Critical role of the lipid rafts in caprine herpesvirus type 1 infection *in vitro*

Annamaria Pratelli*, Valeriana Colao

Department of Veterinary Medicine, University of Bari, Strada per Casamassima Km 3, 70010, Valenzano, Bari, Italy

ABSTRACT

The fusion machinery for herpesvirus entry in the host cells involves the interactions of viral glycoproteins with cellular receptors, although additional viral and cellular domains are required. Extensive areas of the plasma membrane surface consist of lipid rafts organized into cholesterol-rich microdomains involved in signal transduction, protein sorting, membrane transport and in many processes of viruses infection. Because of the extraction of cholesterol leads to disorganization of lipid microdomains and to dissociation of proteins bound to the lipid rafts, we investigated the effect of cholesterol depletion by methyl-β-cyclodextrin (MβCD) on caprine herpesvirus 1 (CpHV.1) in three important phases of virus infection such as binding, entry and post-entry. MβCD treatment did not prejudice virus binding to cells, while a dose-dependent reduction of the virus yield was observed at the virus entry stage, and 30 mM MβCD reduced infectivity evidently. Treatment of MDBK after virus entry revealed a moderate inhibitory effect suggesting that cholesterol is mainly required during virus entry rather than during the post-entry stage. Alteration of the envelope lipid composition affected virus entry and a noticeable reduction in virus infectivity was detected in the presence of 15 mM MβCD. Considering that the recognition of a host cell receptor is a crucial step in the start-up phase of infection, these data are essential for the study of CpHV.1 pathogenesis. To date virus receptors for CpHV.1 have not yet been identified and further investigations are required to state that MβCD treatment affects the expression of the viral receptors.

1. Introduction

Caprine herpesvirus 1 (CpHV.1) is associated with two different syndromes in goats, depending on the age of the animals at the time of infection. In 1- or 2-week-old kids, CpHV.1 may cause a generalized lethal disease affecting mainly the digestive tract. In adult goats, clinical manifestations involve the respiratory or the reproductive tract according to the site of virus entry, although CpHV.1 infects preferentially the genital mucosa. Following primary genital infection, CpHV.1 replicates in the mucosal epithelium and spreads to sacral ganglia where establishes latent infections (Tempesta et al., 1999). Members of the *Herpesviridae* form a large and various family of virus comprising three subfamilies designated alpha-, beta-, and gamma herpesviruses. Based on its antigenic and genetic properties, CpHV.1 belongs to the cluster of ruminant alpha-herpesviruses closely related to bovine herpesvirus 1 (BoHV.1) (Thiry et al., 2006). Virions are composed of a large DNA genome encased in an icosahedral capsid, which is in turn coated with a layer of proteins called the tegument, and in an envelope composed of about a dozen viral proteins and glycoproteins in a lipid bilayer. All herpesviruses have a relatively broad host range for cultured cells, at least for entry. The obvious interferences are that each of these viruses can use multiple cell surface receptors for entry or that each can recognize structural features of receptors conserved among human and animal species (Spear et al., 2000).

The basic fusion machinery for herpesvirus entry includes five viral glycoproteins, designed gB, gC, gD, gH, and gL, although additional viral binding-receptors may also be required, and at least four of these envelope glycoproteins (gB, gD, gH, and gL) are absolutely essential (Shukla and Spear, 2001; Spear and Longnecker, 2003). Most viral fusogens are not functional unless anchored in the membrane of the virion envelope or cell surface; changes in conformation that expose another hydrophobic domain, a fusion peptide, then enable its insertion into a target membrane.

The four essential glycoproteins are thought to act in concert to induce fusion of the viral envelope with a cell membrane. Cell entry receptors are required to trigger this fusion, and the viral ligand for all known Herpes Simplex Virus (HSV) entry receptors is gD. This summary of requirements for entry can be applies to animal alpha-herpesviruses as well, including pseudorabies virus (PRV)

* Corresponding author. Fax: +39 080 4679843.
E-mail address: annamaria.pratelli@uniba.it (A. Pratelli).

and BoHV.1. The molecule that HSV glycoproteins associated with is not necessarily a protein. It could be a lipid moiety since the virus can associate with lipid vesicles that contain no proteins (Bender et al., 2003; Spear et al., 2006). Most, but not all herpesviruses, a notable exception is Epstein Barr Virus (EBV), make their initial contact with cells by binding viral glycoproteins to glycosaminoglycans, usually heparan sulfate, on cell surface proteoglycans. This binding to heparan sulphate, that greatly increases the efficiency of viral entry, may not be essential for virus infection and can be mediated by viral glycoproteins. Shukla and Spear (2001) supposed that interactions of one or more viral glycoproteins with cellular receptors could trigger envelope-membrane fusion or cell-to-cell fusion. Based on these data several studies were focused on the identification of the viral and cellular domains involved in the interaction of virus and cell. For both alpha-herpesviruses and gamma-herpesviruses, binding to cells can be mediated by a virion glycoprotein that is not essential for entry. Entry requires interaction of a viral ligand with another cell surface receptor. The entry receptors discovered to date fall into three classes (Spear et al., 2000). They include herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor family (nectin-1 and nectin-2), two members of the immunoglobulin superfamily, and specific sites in heparan sulfate generated by certain isoforms of 3-O-sulfotransferases (Spear et al., 2000). For HSV, virion gD is the ligand for several cell surface receptors (HVEM, nectins, 3-O-sulfated heparan sulfate), any one of which can mediate entry. The entry process has been studied in detail in many herpesviruses, and in particular the interaction of HSV and PRV with cellular receptors. Spear and Longnecker (2003) have analyzed the interaction of HSV gD with an entry receptor to justify viral entry or cell fusion. The binding of gD to one of its receptors results in a conformational change in gD, enabling its interaction with gB or gH-gL and activation of fusogenic activity. Probably gD is not an integral component of the basic fusion machinery. Moreover, receptors for gB and/or gH-gL may exist, and binding of either to these receptors could also trigger fusion activity, by-passing the requirement for gD. These observations emerge in part from results obtained with PRV, that can use several human and animal members of the nectin family as entry receptors. Also, one of the gamma-herpesviruses, Human Herpesvirus 8 (HHV-8), has been shown to require only gB, gH, and gL for cell fusion, although the possible potentiating activity of other viral proteins has not been ruled out (Pertel, 2002). The entry receptors in epithelial cells have not yet been identified but could include integrins. The viral ligands could be gH and/or BMRF2. For HHV-8 entry, gB can bind to one of the integrins. Any one of these interactions of a viral ligand with an entry receptor is thought to activate the fusion activity of gB and gH-gL (Spear and Longnecker, 2003).

Extensive areas of the plasma membrane surface consist of lipid rafts. The "lipid raft hypothesis" proposes that cell membranes are organized into distinct cholesterol-rich microdomains that are important for signal transduction, protein sorting, and membrane transport (Brown and London, 1998; Ikonen, 2001; Chung et al., 2005). Lipid rafts, enriched in glycosphingolipids, sphingomyelin, cholesterol and associated proteins, play a critical role in different biological aspects of the life cycle of different viruses and in particular, they are involved in many processes of viruses infection (Pratelli and Colao, 2015). The tight packaging of the sphingolipids is maintained by the presence of cholesterol, a major constituent of the lipid rafts, and several proteins partition into these membrane domains (Imhoff et al., 2007). Since lipid rafts are rich in sphingolipid and cholesterol, they are resistant to detergent extraction, which is usually the method of choice to separate physically lipid rafts from other membrane components. On the contrary, the methyl-β-cyclodextrin (MβCD), a derivative of a cyclic oligomer of glucose with a lipophilic property (Pitha et al., 1988), extracts cholesterol out of membranes, disrupts lipid raft formation on cells, and consequently blocks biological processes that depend on lipid rafts (Chung et al., 2005; Pratelli and Colao, 2015).

Since cholesterol-enriched cell and viral membranes are involved in the entry process of several enveloped viruses, we tested the importance of cholesterol on plasma membranes for CpHV.1 entry into mammalian cells using MβCD as cholesterol sequestering drug.

2. Materials and methods

2.1. Cells and virus

The Madin Darby Bovine Kidney (MDBK) cell line derived from a kidney of an apparently normal adult steer (Madin and Darby, 1958). The cells were maintained in Dulbecco Minimal Essential Medium (DMEM) supplemented with 5% foetal calf serum (FCS) and passaged twice a week. The strain Ba-1 (Buonavoglia et al., 1996) of CpHV-1 was used throughout the experiments. The virus was propagated on MDBK cell lines and grown in serum-free medium where the cytophatic effects was detected after 48 h. The most characteristic changes were the development of both enlarged cells, take on a retractile appearance, and syncytia with a variable degree of vacuolation. Rounded and stellate cells might also be seen. The viral titre was determined in 96-well micro-titration plates with MDBK cells and was expressed as 50% tissue culture infectious doses ($TCID_{50}/50\,\mu l$) calculated using the Reed–Muench formula (Reed and Muench, 1938). The infectivity titre of the stock virus was $10^{6.25}\ TCID_{50}/50\,\mu l$.

2.2. Cholesterol sequestering drug and exogenous cholesterol

The MβCD (C4555, Sigma–Aldrich) is a strictly surface-acting drug and in preference to other membrane lipids, it can remove cholesterol selectively and rapidly from the plasma membrane (Barman and Nayak, 2007). Cyclodextrins (CDs) are a family of cyclic oligomers of glucose, that differ in the number of glucose units in the ring, i.e. they are composed of six, seven or eight glucose units in α-, βor γ-CD, respectively (Beseničar et al., 2008). They are highly hydrophilic molecules that can bind and thereby solubilize hydrophobic molecules (Irie et al., 1992). Derivatives of CDs, which are more soluble and less toxic, were in use in pharmacological research for years as carriers of lipophilic drugs and more recently were employed in studying the effect of both cholesterol depletion and lipid raft disassembly. β-CD and its derivative, methyl-β-CD (MβCD) are mostly chosen for these studies as it extracts membrane cholesterol very efficiently and only negligibly binds other lipids (Beseničar et al., 2008).

In order to avoid toxic effect for the cellular system employed, the adequate time of treatment and range concentration were evaluated by a cell viability assay as previously reported (Carro and Damonte, 2013). Briefly, MDBK cells grown in 24-well plates were incubated for 1 h in serum-free DMEM containing or not MβCD up to a concentration of 30 mM, and the number of viable cells were then determined by Trypan blue exclusion. MDBK cells mock treated were used as control. According to the data obtained, the treatment conditions were chosen and to remove the plasma membrane cholesterol from both MDBK cells and CpHV.1, concentrations of 6, 10, 15, 20, 30 mM of MβCD dissolved in DMEM were prepared.

Water-soluble cholesterol (C4951, Sigma–Aldrich) was employed to replenish cholesterol from cellular and viral plasma membranes after extraction using MβCD.

2.3. Measurements of cellular and viral cholesterol levels

Cellular and viral cholesterol were measured using the Amplex® Red Cholesterol Assay Kit (A12216, Invitrogen/Life Technologies) according to manufacturer instructions, as reported by Pratelli and Colao (2015).

For cellular cholesterol determination, confluent monolayer of MDBK grown on six-well plate containing approximately equal cell number were treated with different concentrations of MβCD ranging from 10 to 30 mM. At the same time, monolayers of MDBK cells in six-well plate pre-treated with 30 mM of MβCD, were replenished with various concentrations of exogenous cholesterol ranging from 400 to 800 μg/ml. All the monolayers were then washed three times with DMEM, trypsinised with EDTA, centrifuged at $800 \times g$ at 4 °C for 5 min to remove cellular debris and the pellets were suspended in 50 μl of reaction buffer and homogenized through a 25-gage needle. The cellular cholesterol concentration was determined three times with the Amplex® Red Cholesterol Assay Kit using a spectrofluorometer Victor X3 (PerkinElmer) at 545 nm excitation and 572 nm emission wavelengths. Non-treated MDBK cells were used as control.

To determine viral cholesterol, viral samples containing $10^{6.25}$ $TCID_{50}$/50 μl each of MβCD-treated (6–20 mM) and untreated viral suspensions were centrifuged at $800 \times g$ at 4 °C for 5 min to remove cellular debris and then ultra centrifuged at $10,000 \times g$ at +4 °C for 1 h. In parallel a viral suspension from the same stock virus was pre-treated with 15 mM of MβCD, then replenished with exogenous water-soluble cholesterol by applying final concentrations ranging from 400 to 800 μg/ml and centrifuged as reported. All the pellets were suspended in 50 μl of reaction buffer, homogenized and subjected to cholesterol determination three times with the Amplex® Red Cholesterol Assay Kit as above. MDBK was grown in serum-free medium to avoid that cholesterol measurement was affected by serum cholesterol.

2.4. MβCD treatment of MDBK cells and effect on the virus binding

To recognize the stage at which cell membrane cholesterol was required for CpHV.1 infection, the effect of MβCD treatment on virus binding was evaluated, according to the protocol of Zhu et al. (2010). MDBK cells grown on six-well plates were mock treated or treated with 30 mM MβCD respectively for 30 min at 37 °C. The cells were then washed twice with ice-cold DMEM and infected with 1000 $TCID_{50}$/50 μl CpHV.1 in ice-cold medium. The plates were incubated for 1 h at +4 °C to allow virus attaching to the cells and after extensive washing with DMEM, the plates were frozen and thawed three times. Cell-associated CpHV.1 was then titrated in 96-microplate with MDBK cells as described above. The experiment was repeated three times.

2.5. Depletion and replenishment of cell membrane cholesterol and effect on the virus entry

To investigate the effect of cholesterol depletion on the virus entry, MDBK cells grown in 24-well plates were washed with DMEM and mock pre-treated or pre-treated with MβCD at the concentrations of 6, 10, 15, 20, 30 mM for 30 min at 37 °C. Thereafter the cells were washed with DMEM to remove the drug, inoculated with 50 $TCID_{50}$/50 μl and incubated for 1 h at 37 °C. The inocula were then discarded and, after treatment with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for exactly 1 min to inactivate cell membrane bound but un-penetrated virions as previously described (Chung et al., 2005; Zhu et al., 2010), the cells were further incubated at 37 °C in DMEM without compounds. At 48 h post infection the plates were frozen and thawed three time

and virus yield was determined with virus titration assay in MDBK cells as described above.

For cholesterol replenishment experiments, monolayers of MDBK in 24-well plates mock pre-treated or pre-treated with 30 mM MβCD for 30 min at 37 °C as reported above, were mock supplemented or supplemented with different concentration of exogenous cholesterol ranging from 400 to 800 μg/ml in DMEM and incubated for 1 h at 37 °C (Pratelli and Colao, 2015). Mock cells were replenished with serum-free DMEM. The cells were then washed twice, infected with CpHV.1 for 1 h at 37 °C, treated with citrate buffer and then incubated for 48 h in a CO_2 incubator. The plates were then frozen and thawed three time and virus yield was determined with virus titration assay in MDBK cells as described above. All the experiments were repeated three times.

2.6. Depletion of cell membrane cholesterol and effect at the post-entry stage on virus replication

The effect of cell membrane cholesterol depletion at the post-entry stage on CpHV.1 replication was evaluated as reported by Zhu et al. (2010). MDBK cells seeded in 24-well plates were pre-incubated with 50 $TCID_{50}$/50 μl CpHV.1 for 1 h in a CO_2 incubator. After treatment with citrate buffer, cells were treated with 30 mM MβCD for 30 min at 37 °C (test 1). Two controls were prepared. In the control 1, cells were mock treated before virus infection. In the control 2, cells were pre-treated with 30 mM MβCD and then infected with 50 $TCID_{50}$/50 μl CpHV.1 for 1 h. Both controls were treated with citrate buffer after infection and test 1 and controls were further incubated for 48 h to determine the virus yield with virus titration assay, after extensive freezing and thawing. The experiment was repeated three times.

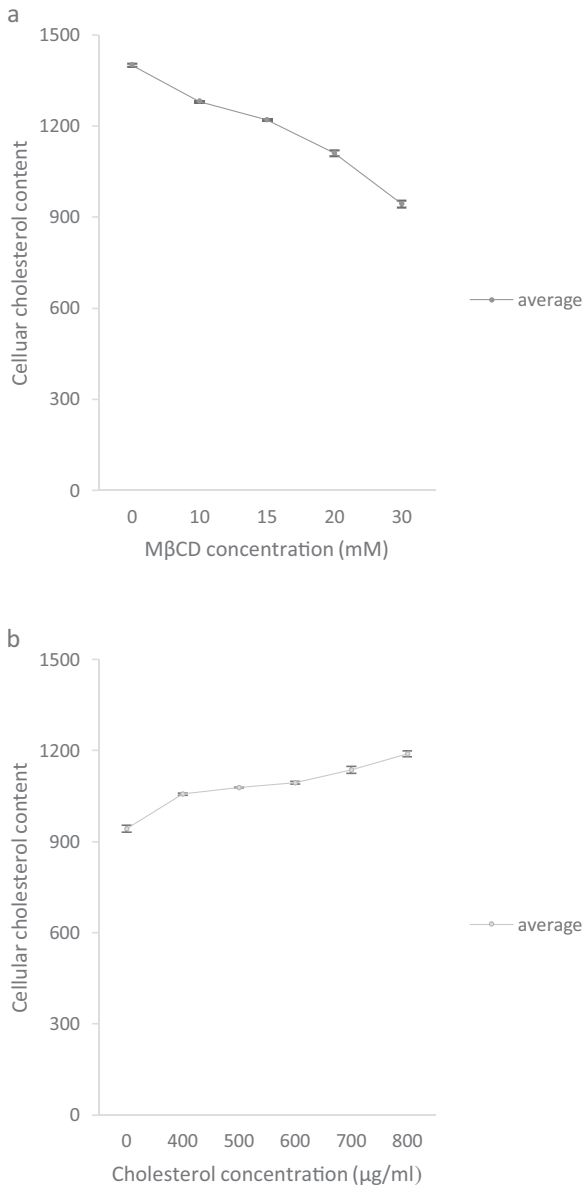## 2.7. Depletion and replenishment of cholesterol from viral envelope and effect on infectivity

To investigate the functional role of cholesterol in the viral envelope for CpHV.1 infectivity, experimental depletion of cholesterol by MβCD was carried out from viral suspensions. For cholesterol extraction, 1000 $TCID_{50}$/50 μl CpHV.1 were incubated with MβCD at the concentration of 6, 10, 15, 20 mM for 30 min at 37 °C. Virus was then pelleted by centrifugation at $10,000 \times g$ for 1 h at +4 °C to remove MβCD and suspended in DMEM. The control was mock treated and pelleted as reported. Virus infectivity was determined in 96-microplate wells with MDBK cells as described above.

Furthermore, to verify whether the effect of cholesterol depletion was reversible, exogenous cholesterol was employed to replenish viral envelope. CpHV.1 was mock treated or treated with 15 mM MβCD for 30 min at 37 °C and the supernatant was removed by ultra centrifugation as described. Virus pellets were suspended with medium or medium containing exogenous cholesterol ranging from 400 to 600 μg/ml, incubated for 1 h at 37 °C then titrated by virus titration assay in 96-microplate wells. The experiment was repeated three times.

3. Results

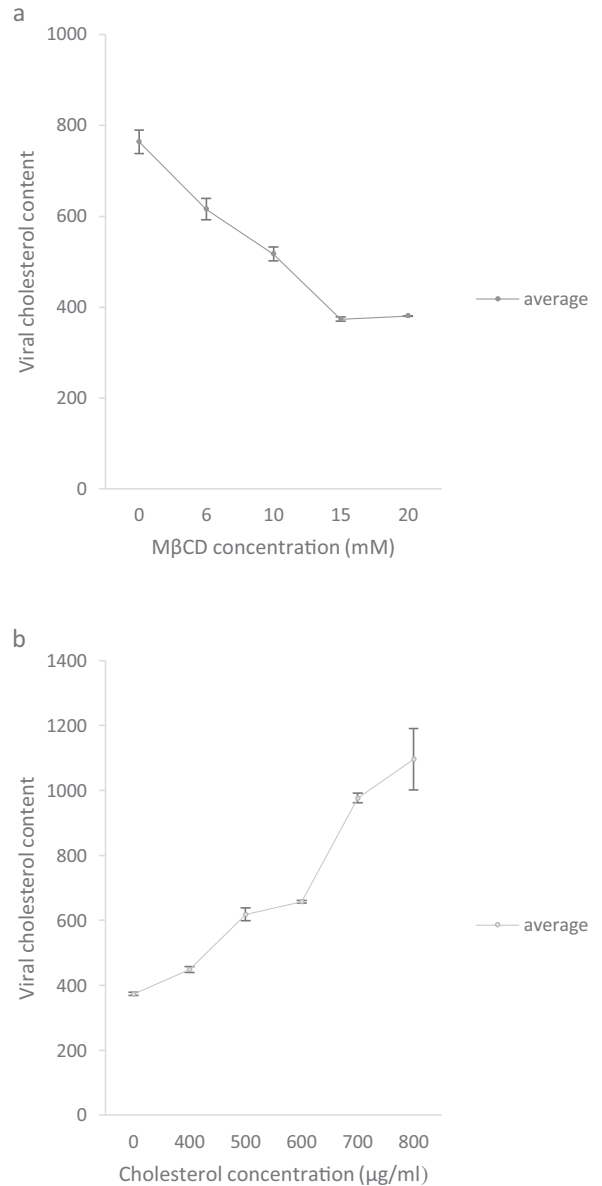### 3.1. Cellular and viral cholesterol measurement

The cholesterol content from both cellular and viral plasma membrane was determined using a cholesterol assay kit. MβCD-treated and untreated MDBK cells were compared. MβCD treatment resulted in a dose-dependent reduction of the cholesterol content and after incubation with 30 mM MβCD, cholesterol content endured a noticeable reduction in comparison to untreated cells (Fig. 1a). MDBK pre-treated with 30 mM of MβCD were analyzed after cholesterol replenishment in increasing amounts.

Fig. 1. Cellular cholesterol determined with a cholesterol assay kit. (a) MDBK cells were treated with different concentration of MβCD and cholesterol was determined in comparison to untreated cells. MβCD treatment resulted in a dose-dependent reduction of the cholesterol content. (b) MDBK cells pre-treated with 30 mM MβCD were tested after cholesterol replenishment in increasing amounts. Exogenous cholesterol partially recovered cellular cholesterol.
Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.
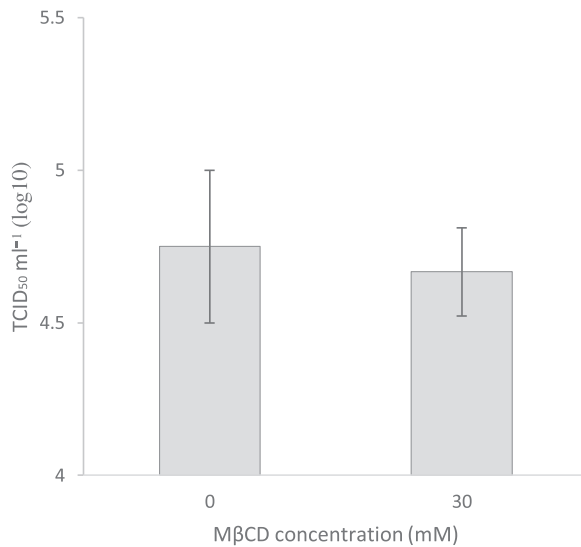

Fig. 2. Viral cholesterol determined with a cholesterol assay kit. (a) Viral suspensions were treated with 6–20 mM MβCD and the cholesterol content in MβCD-treated and untreated CpHV.1 suspensions was determined. A dose-dependent decrease was observed and 20 mM MβCD reduced viral cholesterol. (b) Cholesterol depleted virions were replenished with exogenous cholesterol in increasing amounts. Cholesterol content was partially recovered.
Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.

As shown in Fig. 1b, 800 μg/ml of exogenous cholesterol restore cholesterol values of the cellular membranes nearly to the values determined prior to MβCD treatment.

The cholesterol content in MβCD-treated and untreated CpHV.1 suspensions was determined. Increasing drug concentrations resulted in a dose-dependent decrease of cholesterol content from viral membrane and 20 mM MβCD reduced evidently the viral cholesterol compared to the amount found in untreated virions (Fig. 2a). Cholesterol depleted virions were replenished with exogenous cholesterol in increasing amounts and virus pellets were used for cholesterol measurements. Exogenous cholesterol restored viral cholesterol nearly to the values determined prior to MβCD treatment (Fig. 2b).

The maximum reduction in total cellular cholesterol is to about 70% of its normal level, whereas the maximum reduction in viral cholesterol is to about 45%. The difference is presumably due to the effect of MβCD being limited to the plasma membrane, such that cholesterol is not removed from intracellular membranes.

3.2. Cell membrane cholesterol depletion and effect on the virus binding

Adverse effects of MβCD that might undermine virus infection independently of cholesterol reduction were valuated by measuring cell viability after drug treatment. No detrimental effects were detected by both Trypan blue staining and cell count after 1 h MβCD treatment up to a concentration of 30 mM (data not shown).

Fig. 3. Cell membrane cholesterol depletion at the virus-binding phase. MDBK cells were mock treated or treated with 30 mM MβCD, infected with CpHV.1 in ice-cold medium and then titrated. Cholesterol depletion did not affect CpHV.1 binding to target cells.

Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.

CpHV.1 was employed to infect mock treated and treated MDBK cells for 1 h at +4 °C to verify if cellular membrane cholesterol depletion was essential for CpHV.1 binding to the target cells. Cells were then cultured for virus yield determination with virus titration assay. As reported in Fig. 3, no evident decrease in virus binding to MβCD-treated MDBK cells compared to the mock treated cells was observed.
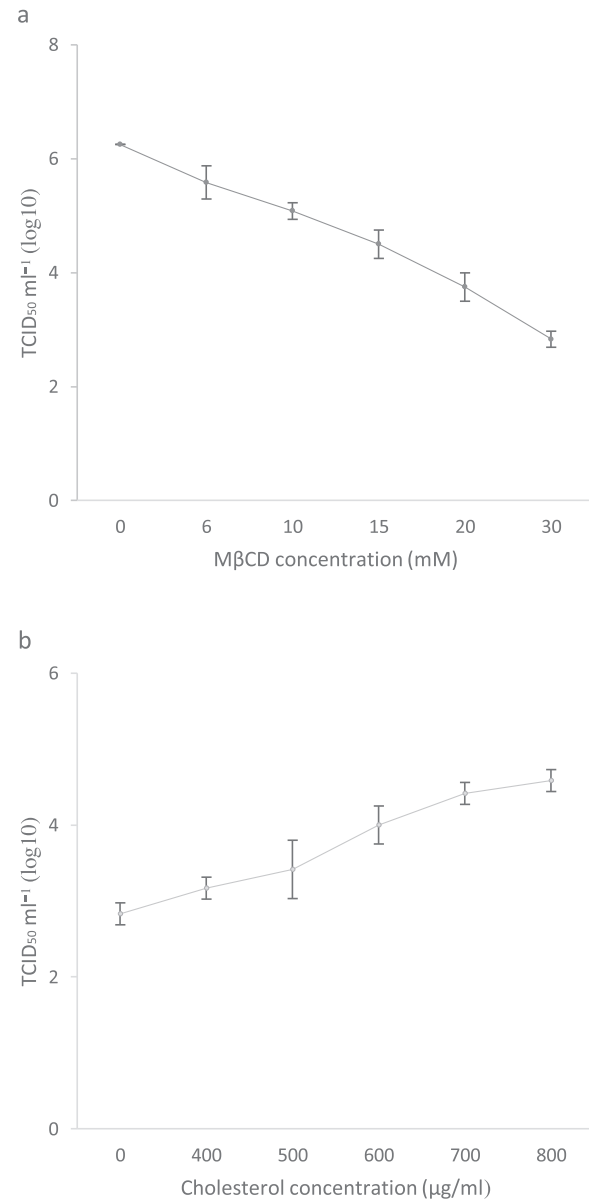
3.3. Pre-treatment of MDBK cells with MβCD and effects on CpHV.1 entry

To investigate if cholesterol on the target cell surface was essential for CpHV.1 entry, MDBK cells were mock pre-treated or pre-treated with non-cytotoxic concentrations of MβCD, and infected with CpHV.1 followed by treatment with citrate buffer to remove the cell bound but un-penetrated virions. Virus yield was determined with virus titration assay. As shown in Fig. 4a, the efficiency of CpHV.1 infection in MDBK cells was compromised by MβCD treatment in a dose-dependent manner. At a concentration of 30 mM the reduction of the infection rate was evident compared to the mock treated control cells and 30 mM was selected and considered appropriate concentration with negligible cell toxicity and efficient cholesterol depletion.

To confirm that the inhibitory effects on CpHV.1 entry in MDBK cells were due to cholesterol depletion, cell membrane cholesterol was replenished with exogenous cholesterol. MDBK cells were first treated or mock treated with MβCD, then exogenous cholesterol ranging from 400 to 800 μg/ml was used for the replenishment and the virus yield was determined with virus titration assay. The inhibitory effect was partially reversed by cholesterol replenishment with 700 μg/ml exogenous cholesterol (Fig. 4b).

3.4. Cell membrane cholesterol depletion at the post-entry stage and effect on CpHV.1 replication

Cell membrane cholesterol depletion at the post-entry stage partially affects virus production. For this purpose, MDBK cells were infected with CpHV.1 for 1 h and, after cell-bound virus inactivation, the cells were treated with 30 mM MβCD for 30 min (test 1) and
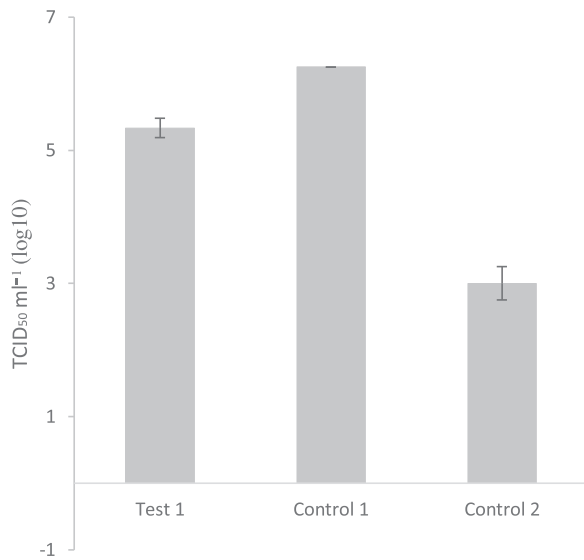

Fig. 4. Depletion (a) and replenishment (b) of cell membrane cholesterol at the virus entry stage. (a) MDBK cells were pre-treated with 6–30 mM MβCD then incubated with CpHV.1. Virus yield was determined with virus titration assay. Cholesterol depletion at the virus entry stage reduced the efficiency of CpHV.1 infection in a dose-dependent manner. b) MDBK depleted cells were supplemented with 400–800 μg/ml cholesterol then incubated with CpHV.1 and titrated. In the cholesterol depleted cells replenished with exogenous cholesterol, the inhibitory effect was partially restored.

Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.

incubated for 48 h. Test 1 compared to the treatment before virus entry (control 2) revealed a moderate but noticeable inhibitory effect, anyhow suggesting that cholesterol was mainly involved during CpHV.1 entry in MDBK cells rather than during the post-entry stage (Fig. 5).

3.5. Viral cholesterol depletion depress CpHV.1 infectivity

The importance of viral cholesterol for CpHV.1 infectivity was analysed by cholesterol depletion from viral envelope. CpHV.1 suspensions were incubated with 6–20 mM MβCD for 30 min at 37 °C or mock treated. The drug was then removed from each suspension by ultracentrifugation and the infectivity was determined by
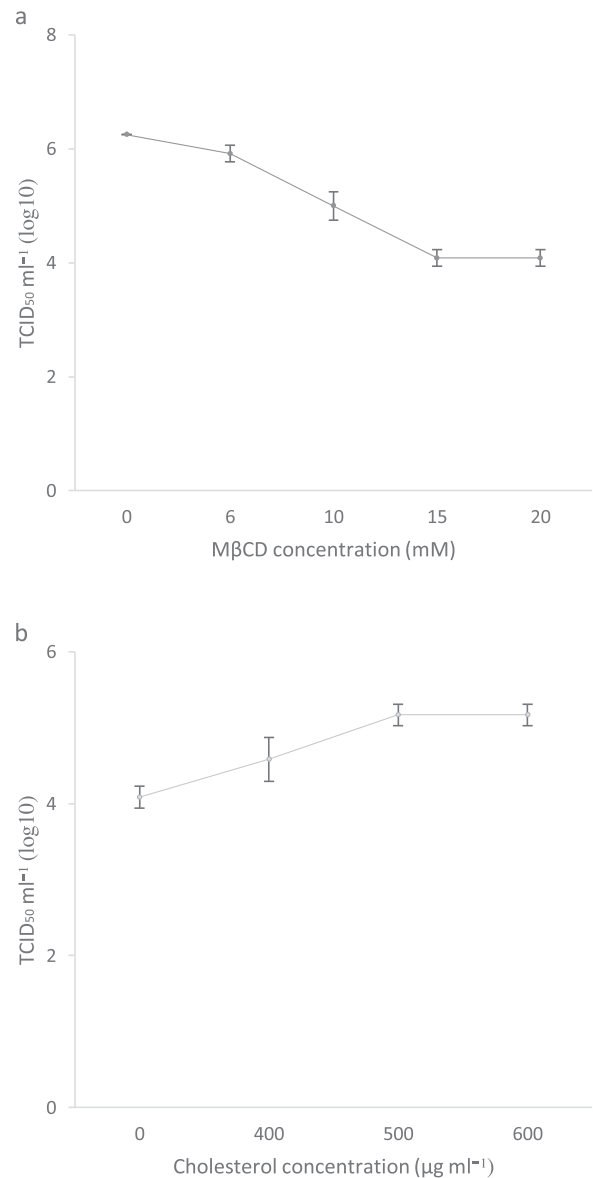
Fig. 5. Cholesterol depletion from cell membrane at the post-entry stage. MDBK cells were infected with CpHV.1 and then treated with 30 mM MβCD (test 1). In the control 1 and in the control 2 MDBK cells were mock treated and treated with 30 mM MβCD before virus infection. Cholesterol depletion at the post-entry stage had only a mild inhibitory effect on CpHV.1 production.
Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.

virus titration assay. As showed in Fig. 6a, the exposure of CpHV.1 to MβCD resulted in a dose-dependent inhibitory effect on the virus infectivity and at a concentration of 15 mM MβCD, virus yield suffered a noticeable reduction. The inhibitory effect of MβCD on the viral envelope was partially restored by cholesterol replenishment. The data reported in Fig. 6b demonstrated that 500 μg/ml of exogenous cholesterol re-established partially virus infectivity, confirming that CpHV.1 envelope cholesterol was also required for virus infectivity.

4. Discussion

Lipid rafts are involved in the regulation of different biological events, including membrane transport and signal transduction pathways. The involvement of these specific membrane microdomains enriched in cholesterol and (glyco) shingolipids in virus entry, assembly and budding was demonstrated both through the localization of the viral structural proteins and through the effects of lipid rafts-disrupting agents in the replication processes of several viruses (Suzuki and Suzuki, 2006). Recent studies demonstrated that lipid rafts play a critical role in the process of viral infection. The involvement of membrane lipid rafts in the entry of non-enveloped viruses was demonstrated for simian virus 40 (SV40), rotavirus, echovirus type 1, enterovirus, rhinovirus and human adenovirus subgroup C (Suzuki and Suzuki, 2006). The essential role of the cholesterol in the replication cycle was also clearly established for many enveloped viruses, and accumulating evidences suggest that enveloped virus entry may require cholesterol in either of the two membrane involved, or in both (Pratelli and Colao, 2015). Cholesterol in both cellular and viral membranes is required for BoHV.1 infection (Zhu et al., 2010), and cell membrane cholesterol is required during virus entry for other viruses in the alfa-herpesvirus subfamily. In particular, HSV entry was inhibited by cholesterol sequestering drug in a dose-dependent manner, and the inhibitory effect was fully reversible by cholesterol replenishment (Bender et al., 2003; Rahn et al., 2011). Desplanques et al. (2008) demonstrated that cholesterol is important for PRV infectivity and stability. Infectivity of Varicella-Zoster Virus (VZV) was

Fig. 6. Cholesterol depletion (a) and replenishment (b) from viral envelope. a) CpHV.1 suspensions containing $10^{6.25}$ TCID$_{50}$/50 μl were treated with 6–20 mM MβCD or mock treated. MβCD treatment resulted in a dose dependent inhibitory effect. b) CpHV.1 suspensions were mock pre-treatment or pre-treatment of with 15 mM MβCD, then mock replenished or replenished with 400–600 μg/ml exogenous cholesterol. Cholesterol replenishment partially re-established virus infectivity.
Experiments were repeated three times and the error bars indicate the standard deviations of the two independent experiments.

sensitive to agents that disrupt lipid rafts by removing cholesterol in a dose-dependent manner and exerted these effects on both target cell and viral membranes. No effect of cholesterol depletion, however, was seen on viral binding (Hambleton et al., 2007). Inhibitor studies further support a role for the fusion process during Human Herpesvirus 6 (HHV-6) entry, suggesting that the cholesterol present in the viral envelope is a key component in viral entry (Huang et al., 2006). Comparative analysis of the lipid compositions of Cyprinus herpesvirus 3 (CyHV-3), a member of the new *Alloherpesviridae* virus family in the *Herpesvirales* order, and common carp brain (CCB) membrane fractions revealed strong similarities between the lipid composition of the CyHV-3 and CCB lipid rafts. A recent study demonstrated that cholesterol-rich lipid rafts are

important for the CyHV-3 replication cycle especially during entry and egress (Brogden et al., 2015).

In the present study, the role of lipid rafts in CpHV.1 infection of MDBK cells was evaluated. Through separate analysis of three important phases of virus infection such as binding, entry and post-entry, a different requirement of cholesterol in cellular and viral membranes for a productive CpHV.1 infection was observed. Interestingly, the cholesterol sequestering drug MβCD under the experimental conditions employed, did not prejudice CpHV.1 binding to MDBK cells. On the contrary, cell membrane cholesterol was required for CpHV.1 replication at the virus entry phase, and a dose-dependent reduction of the virus yield was observed after MβCD treatment. To corroborate that the decrease in the infectivity was the results of the cholesterol depletion, plasma membrane was replenished with exogenous cholesterol and the inhibitory effect was reversed. The requirement for host cholesterol during post-entry step was also evaluated and MDBK cells were treated with cholesterol sequestering drug after virus entry. The analysis revealed that virus replication was inhibited, but comparing this reduction with control 2 (cells pre-treated with MβCD and then infected) the effect was moderate, suggesting that cholesterol is mainly required during virus entry rather than during the post-entry stage.

Virus cholesterol depletion resulted in a reduction of infectivity for several enveloped viruses belonging to quite different viral families: Human Immunodeficiency Virus (HIV) (Guyader et al., 2002), BoHV.1 (Zhu et al., 2010), Human Herpesvirus 6 (HHV-6) (Huang et al., 2006), VZV (Hambleton et al., 2007), PRV (Desplanques et al., 2008), Transmissible Gastroenteritis Virus (TGEV) (Ren et al., 2008), Canine Coronavirus (CCoV) (Pratelli and Colao, 2015), influenza virus (Sun and Whittaker, 2003), Duck Hepatitis B Virus (DHBV) (Funk et al., 2008), Canine Distemper Virus (CDV) (Imhoff et al., 2007), Dengue Virus (DENV) (Carro and Damonte, 2013), Hepatitis C Virus (HCV) (Aizaki et al., 2008) and Lassa virus (Schlie et al., 2010). CpHV.1 was susceptible to the inactivating effect of MβCD treatment in a dose-dependent manner and the loss of infectivity was reversed with cholesterol replenishment confirming that cholesterol depletion was responsible for the decrease of CpHV.1 infectivity.

The susceptibility of CpHV.1 infectivity to MβCD treatment may be related to the modality of envelope development. Enveloped viruses assemble their capsids in the cytoplasm and then become enveloped by budding from the plasma membrane or into cytoplasmic membranes. Viruses that assemble their capsids in the nucleus have the fundamental problem of transporting capsids across the nuclear membrane. Relatively small non-enveloped viruses, such as polyomaviruses, can move through nuclear pores, while larger non-enveloped viruses, such as adenoviruses, disrupt the nuclear envelope. Herpesvirus capsids are too large to move through nuclear pores and, consequently developed a rather complex process of maturation. The virus promotes primary envelopment of capsids by budding at the inner leaflet of the nuclear membrane and translocation of capsids into the cytoplasm after loss of the primary envelope by fusion with the outer leaflet of the nuclear membrane. Once released in the cytoplasm, non-enveloped capsids acquire a secondary envelope by budding into vesicles of the trans-Golgi network and these mature virions are then secreted from cells (Mettenleiter, 2002). However, the intracellular distribution of cholesterol is not uniform, with a major fraction displayed in the plasma membrane (Lange, 1991), resulting in part from its close association with sphingomyelin.

Another point of reflection arises from the results reported in the present study. The cholesterol-sequestering drug inhibited CpHV.1 infection, underlining the requirement for both host and viral cholesterol. As observed for HIV.1 (Popik et al., 2002) it is possible to suppose that virus and/or cellular receptors are

associated with lipid rafts. Recognition of a host cell receptor by a virus is the first and perhaps the most crucial step in initiating the disease process. The cellular receptors of several enveloped and non-enveloped viruses was identified and most of these receptors are glycoproteins, although viruses can use lipids or carbohydrates. Heparan sulfate and the new receptors identified by expression cloning will be referred to as coreceptors for entry because, in general, alpha-herpesvirus entry is facilitated by, if not dependent on, interactions of viral proteins with at least two, perhaps more, cell surface receptors (Spear et al., 2000). For instance, alteration of the lipid composition of the plasma membrane can modulate CD81-dependent HCV entry into host cells. Consequently, the depletion of cholesterol from the plasma membrane or the alteration of the sphingomyelin/ceramide ratio of the plasma membrane, affects HCV entry by reducing the cell surface expression of the tight-junction protein CD81 (Kapadia et al., 2007; Popescu et al., 2014). Unfortunately, virus receptors for CpHV.1 have not yet been identified. Until their identification, it is premature to state that MβCD treatment affected the interaction between CpHV.1 and the cellular receptors or affected the expression of the receptors, but it could be a suitable and assessable theory to support in the future.

Acknowledgements

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