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ORIGINAL ARTICLE

Encephalomyocarditis virus may use different pathways to initiate infection of primary human cardiomyocytes

Saliha Hammoumi · Monique Guy · Marc Eloit · Labib Bakkali-Kassimi

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Abstract Encephalomyocarditis virus (EMCV) can infect a wide range of vertebrate species including swine and non-human primates, but few data are available for humans. We therefore wanted to gain further insight into the mechanisms involved in EMCV infection of human cells. For this purpose, we analyzed the permissiveness of primary human cardiomyocytes towards two strains of EMCV; a pig myocardial strain (B279/95) and a rat strain (1086C). In this study, we show that both strains productively infect primary human cardiomyocytes and induce complete cytolysis. Binding and infection inhibition experiments indicated that attachment and infection are independent of sialic acid and heparan sulfate for B279/95 and dependent for 1086C. Sequence comparison between the two strains and three-dimensional analysis of the capsid revealed that six of the seven variable residues are surface-exposed, suggesting a role for these amino acids in binding. Moreover, analysis of variants isolated from the 1086C strain revealed the importance of lysine 231 of VP1 in the attachment of EMCV to cell-surface sialic acid residues. Together, these results show a potential for EMCV strains to use at least two different binding possibilities to initiate infection and provide new insights into the mechanisms involved in primary human cell recognition by EMCV.

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Introduction

Encephalomyocarditis virus is a single-stranded positive-polarity RNA cardiovirus belonging to the family *Picornaviridae*. It can naturally infect a wide range of vertebrate species including swine and non-human primates [1, 11, 13, 16, 26], reptiles and insects [9]. Rodents are considered to be natural hosts of EMCV and are thought to be the primary reservoir and disseminators of the virus [28]. The primary route of transmission for EMCV is believed to be fecal-oral [33]. Transplacental vertical transmission is reported to occur naturally in swine and baboon [13]. Whether such infections remain asymptomatic or produce clinical symptoms depends on many factors, including the virus strain, the infected species, the viral dose, and the age and immune status of the infected animal. EMCV infection causes acute myocarditis and sudden death in piglets, and reproductive failure in sows [19, 21]. Infection of older pigs is asymptomatic. While it is generally asymptomatic in rodents, EMCV can cause insulin-dependent diabetes mellitus and nervous disorders in mice [6]. The virus has been isolated from various organs, including heart, tonsils, kidney, lungs, liver, spleen, small intestine and mesenteric lymph nodes, from naturally and experimentally infected animals [9, 24]. The target organ involved in the infection is also dependent on the species and viral strain.

EMCV has rarely been recognized as a cause of human illness. Strains of EMCV have been reported from children suffering from aseptic meningitis, poliomyelitis-like disease and Guillain-Barré syndrome, although a causal relationship between EMCV and the symptoms has not been established [10]. An accidental infection by Mengo virus occurred in a man who cared for a paralyzed cynomolgus macaque. The patient developed signs of acute encephalitis, and virus was recovered from his

serum [9]. Several serological studies have shown the presence of antibodies against EMCV in the healthy human population, suggesting that human EMCV infection is relatively common, but most cases are asymptomatic or unrecognized [7, 17, 30]. In vitro studies have shown that primary human cardiomyocytes are permissive to an EMCV MN-30 strain previously isolated from an aborted pig foetus. On the other hand, human renal epithelial cells, aortic endothelial cells, bone marrow progenitor cells, peripheral blood mononuclear cells and hepatocytes were non-permissive to this strain [4]. Other reports have described ultrastructural alterations of cultured human fetal pancreatic cells or of tissue cultures from human foetal brain infected with two variants of EMCV (M and E) [2, 31]. However, a difference in virulence between the two variants has been observed. Indeed, 100 plaque-forming units (PFU) were sufficient to induced alterations of pancreatic cells with the M variant while 10 000 PFU were needed with the E variant to induce similar alterations. These observations suggest that susceptibility of human cells to EMCV infection may vary according to the cell type and to the strain or the variant.

The first step in any infection process is the attachment of the virus to a cell-membrane molecule representing the cellular receptor or co-receptor for the virus. This virus-cell interaction is considered a major determinant of viral host range, tissue tropism and pathogenesis. The mechanisms by which EMCV binds to and enters permissive human cells are not well understood. EMCV is believed to bind to non-permissive human erythrocytes by interaction with sialic acid residue of glycophorin A, the major sialoglycoprotein of the erythrocyte surface membrane [5, 29]. Jin et al. [14] reported the identification of a cell surface 70-kDa sialoglycoprotein as an attachment molecule for EMCV on two human permissive cell lines, HeLa and K562. These data suggest a role of sialic acid in EMCV binding to human cell lines. Whether sialic acid is also involved in EMCV attachment to permissive primary human cells or to other cell types remains to be established. Furthermore, it is known that strains or variants of picornaviruses can use different cell-surface molecules for attachment. For example, strains of the two neurovirulence groups of Theiler's virus, another cardiovirus, use different carbohydrate co-receptors for attachment: heparan sulfate for high-neurovirulence strains and sialic acid for the low-neurovirulence strains [27]. Therefore, it is reasonable to expect that strains or variants of EMCV that induce different pathologies may use different molecules to initiate infection.

To gain further insight into the mechanisms involved in EMCV infection of human cells, we analyzed the permissiveness of primary human cardiomyocytes towards two strains of EMCV: a pig myocardial strain (B279/95) and a rat strain (1086C). We report here that the two strains productively infect human cardiomyocytes but differ in their virulence. This difference in virulence is likely due to their binding efficiency. We analyze the cell-surface molecules implicated in their attachment and show that the two strains use different mechanisms to initiate infection, demonstrating the flexibility of EMCV in receptor usage.

Materials and methods

Cell culture

Cryopreserved primary human cardiomyocytes (CMC) were obtained from Clonetics (Cambrex, ref CC-2582) and used at the first or second passage. The cells were grown in the media and under the conditions recommended by the manufacturer.

Virus stocks

EMCV strains were kindly provided by Dr. Frank Koenen (Department of Virology, Section of Epizootic Diseases, CODA-CERVA, Groeselenberg 99, B-1180 Ukkel Belgium). The B279/95 strain was isolated from a piglet that died of myocarditis [20]; the 1086C strain was isolated from a rat (Dr. Frank Koenen, personal communication). The two strains were propagated on BHK-21 cells. Variants of the 1086C strain were isolated by limiting dilution in 96-well plates of BHK-21 cells. Each variant was subjected to five passages on BHK-21 cells, and virus stocks were prepared as clarified lysates as described previously [12].

Virus titration

Virus titer was determined by plaque assays using a BHK-21 cell monolayer. Cells were seeded in 35-mm dishes (1.5×10^5 cells) for 24 h at 37 °C. Medium was then removed, 0.5 ml of serial 10-fold dilutions of virus suspension was added, and the cells were incubated for 1 h at 37 °C. Subsequently, supernatant was removed and 2 ml of MEM containing 2% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 1.5% low-melting-point agarose were added. The dishes were incubated at 37 °C for 3 days and stained with crystal violet solution containing 4% paraformaldehyde. After removal of the agarose plug, plates were washed with water. Virus concentrations were determined as PFU ml^{-1}.

Reagents

Neuraminidase from *Vibrio cholerae*, heparinase I and heparinase III from *Flavobacterium heparinum*, and heparan sulfate (HS) were obtained from Sigma. Heparinase I and III were diluted in PBS with Ca^{2+} and Mg^{2+} containing 0.01% BSA. Anti-EMCV monoclonal antibody (mAb) 16E4 was produced in our laboratory. Goat anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) conjugate was obtained from Biosys.

Human cell infection

Virus production was analyzed by infection of subconfluent CMC cells in 96-well plates at a multiplicity of infection (MOI) of 1000 PFU per cell. Virus was allowed to adsorb for 1 h at 4 °C. Then, the monolayer was washed twice with PBS and incubated with 100 μl of cell culture medium at 37 °C under a 5% CO_2 atmosphere. At 0, 2, 4, 6, 8, 16 and 24 hours postinfection (h.p.i.), one plate was frozen for virus titration. At the end of the experiment, the plates were subjected to another round of freeze-thawing to release intracellular virus, and the virus titer in each well was determined on a BHK-21 cell monolayer as described above.

For analysis of infection, subconfluent CMC cells in 24-well plates were infected at different MOI. After 1 hour of adsorption at 37 °C, cell monolayers were washed with PBS and 1 ml of cell culture medium was added before incubation at 37 °C in a 5% CO_2 atmosphere. Plates were observed for cytopathic effect at 24 h.p.i., and cell viability was evaluated by colorimetric assay using cell proliferation reagent WST-1 (Roche). Briefly, at 24 h.p.i., 100 μl of WST-1 was added to each well. After 2 h of incubation at 37 °C in a 5% CO_2 atmosphere, the plates were shaken vigorously for one minute, and the absorbance of the wells was read using a microplate reader (ELISA). Cell viability was calculated as a percentage of uninfected cell controls.

Cell binding assays

Cells were maintained on ice during all of the reactions in order to inhibit virus uncoating, and cold solutions were used. Binding of virus to cells was determined using flow cytometry as described previously [12]. Cells were seeded in 24-well plates (10^6 cells per well), washed three times with PBS and incubated with virus for 1 h whilst being agitated. Unbound virus was removed by washing three times with PBS, and cells were incubated with anti-EMCV 16E4 mAb for 30 min whilst being agitated. After two washes with PBS and one with PBS containing 0.05% Tween 20, the cells were incubated with goat anti-mouse IgG-FITC conjugate for 30 min whilst being agitated. Cells were rinsed twice with PBS containing 0.05% Tween 20 and once with PBS. The cells in each well were then detached by pipetting with 300 μl of PBS and fixed with 1% paraformaldehyde (PFA). Samples were analyzed on a FACSCAN flow cytometer using CellQuestPro software (Becton Dickinson). Each experiment was done in duplicate. Mean fluorescence intensity of virus binding was calculated against that of uninfected cells incubated with mAb 16E4 and secondary antibody.

Inhibition assays and enzyme treatment

For virus binding inhibition assays, cell monolayer grown in 24-well plates were washed with PBS and then incubated for 1 h at 37 °C with neuraminidase at 10 nU per cell, heparinase I at 25 μU per cell, or heparinase III at 6.25 μU per cell. After three washing steps with PBS, the virus-binding assay was performed on the cells as described above.

To examine the effect of enzymatic treatment of cells on infection, CMC cells grown in 96-well plates were incubated with one of the enzymes or with a combination of the enzymes for 1 h at 37 °C. Cells were washed with PBS and infected with different virus strains or variants. Cell viability was measured at 24 h.p.i. using cell proliferation reagent WST-1 (Roche). Infections were carried out in quadruplicate with uninfected and treated or infected and untreated cells as controls. Viability of cells was calculated as a percentage of the uninfected cell control, and percent protection was defined as the difference in viability of infected cells with and without treatment. Statistical analysis was performed using a one-way ANOVA for repeated measures, followed by Bonferroni's post hoc t-test with significance set at a "p" value less than 0.05.

The effect of soluble heparan sulfate on attachment and infection was monitored by incubation of the virus with HS for 1 h at 37 °C before binding and infection assays.

Sequence analysis

For sequencing of EMCV strains and variants, infected BHK-21 cells were used for RNA extraction using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. RNA was used for amplification of EMCV genomic fragments using a one-step RT-PCR kit (QIAGEN) and EMCV-specific primers (not shown). Amplified DNA fragments were purified and sequenced directly on both strands by a dye chain termination method. Nucleotide sequences were analyzed on an IBM-compatible personal computer using the program Lasergene99 (DNASTAR, USA). The complete capsid nucleotide sequences of B279/95 and 1086C strains were submitted to the GenBank database and assigned accession numbers DQ835184 and DQ835185, respectively.

Results

Permissiveness of CMC cells to B279/95 and 1086C strains

Human cardiomyocytes have been reported to be permissive to an EMCV MN-30 strain isolated from an aborted swine foetus [4]. We tested the susceptibility of CMC cells towards two different strains of EMCV, a highly virulent porcine myocardial strain (B279/95) and a rat strain (1086C). Each strain was assayed for virus production and cell lysis. The CMC cells were infected with the B279/95 strain or the 1086C strain at a high multiplicity of infection, and virus production at different times postinfection was determined and compared. Virus production was first detected 4 h after inoculation, and maximal titers were reached by 8 h for both strains (Fig. 1a). At 24 h after infection, the cytopathic effect (CPE) was more pronounced in cells infected with the B279/95 strain than in 1086C-infected cells. This difference in CMC infection was clearly demonstrated when the MOI was decreased. At an MOI of 30 PFU per cell, only 20% of B279/95-infected CMC cells were viable 24 h.p.i., compared to 94% for 1086C-infected cells, whereas the two strains produced similar CPE on BHK-21 cells (Fig. 1b).

Since the first step in virus infection is the attachment of viral particles to a cell-surface receptor or coreceptor, we investigated if the difference in development of cytopathic effect observed between the two strains resulted from a difference in their affinity for CMC cells. To answer this question, the binding properties of B279/95 and 1086C on CMC cells were examined by flow cytometry and compared. An important difference in the attachment efficiencies of B279/95 and 1086C was observed. The data in Fig. 2 indicate that B279/95 binding on CMC cells is tenfold greater than 1086C binding, while the two viruses show similar binding to BHK-21 cells (data not shown).

CMC cell-surface residues involved in B279/95 and 1086C binding and infection

The difference in binding observed between the two EMCV strains suggests either a difference in receptor type used that could be more abundant for B279/95, or that the two strains use the same receptor, but the affinity of strain 1086C for this receptor is weaker. As the involvement of sialic acids in EMCV binding has been demonstrated previously for some human cells, such as erythrocytes, HeLa and K562 cells [14, 29], we investigated the role of sialic acids in B279/95 and 1086C binding to and infection of CMC cells. We used flow cytometry analysis and the WST-1 assay to examine the influence of treatment of cardiomyocytes by neuraminidase on binding and infection, respectively. For these studies, it was first necessary to determine the quantity of neuraminidase that releases a maximum of cell-surface sialic acids but is not toxic for cells. We found that treatment 10 nU of neuraminidase per cell from *Vibrio cholerae* resulted in 90% cell survival and reduced cell-surface sialic acid by 50% as measured by WGA-FITC binding (not shown). More concentrated neuraminidase dramatically reduced the number of living cells. Using this concentration, neuraminidase treatment of CMC cells showed no inhibitory effect on B279/95 binding or infection. In contrast, 1086C binding was reduced by more than 60%, and infection, by 27% ($p < 0.05$, CI 95 %) (Fig. 3). These results indicate that cell-surface sialic acids are involved in attachment and infection of the 1086C strain but not of the B279/95 strain.

As many viruses, including picornaviruses, have been found to use heparan sulfate (HS) as a receptor or coreceptor, we examined the role of HS in B279/95 attachment and infection of cardiomyocytes. We assessed B279/95 binding and infection of CMC cells incubated with either heparinase I, which degrades 2-O- or 6-O-sulfated and N-sulfated residues in heparin (relative activity 3:1) or with

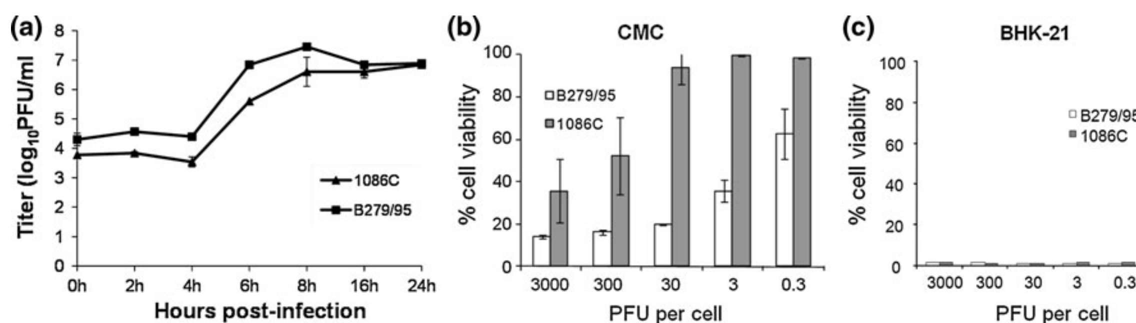

Fig. 1 EMCV production and cytopathic effect on CMC cells. (**a**) Growth curves of strains B279/95 and 1086C on CMC cells. Cells were infected with virus at an MOI of 1000 PFU per cell. Virus titers were determined by plaque assays on BHK-21 monolayers. (**b**) Cytopathic effect induced by strains B279/95 and 1086C on CMC and BHK-21 cells at 24 h p.i. Cells were infected with virus at different MOI. At 24 h p.i., cell viability was evaluated by colorimetric assay using the cell proliferation reagent WST-1. Cell viability was calculated as a percentage of the uninfected-cell control

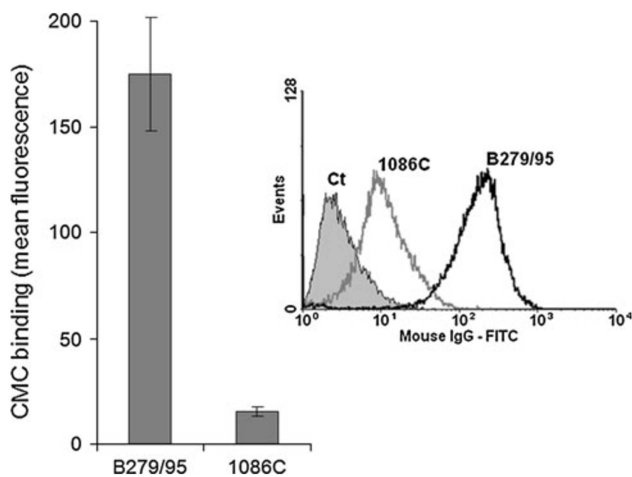

Fig. 2 Binding of B279/95 and 1086C to CMC cells. CMC cells were incubated with the indicated strain (MOI of 100), anti-EMCV MAb 16E4, and goat anti-mouse Ig-FITC for 30 min at 4 °C, and bound virus was detected using a FACSCAN flow cytometer

heparinase III, which acts on 6-O-sulfated, N-sulfated or N-acetylated residues in HS only, or HS. Treatment with either enzyme resulted in reduction of B279/95 binding in a dose-dependent manner (Fig. 4a). Incubation of cells with heparinase I at 25 μU per cell and with heparinase III at 6.25 μU per cell reduced binding by 36% and 42%, respectively. However, no significant inhibition of infection was observed after treatment of cells with each enzyme or a mixture of both (Fig. 5a). Furthermore, when B279/95 was incubated with soluble heparan sulfate before infection, a dose-dependent inhibition effect was observed on virus binding and infection (Fig. 4b; 5b). However, while 1000 μg of HS ml^{-1} inhibited B279/95 binding by 95%, infection was inhibited by only 23% ($p = 0.002$, CI 95 %). These data indicate that HS plays a more profound role in B279/95 binding than in infection of CMC cells.

To monitor if interaction with HS is a particularity of the B279/95 strain, we performed similar binding and infection inhibition assays with the 1086C strain. As shown in Fig. 4a, 1086C binding was also reduced after treatment of cells with heparinase. The inhibition with heparinase I was more important compared to what was observed with the B279/95 strain: 60% inhibition with heparinase I and 46% with heparinase III. No significant reduction of infection was observed when the cells were treated with heparinase I, heparinase III or both heparinases in comparison to untreated virus-infected cells (Fig. 5a). Furthermore, treatment of cells with a combination of neuraminidase and heparinase I, heparinase III or both heparinases before infection with 1086C strain resulted in an inhibition of infection greater than that observed after treatment with neuraminidase alone. The cell viabilities at 24 h.p.i were 85.71%, 46.95% and 65.90 %, respectively ($p < 0.001$, CI 95 %) compared to 31.5% observed after treatment with neuraminidase alone. This cumulative inhibition effect was not observed with the B279/95 strain (Fig. 5a). Incubation of 1086C with HS before infection resulted in reduction of virus binding and infection in a similar manner to that observed with the B279/95 strain. Incubation with 1000 μg of HS ml^{-1} reduced 1086C binding by 90% (not shown) and infection by 19% ($p = 0.001$, CI 95 %) (Fig. 5b).

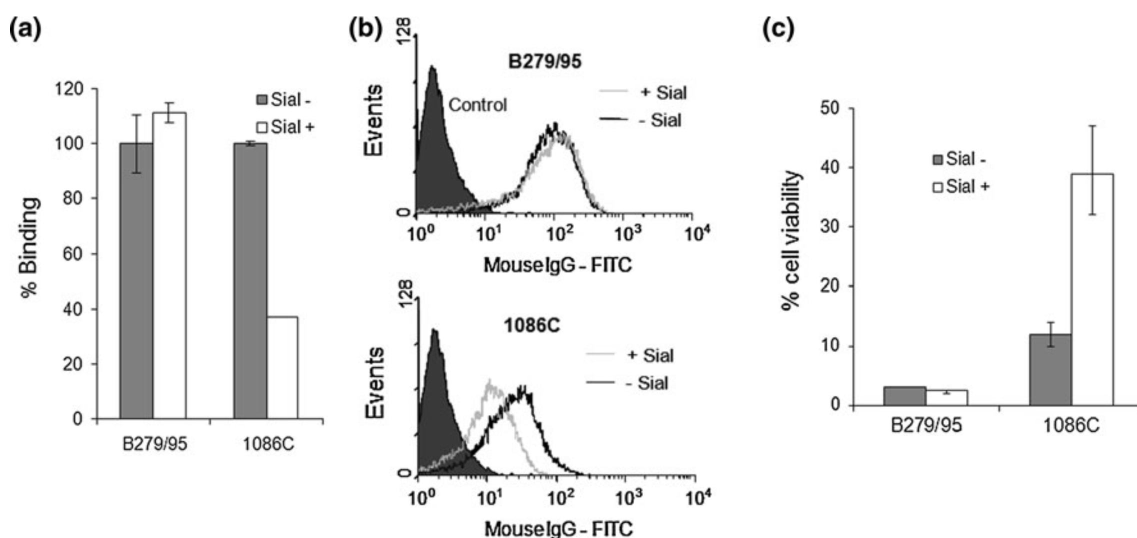

Fig. 3 Effect of sialidase on EMCV binding and infection. CMC cells (10^6) were treated with 10 nU of neuraminidase from *Vibrio cholerae* per cell for 1 h at 37 °C prior to analysis of virus binding (**a** and **b**) and infection (**c**) (MOI 1000). Bound virus was analyzed by flow cytometry, and CPE was analyzed by the WST-1 assay. Binding (**a** and **b**) and infection of cells (**c**) were calculated relative to binding and infection in the absence of sialidase treatment

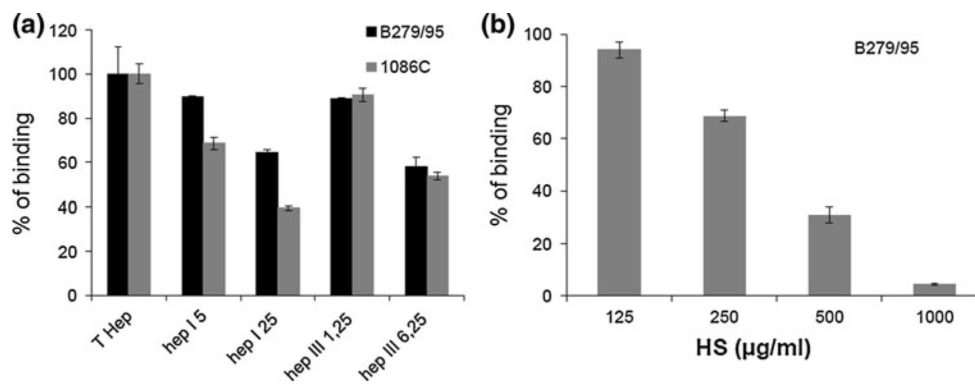

Fig. 4 Effect of heparinase and heparan sulfate on EMCV binding. **a**) CMC cells (10^6) were treated with heparinase I (5 and 25 µU/cell) or III (1.25 and 6.25 µU/cell) for 1 h at 37 °C prior to virus binding (MOI 1000). Bound virus was analyzed by flow cytometry, and inhibition of binding was calculated relative to binding in the absence of enzyme treatment. **b**) Virus was incubated with increasing concentrations of HS for 1 h at 37 °C before binding of CMC cells (MOI of 1000)

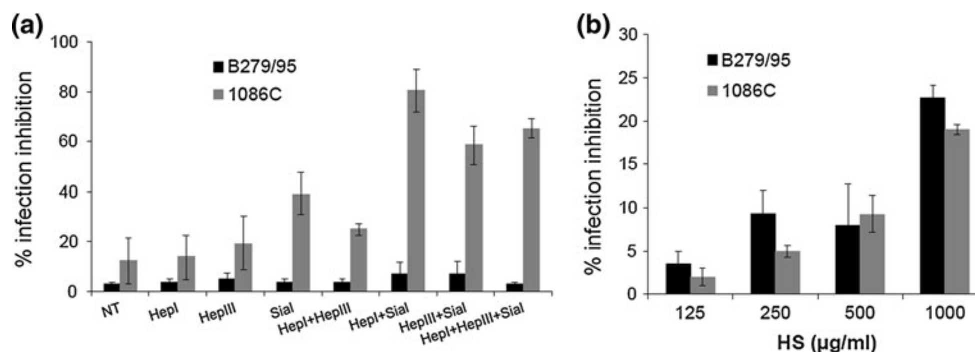

Fig. 5 Effect of heparinase and sialidase on EMCV infection. **a**) CMC cells (10^6) were treated with heparinase I (25 µU µU/cell), heparinase III (6.25 µU µU/cell), neuraminidase (10 nU per cell), or a combination of the enzymes for 1 h at 37 °C prior to infection with B279/95 or 1086C (MOI 1000). **b**) Virus was incubated with increasing concentrations of HS for 1 h at 37 °C before infection of CMC cells (MOI of 1000). CPE was analyzed by the WST-1 assay, and inhibition of infection was calculated relative to infection in the absence of enzyme treatment

Amino acid residues involved in EMCV binding and infection of CMC cells

In order to identify the amino acid residues that may play a role in EMCV binding and infection, we determined and compared the capsid coding region of the two EMCV strains. Sequence comparison revealed a difference in seven amino acid residues between 1086C and B279/95, three in VP1 (K54E, N64T, H81Y), two in VP2 (V141A, K145R) and two in VP3 (D60N, S92T). None of the mutations were located in the “pit”, the putative receptor-binding site of EMCV. However, with the exception of amino acid S92 of VP3, all of these residues were well exposed on the capsid surface (Fig. 6), suggesting their possible interaction with cell-surface residues.

Furthermore, we isolated 14 variants of the 1086C strain on BHK-21 cells and compared their binding to cardiomyocytes and their ability to induce cytolysis. The data revealed that the variants can be divided into two groups: group I (variants Cl1, Cl2, Cl7, Cl9), whose binding was equivalent to that of the initial 1086C strain and induced a total destruction of the cell monolayer by 24 h.p.i., and group II (Cl3, Cl4, Cl5, Cl6, Cl8, Cl10, Cl11, Cl12, Cl13, Cl14), which showed less binding and were less virulent (data not shown). One variant from the group I (Cl7) and one from group II (Cl3) were selected, and their binding characteristics were analyzed. In the absence of enzymatic treatment, the Cl7 variant bound 10-fold more efficiently than the Cl3 variant (Fig. 7a) and induced more than 75% cell destruction at 24 h.p.i., compared to 34% for variant Cl3 (Fig. 7b). The binding and infection of variant Cl3 was not significantly ($p > 0.05$) inhibited by treatment of cells with neuraminidase, whereas the attachment of variant Cl7 was reduced by more than 70%, and infection, by 47% ($p < 0.001$, CI 95 %) (Fig. 7a, b).

In order to identify the amino acid residues that may be involved in attachment of the Cl7 variant to sialic acid, we determined and compared the capsid amino acid sequences of the two variants. Only one amino acid residue situated in the VP1 protein at position 231 was different between the

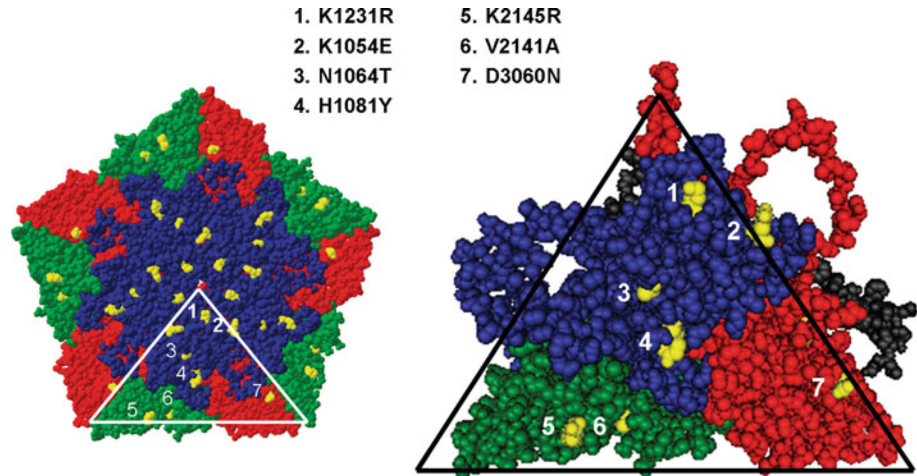

Fig. 6 Amino acid residues involved in EMCV binding and infection. Residues are shown on the pentamer (left) and the protomer (right) in a space-filling model generated with VIPERdb and Swiss-PdB software based on structural data obtained with Mengo virus [22]. Residue 1: mutation between Cl3 and Cl7 variants of 1086C strain. Residues 2 to 7: mutations between strains B279/95 and 1086C. VP1 is indicated in blue, VP2 in green, VP3 in red and VP4 in grey

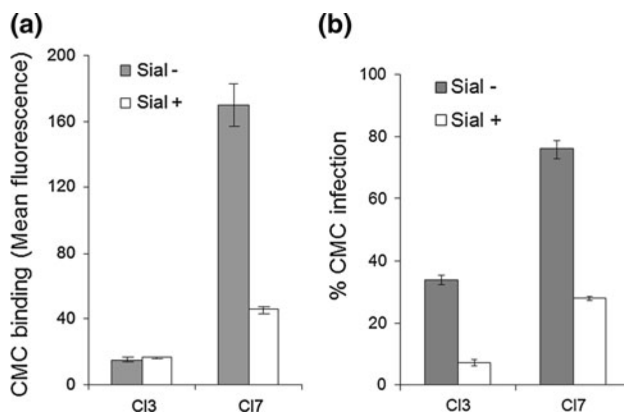

Fig. 7 Effect of sialidase on Cl3 and Cl7 binding (**a**) and infection (**b**). CMC cells (10^6) were treated with 10 nU of neuraminidase per cell for 1 h at 37 °C prior to virus binding (**a**) and infection (**b**) (MOI 1000). Bound virus was analyzed by flow cytometry, and CPE was analyzed using the WST-1 assay

two variants. This residue was a lysine in variant Cl7 and an arginine in variant Cl3. It is localized at the surface of the capsid and outside the putative receptor site (Fig. 6). This mutation was also found in all other non-virulent variants (not shown).

Discussion

The data reported here show that human cardiomyocytes are permissive to a highly virulent myocardial pig strain (B279/95) of EMCV, and infection is associated with cell destruction. These results are in accordance with the data reported by Brewer et al. [4], which demonstrated the permissiveness of CMC cells to an abortive pig strain of EMCV (MN-30). The strain B279/95 replicated in CMC cells and induced complete cytolysis at 24 h.p.i. However, the cytopathic effects were dependent on the MOI used, whereas for BHK-21 cells, a very low MOI was sufficient to induce complete lysis. One explanation for this difference between the two cell lines is that EMCV is adapted to BHK-21 cells, since it is produced in them. Another explanation may be related to interferon. BHK-21 cells do not produce interferon, which is one of the first defenses of cells against viruses. This might explain the intensity of the cytopathic effect on BHK-21 monolayers. In contrast, for cardiomyocytes, the first infected cells can synthetize interferon in order to reduce infection of the adjacent cells. This is also one of the reasons why BHK-21 cells are often used for the production of many viruses. Moreover, BHK-21 cells were obtained from a hamster, and rodents are a reservoir for EMCV. Therefore, the high sensitivity of these cells could also be related to the species tropism.

Strain B279/95 was isolated from a piglet that died of myocarditis [20]. Consequently, the cytolysis and significant viral production observed in cardiomyocytes may be expected. However, this strain can infect and produce cytopathic effects in a variety of human cells, including primary astrocytes and renal epithelial cells (not shown). The findings of Brewer et al. [4] suggest that strain B279/95 is more virulent than MN-30. Brewer et al. have shown that CMC cells are permissive to strain MN-30, while human renal epithelial cells are resistant. These results indicate that EMCV strains may differ in their ability to infect human cells. From a medical point of view, this B279/95 strain could present a high risk for humans in the case of pig-to-human xenotransplantation if we consider the routine immunosuppression of recipients.

Our data also show that a strain isolated from a rat infects human cardiomyocytes and induces cell lysis. However, this strain is less virulent than the B279/95 strain, since a high MOI is needed to achieve complete cell lysis. This high MOI is certainly not encountered under *in vivo* conditions, and this strain would probably not be infectious under such conditions. However, in order to gain

further insight into the mechanisms involved in EMCV infection of human cells by comparing the two strains, we carried on the infection studies using high MOI. The low virulence of the 1086C strain is probably due, at least in part, to its low efficiency of binding to CMC cells, which was 10-fold lower than that of B279/95. The binding differences observed between the two strains suggest either a difference in receptor type used, being more abundant for B279/95, or that the two strains use the same receptor present on CMC cells but the affinity of strain 1086C for this receptor is weaker.

The initial step in virus infection is often the binding to an attachment molecule, a coreceptor, followed by binding to a high-affinity entry protein. EMC virus attachment to permissive human cell lines K562 and HeLa and non-permissive human erythrocytes has been reported to be mediated by a cell-surface sialic acid [14, 29]. Here, we showed that neuraminidase treatment of primary human cardiomyocytes prior to binding assays reduced the attachment and infection of strain 1086C but not of strain B279/95. This result indicates that cell-surface sialic acid residues mediate attachment and infection of 1086C on cardiomyocytes, whereas the attachment of B279/95 to these cells is independent of sialic acid. Furthermore, analysis based on soluble HS inhibition of attachment and infection and enzymatic removal of HS established that the two strains are able to use HS for binding to CMC cells. Since these treatments did not significantly reduce virus infection, this suggests that the HS binding is not absolutely necessary for virus infection. However, the fact that treatment of cells with a combination of heparinase and neuraminidase increased the inhibition of 1086C infection in comparison to treatment with neuraminidase alone suggests that HS binding is cooperatively linked to the process of 1086C infection. Infection by B279/95 was not inhibited by neuraminidase or heparinases or a combination of both, suggesting that this strain uses a different coreceptor to initiate infection or binds directly to a protein entry receptor. These results imply the existence of at least two different kinds of binding before the entry of EMCV into human cardiomyocytes. A similar difference in the binding properties of different strains has already been reported for another cardiovirus. Whereas persistent strains of Theiler's virus (BeAn and DA) bind to sialic acid, the highly virulent strain GDVII uses heparan sulfate to bind and infect permissive cells [32]. It is uncertain if there is a correlation between the nature of binding of EMCV strains and their virulence. Further *in vivo* experiments are needed to answer this question.

Genetic analysis of the capsid coding region showed seven amino acid differences between the B279/95 and 1086C strains, three in VP1 (K54E, N64T and H81Y) and two in VP2 (V141A and K145R) and VP3 (D60N and S92T). Since 95% of the amino acid sequences of EMCV and Mengo virus are identical, it seems reasonable to predict that the three-dimensional structure is conserved between the two viruses. Considering the three-dimensional structure of the Mengo virus capsid [22], with the exception of mutation S92T, all of the amino acids that differ between the two EMCV strains are exposed on the outer surface, suggesting a role of these residues in EMCV binding to human cells. Since none of these amino acids are located in or near the putative receptor-binding site, which is a depression called a "pit" between VP1 and VP3, it is possible that these amino acids interact with a coreceptor molecule rather than with the receptor.

Amino acid 64 of VP1 is located in the βB-βC loop. For Mengo virus, this region has been reported to constitute a recognition site for neutralizing antibodies [18]. Indeed, mutants of Mengo virus that were able to escape neutralization by a selected neutralizing monoclonal antibody have been found to have mutations in this region: K49Q and N62D of VP1. For EMCV, the mutation N62D has been observed for the B279/95 strain after attenuation of the virus by serial passages on BHK-21 cells [8]. Furthermore, diabetogenic (PV2) and non-diabetogenic variants (PV7) of EMCV have been reported to differ by one mutation at residue 63 of the VP1 (Q63E/K) [25]. These results suggest that amino acid 64 may contribute to determining EMCV tropism and virulence.

Other mutations affecting the surface of the capsid are localized in VP2 puff B (V141A and K145R) and in VP3 "knob" (D60N). These two regions are localized on the surface of Mengo virus and constitute recognition sites 1 and 2, respectively, for neutralizing antibodies. Mengo virus mutants selected to escape chosen neutralization monoclonal antibodies have shown mutations in VP2 (N144S and R145K) and in VP3 (A61V) [18]. For Theiler's virus, it was shown that mutations responsible for adaptation to L929 cells occurred in two clusters of amino acids known to be part of neutralization epitopes [15]. By analogy, it is probable that the regions corresponding to the neutralization sites of EMCV also contribute to the interaction of the virus with cell-surface molecules and influence virus tropism.

Analysis of variants isolated from the 1086C strain revealed that variants showing a difference in their ability to use sialic acid for binding to cardiomyocytes differ by one amino acid in the capsid coding region at residue 231 of the VP1 protein. All of the variants that used sialic acid had a lysine residue rather than arginine. These results, which were obtained due to the use of a high MOI, confirmed that strain 1086C is sialic acid dependent. This strain is a mixture of variants that can adsorb efficiently to cardiomyocytes using sialic acid (group I) and variants that do not bind specifically to cardiomyocytes because they do not bind to

sialic acid (group II). These results also support a role of residue K231 of VP1 in the interaction of EMCV with sialic acid residues and are consistent with an observation previously reported for Mengo virus. Mengo virus mutants that had lost their ability to hemagglutinate human erythrocytes have been found to have two consecutive mutations in VP1: K231R and P232S [23]. The fact that agglutination of human erythrocytes by Mengo virus is dependent on the presence of sialic acid on the erythrocyte surface [3] suggests that residues K231 and P232 of VP1 are involved in the interaction of the virus with sialic acid. As for Mengo virus, the attachment molecule for EMCV on human erythrocytes is glycophorin A, the major sialoglycoprotein of the erythrocyte surface, and sialic acid is the component involved in virus binding. Since the capsid sequences of the two viruses are well conserved, it is likely that same residues are used by the two viruses for interaction with cell-surface sialic acid. The non-erythrocyte origin of cardiomyocytes suggests the involvement of another cell-surface molecule other than glycophorin A in EMCV binding to those cells. EMCV virus attachment to the permissive human cell lines HeLa and K562 has been reported to be mediated by a cell-surface 70-kDa sialoglycoprotein [14]. Whether this protein is also involved in the attachment of EMCV to human cardiomyocytes remains to be established.

In conclusion, our results indicate that a pig myocardial strain and a rat strain of EMCV productively infect primary human cardiomyocytes and induce cell destruction. It also shows flexibility in receptor usage for EMCV. In addition to sialic acid, cell-surface heparan sulfate appears to contribute to the binding of EMCV to human cardiomyocytes but does not seem to be necessary for infection. Since attachment and entry of EMCV are still poorly understood, a possible role for heparan sulfate during cell infection should be taken in consideration in further studies. Furthermore, analysis of B279/95 infection suggests that this strain uses an alternative attachment mechanism that requires an interaction with neither sialic acid nor heparan sulfate. The capsid mutations identified in these experiments will help to understand better the structural requirements for EMCV attachment to human cells.

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