (Johnson and Schlesinger, 1980; Johnson and Spear, 1982; Griffiths *et al*.,

1983). In vertebrate cells monensin exerts a variety of effects on maturation and release of Sindbis and Semliki Forest viruses, inhibiting virus budding from the cell surface. In addition, the transport of envelope glycoprotein precursors is totally or partially blocked from the medial to the trans-Golgi compartment. The drug prevents trimming of the high mannose oligosaccharides in SFV-infected BHK cells (Quinn *et al*., 1983). Johnson and Schlesinger (1980) have also reported that in SINV-infected BHK cells the proteolytic cleavage of the virus glycoprotein PE2 to E2 and E3 was inhibited by monensin, and that the movement of viral proteins from the Golgi apparatus to the cell surface was also impaired. This would explain the switch from budding at the plasma membrane level to budding into internal vesicles as observed for monensin-treated TC7 cells.

Monensin causes a transmembrane exchange of sodium ions for protons and consequently alters the pH in intracellular compartiments such as Golgi apparatus, lysosomes and endosomes. At the same time, the Golgi cisternae appear to swell, presumably due to osmotic uptake of water resulting from the inward movement of ions (Tartakoff, 1983; Griffiths, 1983). For influenza virus there are dramatic pH-induced changes in its structure. A conformational change in the hemagglutinin (HA) glycoprotein has been observed to occur at pH values corresponding to those optimal for the membrane fusion activity of the virus (Skehel *et al.*, 1982). The effect of monensin is positive for influenza virus. Treatment of virus-infected cells with monensin overcomes the effect of the amantadine block of the M_2 ion channel and keeps HA in its pH neutral form (Sugrue *et al.,* 1990). From these experiments it has been proposed that the M_2 ion channel equilibrates the pH of the lumen of the trans-Golgi network (TGN) with that of the cytoplasm, and that there is a similarity in the mechanism and the M_2 ion channel (Ciampor *et al.,*1992a,b; Grambas *et al.,* 1992; Sugrue *et al.,* 1990).

Apparently monensin does not interfere with the Mayaro virus morphogenesis in *A. albopictus* cells (clone C6/36). Differences in the morphogenesis of SINV in different *A. albopictus* cell clones have been observed (Miller and Brown, 1992). In *A. albopictus* cell clones C6/36 and C7-10, the virus matures both at the cell surface and in internal vesicles. In the clone U4.4, however, virus maturation appears to occur only in internal vesicular compartments.

The process of alphavirus assembly in vertebrate cells seems to be extremely rapid and efficient (Strauss and Strauss, 1994). Cleavage of P62 takes place between the TGN and the plasma membrane (Sariola *et al*., 1995). This explains the action of monensin in BHK-21 cells, which blocks SINV maturation and prevents the cleavage of protein PE2 to E2. In contrast, in invertebrate cells, the P62 processing starts early, probably in a pre-Golgi compartment (Naim and Koblet, 1990). If the routes of maturation and demands for processing the virus proteins differ in vertebrate and invertebrate (mosquito) cells, it could explain the apparent insensitivity to monensin of the virus-infected *A. albopictus* infected cells as described in this paper. We also could suggest that there are different pathways in vertebrate and invertebrate cells for transporting proteins to the plasma membrane, one of which would not be affected by monensin. This study points out that a better understanding in terms of intracellular transport and maturation of cellular proteins may help us in the comprehension of the Mayaro virus replicative cycle in vertebrate and invertebrate cells.

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