Efficient infection of buffalo rat liver-resistant cells by encephalomyocarditis virus requires binding to cell surface sialic acids

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In contrast to the production of virus and cell lysis seen in baby hamster kidney cells (BHK-21) infected with the strain 1086C of encephalomyocarditis virus (EMCV), in buffalo rat liver cells (BRL) neither virus replication nor cytopathic effects were observed. After 29 passages in BRL cells, each alternating with boosts of the recovered virus in BHK-21 cells, the virus acquired the ability to replicate effectively in BRL cells, attaining virus titres comparable to those in BHK-21 cells and producing complete cell destruction. The binding of virus on BRL cells was increased after adaptation and was similar to that observed on BHK-21 cells. Treatment of BRL cells with sialidase resulted in an 87 % reduction in virus binding and inhibition of infection. Sequence analyses revealed three mutations in the VP1 amino acid sequence of the adapted virus at positions 49 (Lys→Glu), 142 (Leu→Phe) and 180 (Ile→Ala). The residue 49 is exposed at the surface of the capsid and is known to be part of a neutralization epitope. These results suggest that the adaptation of EMCV to BRL cells may have occurred through a mutation in a neutralizing site that confers to the virus a capacity to interact with cell surface sialic acid residues. Taken together, these data suggest a link between virus neutralization site, receptor binding and cell permissivity to infection.

INTRODUCTION

Encephalomyocarditis virus (EMCV) belongs to the genus Cardiovirus within the family Picornaviridae that has a worldwide distribution (Stanway et al. 2005). The virus was first isolated from non-human primates and it is now recognized as a cause of mortality in many host species. Rodents are considered to be natural hosts of EMCV and thought to be the primary reservoir and disseminators of the virus (Acland & Littlejohns, 1975; Koenen et al. 1999; Seaman et al. 1986).

The primary route of transmission for EMCV is believed to be faecal–oral, through ingestion of food or water contaminated with virus (Zimmerman et al. 1993). Transplacental vertical transmission has been reported to occur naturally in swine and baboons (Hubbard et al. 1992; Kim et al. 1989). Whether an infection remains asymptomatic or induces clinical signs depends on virus factors, including viral dose, virus strain and host factors including species, age and immune status of the infected animal. EMCV infection causes acute myocarditis and sudden death in piglets, and reproductive failure in sows. Infection of older pigs is asymptomatic. Although generally asymptomatic in rodents, EMCV infection can cause insulin-dependent diabetes mellitus or nervous disorders in mice (Cerutis et al. 1989). The virus has been isolated from various organs including heart, tonsils, kidneys, lungs, liver, spleen, small intestine and mesenteric lymph nodes, from naturally or experimentally infected animals (Billinis et al. 2004; Dea et al. 1991; Maurice et al. 2002; Papaioannou et al. 2003; Spyrou et al. 2004). Many isolates have been reported to be antigenically indistinguishable (Kim et al. 1991). However, the pathogenicity of EMCV in various animal species was found to be strain-dependent (Kim et al. 1989). The mechanisms involved in EMCV virulence and pathogenicity are unknown.

The first step in any infection process is the attachment of the virus to a cell membrane molecule representing the cellular receptor or coreceptor for the virus. This virus–cell interaction and the distribution of a virus receptor are considered to be major determinants of viral host range, tissue tropism and pathogenesis. Vascular cell adhesion molecule 1 has been identified as a receptor for the D variant of EMCV on murine vascular endothelial cells.
(Huber, 1994). Jin et al. (1994) identified a cell surface 70 kDa sialoglycoprotein as an attachment molecule for EMCV on two permissive human cell lines, HeLa and K562. EMCV is believed to bind to non-permissive human erythrocytes by interaction with sialic acid residues of glycophorin A, the major sialoglycoprotein of the erythrocyte surface membrane (Burness & Pardoe, 1983; Tavakkol & Burness, 1990). Together, these data point to a crucial role for sialic acid in binding of EMCV to cells.

Previous experiments have demonstrated that while EMCV replicates in numerous animal and human cells, a panel of rat cells were relatively resistant to EMCV infection (Kelly et al., 1983; Su et al., 2003). In an effort to gain understanding of the molecular mechanisms underlying resistance to EMCV infection and expansion of the host range, we examined the step(s) in the EMCV replication cycle that were blocked in buffalo rat liver-resistant cells. We analysed the virus changes that led to the adaptation of these cells by comparing the genomic sequences between the parental and the adapted viruses. We demonstrated that the block in EMCV replication in these cells occurs at the level of virus attachment. Adaptation of EMCV to produce a rapid cytopathic effect (CPE) in BRL (buffalo rat liver) cells correlated with an increase in virus binding to cell surface sialic acid residues and introduction of amino acid mutations in the VP1 capsid protein. One of these mutations occurred at an amino acid residue exposed at the surface of the viral capsid and known to be part of neutralizing epitope, suggesting a close relationship between a receptor-binding site and a virus neutralization epitope.

**METHODS**

**Cells and virus.** BHK-21 cells (baby hamster kidney) were maintained in Eagle’s minimum essential medium with Earle’s salts supplemented with 1 mM l-glutamine (Gibco-BRL), 1 % sodium pyruvate (100 mM; Gibco-BRL), 1 % non-essential amino acids (100 μl; Gibco-BRL), 100 U penicillin (Gibco-BRL) ml⁻¹, 100 μg streptomycin (Gibco-BRL) ml⁻¹ and 10 % fetal calf serum (FCS; Gibco-BRL).

BRL cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10 % FCS, 4500 mg glucose ml⁻¹, 1 mM pyruvate (100 mM; Gibco-BRL), 1 % non-essential amino acids (100 μl; Gibco-BRL), 100 U penicillin (Gibco-BRL) ml⁻¹ and 10 μg streptomycin ml⁻¹.

The EMCV 1086C strain was kindly provided by Dr Frank Koenen (Department of Virology, Section of Epizootic Diseases, CODA-CERVA, Groeselenberg 99, B-1180 Ukkel, Belgium). The strain was initially isolated from a rat (Dr Frank Koenen, personal communication) and was propagated in BHK-21 cells. The virus stock was propagated alternately in BHK-21 and BRL cell monolayers (Gammoumi et al., 2006). The BRL cells were incubated with mAb 16E4 and secondary antibody from a FACScan flow cytometer using CellQuestPro software (Becton Dickinson). Each experiment was done in duplicate. For each sample 1 x 10⁴ individual cells were recorded using a dot-plot combination of low-angle forward scattered and right-angle scattered laser light. Mean fluorescence intensity of virus binding was determined by subtracting the mean fluorescence intensity of the uninfected cells incubated with mAb 16E4 and secondary antibody from the mean fluorescence intensity of infected cells.

**Adaptation of 1086C strain to BRL cells.** The EMCV 1086C strain was propagated alternately in BHK-21 and BRL cell monolayers grown in 25 cm² tissue culture flasks until a variant virus population promoting CPE on BRL cells had emerged. After each cell passage, infected cells were subjected to three freeze–thaw cycles and clarified by centrifugation for 15 min at 2500 g at 4 °C. The BRL cells were infected at an m.o.i. of 100 TCID₅₀ per cell and incubated for 48 h and the BHK-21 cells were inoculated with 0.5 ml clarified suspension of infected BRL cells and incubated for 24 h. At each passage, the virus titre was determined on BHK-21 cells. The adapted virus was then propagated on BRL cells and subjected to 15 cell passages. The recovered virus was named 1086C Ad.

**RNA transfection.** Total RNA was extracted from the culture supernatant of infected and uninfected BHK-21 cells using the RNeasy Mini kit (Qiagen) according to the manufacturer’s recommendation. Viral RNA was used to transfected BHK-21 or BRL cell monolayers grown in 24-well plates by using TransFast reagent (Promega). The transfection was performed according to the manufacturer’s instructions, by using 2 vols TransFast for 1 μg RNA.

**Plaque formation assay.** Cells were seeded in 35 mm dishes (1.5 x 10⁵ cells per well) for 24 h at 37 °C. Medium was then removed, 0.5 ml 10-fold serial dilutions of virus suspension was added and the cells were incubated for 1 h at 37 °C. Subsequently, supernatant was removed and 2 ml growth medium containing 1.5 % low-melting point agarose was 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 expressed as a number of p.f.u. ml⁻¹.

**Indirect immunofluorescence assay.** Cells were seeded at 1–2 x 10⁴ cells ml⁻¹ in 96-well plates and infected with virus for 1 h at 37 °C. Monolayers were then incubated with growth medium for 5 h at 37 °C. Growth medium was then removed and monolayers were washed three times with PBS before fixation with 90 % cold acetone for 20 min at −20 °C. Monolayers were then washed three times with PBS and incubated with anti-EMCV monoclonal antibody (mAb) for 1 h at 37 °C. After three washes with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Biosys) was added and the cells were incubated for 30 min at 37 °C. Monolayers were then washed three times with PBS and once with distilled water. Cells were observed by epifluorescence by using an Olympus IX-71 fluorescence microscope equipped with an FITC filter.

**Virus-binding assay using flow cytometry.** Several anti-EMCV mAbs were produced as described previously (Bakkali et al., 1994) and tested by flow cytometry for their reactivity with the 1086C strain adsorbed in BHK-21 cells. The neutralizing mAb (mAb 16E4) that gave the best signal was selected.

Cells were maintained on ice during the assay in order to inhibit virus uncoating and all solutions were chilled. Cells were seeded in 24-well plates (5 x 10⁵ cells per well), washed three times with PBS and incubated with virus at an m.o.i. of 10⁻⁴ p.f.u. per cell for 1 h under agitation. Unbound virus was washed by rinsing three times with PBS and incubated with anti-EMCV 16E4 mAb for 30 min under agitation. After washing twice with PBS and once with PBS containing 0.05 % Tween 20, cells were incubated with goat anti-mouse IgG–FITC conjugate for 30 min under agitation. Cells were rinsed twice with PBS containing 0.05 % Tween 20 and once with PBS and then fixed with 1 % paraformaldehyde. Samples were analysed by a FACScan flow cytometer using CellQuestPro software (Becton Dickinson). Each experiment was done in duplicate. For each sample 1 x 10⁴ individual cells were recorded using a dot-plot combination of low-angle forward scattered and right-angle scattered laser light. Mean fluorescence intensity of virus binding was determined by subtracting the mean fluorescence intensity of the uninfected cells incubated with mAb 16E4 and secondary antibody from the mean fluorescence intensity of infected cells.
Effect of sialidase treatment on virus infection and attachment. BRL cell monolayers grown in 24-well plates at $5 \times 10^5$ cells per well were washed with serum-free medium and then incubated for 1 h at 37 °C with *Vibrio cholerae* sialidase (Sigma) at a final concentration of 25 mU ml$^{-1}$ in PBS pH 5.4. Untreated cells were incubated in the same conditions with PBS pH 5.4 alone. After three washing steps, cells were infected for 1 h at 37 °C at an m.o.i. of 2.5 p.f.u. per cell. Cells were washed again three times and complete medium was added. CPE was monitored by light microscopy after 24 h of incubation at 37 °C. The effect of sialidase on virus infection was also monitored in a plaque reduction assay. In this experiment, a plaque formation assay was performed as described above on sialidase-treated cells and for comparison on untreated cells.

The effect of sialidase on virus attachment was analysed by performing virus-binding assays on cells treated with sialidase, as described above. The results are expressed as the percentage of binding on sialidase-treated cells relative to binding on untreated cells.

**Sequencing.** RNA was extracted from BHK-21 cells infected with the 1086C strain or from BRL cells infected with the adapted virus by using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was used for amplification of EMCV genomic fragments using one-step RT-PCR kit (Qiagen) and EMCV-specific primers (data not shown). Amplified DNA fragments were purified and both strands were sequenced directly by using the dye chain termination method. Nucleotide sequences were deduced from two independent PCR fragments and analysed on an IBM compatible personal computer by using program Lasergene99 (DNASTAR). The nucleotide sequence was submitted to the GenBank database and assigned the accession number DQ835185.

**RESULTS**

**BRL cells are resistant to EMCV infection**

Infection of BHK-21 cells with the 1086C strain at an m.o.i. as low as 1 p.f.u. per cell resulted in complete destruction of cell monolayers within 24 h. In contrast, no CPE was observed 72 h post-infection when the virus was inoculated into BRL cells, even at a high m.o.i. In a plaque formation assay, the virus produced large plaques on BHK-21 cells, whereas no plaques were observed on BRL cells (Fig. 1a).

To determine whether BRL cells supported virus growth without CPE, BHK-21 and BRL cells were infected with EMCV at an m.o.i. of 10. The cells were harvested every hour during a 9 h period and the virus titre was determined by end-point dilution on permissive BHK-21 cells. Virus production in BHK-21 cells was observed as early as 4 h after infection and maximum titre was reached by 8 h post-infection (Fig. 1b). In the BRL cells, the virus titre remained relatively stable after 9 h of incubation, most likely corresponding to the input virus. Furthermore, examination of cells by immunofluorescence following virus inoculation (m.o.i. of 100) revealed that in infected BHK-21 cells viral antigens were detected in >90% of cells, whereas in BRL cells only a few cells (<1%) expressed viral antigens (Fig. 1c). These results indicate that BRL cells are resistant to infection by the EMCV 1086C strain.

**Restriction in BRL cells infection occurred at an early stage of viral cycle**

The block in EMCV infection in BRL cells may occur at one or several stages of viral cycle. The first step of EMCV infection consists of the attachment of virus particles to a cell surface receptor or coreceptor and internalization of viral RNA. In order to determine whether this stage of virus cycle is a limiting step in infection of BRL cells, cells were transfected with viral RNA and then monitored for the production of viral antigens, the production of infectious particles and CPE. In both BHK-21- and BRL-transfected cells, viral antigens were detected by immuno-
fluorescence as early as 5 h post-transfection (Fig. 2a). Analysis of infectious virus production in transfected BHK-21 and BRL cells revealed comparable kinetics of virus production between the cell types (Fig. 2b). The kinetic curves represent a single replicative cycle since more than 90% of the cells were infected at 5 h post-transfection (Fig. 2a). Infectious virus was produced as early as 2 h after transfection and maximum titre was reached by 8 h after transfection. At 24 h after transfection, similar quantities of infectious virus were detected in cell culture supernatant of both cells, indicating that virus particles are released from BRL cells. However, no plaque formation was observed in transfected BRL cells grown under agar, whereas in transfected BHK-21 cells the progeny virus produced large plaques (data not shown).

Taken together, these results indicate that EMCV genomic RNA is able to replicate in BRL cells and to produce infectious virus, but then the progeny virus is unable to spread from cell to cell. Thus, EMCV infection of BRL cells is blocked at an early stage of virus cycle, that is, virus attachment and/or internalization.

In order to localize the block in BRL infection we compared the binding efficiency of EMCV to BHK-21 and BRL cells. As shown in Fig. 2(c), EMCV bound 10 times more efficiently to BHK-21 cells than to BRL cells. This result suggests that the resistance of BRL cells to EMCV infection is likely due to a low attachment of virus particles to cell surface molecules.

**EMCV adaptation to BRL cells**

To gain a better understanding of the mechanism(s) involved in the block of EMCV infection, we attempted to adapt the 1086C strain to BRL cells, in order to study the viral modifications that led to this adaptation. First, assays of adaptation by successive passages of EMCV in BRL cells were unsuccessful. Virus titre decreased after each passage and infectious virus was no longer recovered from inoculated cells after the fourth passage. To circumvent this complication, the 1086C strain was repeatedly passaged in BRL cells with alternate passages in BHK-21 cells to boost viral yield. To monitor virus adaptation, the virus titre was determined after each cell passage. As shown in Fig. 3(a), the titre of infectious virus produced in BRL cells increased with passages and approached that obtained in BHK-21 cells. At the fourth alternate passage, CPE became evident in infected BRL cells 48 h after inoculation. Nevertheless, the produced virus was unable to produce CPE in BRL cells when it was inoculated directly into these cells. After 29 alternate passages, the virus was successfully propagated 15 times in BRL cells and, at each cell passage, complete destruction of cells was observed 24 h after inoculation. Plaque formation assays showed that the adapted virus produced plaques in infected BRL cells, demonstrating its ability to spread from cell to cell (Fig. 3b). Furthermore, the growth kinetics of the adapted virus in BRL cells and in BHK-21 cells, infected at an m.o.i. of 10, were similar (Fig. 3c). Virus production was detected...
after 4 h of infection and virus titre showed a 3 log increase to reach a maximum titre by 8 h. At 5 h after inoculation, viral antigen was detected by immunofluorescence in >90% of inoculated BRL cells (Fig. 3d). These results demonstrate the ability of the adapted virus to infect BRL cells and to replicate in these cells.

**Adaptation correlated with an increase in virus binding to sialic acid residues**

RNA transfection experiments and cell-binding assays suggested that restriction in BRL cell infection is likely due to a low attachment of the virus to the cell surface. Thus, we speculated whether the adaptation of 1086C to BRL cells resulted in an increase in virus binding. As shown in Fig. 4, the binding of the adapted virus to BRL cells was similar to that observed in BHK-21 cells. Furthermore, compared with the initial binding of the parental 1086C strain, the attachment of the adapted virus to BRL cells was 10-fold higher than the initial binding. This result indicates that the adaptation of 1086C strain to BRL cells correlated with enhanced virus binding to cell surface molecules.

Sialic acid residues have been reported to be involved in EMCV binding to mammalian cells. To determine whether the adapted virus uses sialic acid for the attachment to and infection of BRL cells, cells were digested with *V. cholerae* sialidase, which hydrolyses both α(2-3) and α(2-6) linkages of sialic acid, before binding and infection assays. Sialidase treatment resulted in 87% reduction in virus binding (Fig. 5a and b) and inhibited virus plaque formation (Fig. 5c) and CPE production (Fig. 5d). These results indicate that BRL cell surface sialic acid residues are required for the attachment and infection of the adapted virus. Binding
inhibition assays using BHK-21 cells revealed that the attachment of the parental 1086C strain to BHK-21 cells was not inhibited by sialidase treatment, whereas binding of the adapted virus was reduced by more than 75 % (Fig. 5a and b). This indicates that the parental virus did not bind to sialic acids, suggesting that the capacity of the adapted virus to bind sialic acid resulted from virus adaptation.

**Characterization of the genomic mutations selected during the adaptation of EMCV to BRL-resistant cells**

In order to identify the genomic modifications that led to the adaptation of 1086C strain to BRL cells, we determined and compared the complete coding sequence of the virus before and after adaptation. Nucleotide sequence comparison revealed nine mutations in the adapted virus sequence: one in VP2 (T123C), two in VP3 (C397T and C168T), four in VP1 (A145C, G426T, A538G and T539C) and two in the 3D protein (C531T and T552C). Only mutations in VP1 led to an amino acid substitution. Thus, lysine 49 was replaced by glutamine, leucine 142 by phenylalanine and isoleucine 180 by alanine. None of these mutations are located in the ‘pit’: the presumed receptor-binding site. However, amino acid Lys49 is located on the outer surface of the capsid in the loop connecting βB and βC strands (Fig. 6). In contrast, residues 142 and 180 are not surface exposed and are located in the βE strand and the βF–βG loop, respectively.

**DISCUSSION**

Previous studies have demonstrated that while EMCV replicates in numerous animal and human cells, a panel of rat cells were relatively resistant to EMCV infection and replication. Indeed, limited replication of virus was observed in rat myocardial cells, rat C6 glioma cells and rat embryo cells, while rat HTC hepatoma cells were completely refractory to EMCV replication. None of the rat cell lines developed cytopathic changes following virus inoculation and only a small percentage of cells demonstrated viral antigen 24 h after infection (Donta et al., 1986; Kelly et al., 1983). Plagemann & Swim (1966) have also reported resistance in other rat cells, Novikoff hepatoma cells (N1S1), to Mengo virus, which is now considered to be a strain of EMCV. In the present study we show that, in contrast to BHK-21 cells, no CPE was observed in buffalo rat liver cells (BRL) 72 h after inoculation with the 1086C strain of EMCV. Furthermore, viral antigen and viral production were not detected in the inoculated cells. These results demonstrate the inability of EMCV to infect BRL cells. These observations are confusing if we consider that rats are the natural host and reservoir of EMCV and virus has been isolated from different organs of experimentally infected animals. One explanation of this cell resistance may be an alteration in cellular receptor that may occur when cells adapt to growth in established tissue culture. The finding that transfection of BRL cells with viral RNA resulted in production of viral antigen and release of infectious progeny virus indicated that the restriction of EMCV replication in BRL cells depends only on its inability to penetrate into the cell. Thus, the block in virus infection of these cells occurs at an early stage of virus replication, namely, attachment to cell surface and/or uncoating. Analysis of binding by flow cytometry showed that the 1086C strain exhibited low binding to BRL cells compared with the permissive BHK-21 cells. This finding suggests that the low binding likely contributes to resistance of BRL cells. However, we cannot rule out the fact that the uncoating step may also be a limiting step in BRL cell infection. Indeed, after comparing the susceptibility of murine and rat cells to the M strain of EMCV, Donta et al. (1986) have reported that the block in infection of rat glial cells (C6) probably occurs at the level of uncoating. But in this case, the binding of the M strain was similar in murine and rat cell lines.

To characterize the mechanism(s) responsible for restriction of the EMCV growth in BRL cells more fully, we tried to obtain a host range mutant adapted to promote a rapid and complete CPE in these cells. The first attempts to adapt the 1086C strain by successive blind passages in BRL cells were unsuccessful. We observed a diminution in the quantity of virus recovered from infected BRL cells after each cell passage and the virus was lost after the fourth passage (data not shown). A similar observation has been
reported by Kelly et al. (1983) concerning adaptation of the M strain of EMCV to rat embryo cells. This failure in virus adaptation is probably due to selection of a very low quantity of viral particles during the first passage that is diluted during the next passages. To overcome this difficulty, we attempted the adaptation by successive passages of the 1086C strain in BRL cells each alternating with a passage in BHK-21 cells to amplify the recovered virus. By using this method, an increase in the virus quantity recovered from BRL cells was observed after each passage, indicating progressive adaptation of EMCV to these cells. After 29 alternate passages, the variant obtained caused complete CPE in BRL cells 24 h after infection and produced high levels of infectious virus. Furthermore, the kinetics of virus production in BRL cells were similar to those observed in BHK-21 cells, and the virus was able to spread from cell to cell as shown by the plaque formation assay. The adapted virus was propagated 15 times in BRL cells without losing its infectivity, demonstrating its stability.

Binding experiments indicated that the attachment of the 1086C strain to BRL cells was increased after adaptation to reach a level similar to that observed in BHK-21 cells. Since the transfection experiments with viral RNA have shown that the virus is able to replicate in BRL cells, this observation strongly suggests that adaptation likely occurred through mutation of the virus capsid, which enhanced virus binding to a cell surface receptor. Indeed, sequence analyses of the adapted virus revealed three mutations in the amino acid sequence of the VP1 protein: lysine 49 was replaced by glutamine, leucine 142 by phenylalanine and isoleucine 180 by alanine. The three-dimensional structure of the capsid of Mengo virus has previously been determined by X-ray crystallography (Luo et al., 1987). Since 95 % of the amino acid sequence of EMCV and Mengo virus is identical, it seems reasonable to predict that the three-dimensional structure of the capsid is conserved between the two viruses. According to the three-dimensional structure of the Mengo capsid, none of the three mutations are located in the putative receptor site. Mutation 142 is located in the \( \beta \)E strand and mutation 180 in the \( \beta F-\beta G \) loop. These 2 aa are located in the inner surface of the capsid and are thus unlikely to be involved in virus binding. However, mutation 49 is located in the loop

![Fig. 5. Inhibition of 1086C Ad binding (a, b) and infection (c, d) by pretreatment of BRL cells with sialidase. Cells (5\( \times \)10^5) were pretreated with sialidase at 25 mU ml^-1 before binding or infection assays. Binding was analysed by flow cytometry and the results are shown as mean percentage of bound virus of two experiments carried out in parallel (a) or as a histogram of cell-associated fluorescence (b). (c) Plaque inhibition assays on BRL cells pretreated with sialidase. (d) Inhibition of 1086C Ad infection by sialidase treatment. V. cholerae sialidase-treated (right panels) and untreated (left panels) BRL cell cultures were infected at an m.o.i. of 2.5 p.f.u. per cell. The picture illustrates CPE on the culture observed at 24 h post-infection by light microscopy. Original magnification \( \times 20 \).](http://vir.sgmjournals.org)
Fig. 6. Capsid mutations responsible for virus adaptation to BRL cells. (a) Shown is a pentamer of the EMCV capsid (seen from exterior) in a space-filling view generated with Swiss-PdbViewer software. The capsid fivefold axis is indicated by an arrow. The triangle shows the capsid unit (protomer) and the capsid viral proteins VP1, VP2 and VP3. The residue 49 of VP1 is shown in white on each protomer. The residues 142 and 180 of VP1 are located in the interior of the protomer and are not visible in this picture. (b) Position of Lys49, Leu142 and Ile180 on a ribbon diagram of the VP1 protein.

Previous studies have shown that cell surface sialic acid residues are involved in the interaction of EMCV with different mammalian cells (Burness & Pardoe, 1983; Jin et al. 1994; Tavakkol & Burness, 1990). Our finding that the binding to and the infection of BRL cells by the adapted virus are inhibited after the treatment of cells with sialidase also demonstrated that sialic acids play a role in EMCV infection of these cells. This is in agreement with previously reported results showing that EMCV binding to rat C6 glioma cells was markedly reduced following exposure of cells to sialidase (Donta et al., 1986). The fact that the binding of the 1086C parental virus to BHK-21 cells was not reduced after sialidase treatment, whereas the binding of the adapted virus was reduced by more than 75%, suggests that adaptation resulted in a selection of virus particles with binding affinity to cell surface sialic acid residues and that the parental virus binds to a different molecule not affected by sialidase treatment. These observations suggest that the mutation K49Q might lead to the acquisition of a new receptor-binding region in the capsid of the adapted virus. Changes in receptor specificity during the course of passage in cell culture have been documented for picornaviruses. Jnaoui et al. (2002) have shown that adaptation of the Theiler’s GDVII virus to CHO-K1 cells correlated with the appearance of sialic acid binding. Similar to our study, this binding capacity was found to result from the mutation of a single amino acid (VP1, 51) located in VP1 CD loop. Although the parental foot-and-mouth disease virus enters cells through an RGD-dependent integrin, multiply passaged virus acquires the capacity to bind heparin and to infect human K-562 cells, which do not express integrin $\alpha$5$\beta$1 (Baranowski et al., 2000). The virus binds to two separate receptors with two separate regions of the capsid that both overlap with neutralization epitopes. Enterovirus 70 variant derived by passage in rhesus monkey kidney cells (EV70-Rmk)
replicates poorly in HeLa cells and utilizes a sialylated molecule other than DAF as cell receptor. In contrast, passage of this variant in HeLa cells leads to isolation of virus (EV70-Dne) that is dependent upon DAF for cell entry and does not replicate in rhesus monkey kidney cells (Kim & Racaniello, 2007). Human rhinovirus type 89 is adapted to use heparin sulfate for cell entry after passages in non-permissive and ICAM-1 receptor-deficient cells (Reischl et al., 2001, Vlasak et al., 2005). Similar to the EMCV-adapted mutant, one consistent amino acid change was found within the BC loop of VP1, which is involved in the binding of minor-group viruses to the low-density lipoprotein receptors. Other mutations were also found in capsid regions that are not surface exposed. The authors have hypothesized that the mutations in buried sites of the capsid may contribute to a loss of virus stability at low pH and at elevated temperature, which might be required for efficient uncoating in the absence of ICAM. Given the buried location of the mutations leucine 142 by phenylalanine and isoleucine 180 by alanine that we found in the BRL cell-adapted virus, we can speculate by analogy to HRV89 that these mutations may induce a reduction in EMCV stability allowing for easier uncoating. Further studies are needed to understand further the requirement of these mutations to EMCV infection in BRL cells.

In conclusion, we adapted EMCV to replicate and to produce CPE in buffalo rat liver-resistant cells. This adaptation appears to result from an increase in virus binding and the acquisition of the ability to bind cell surface sialic acid residues. Adaptation is accompanied by the accumulation of mutations in virus neutralizing site, suggesting a connection between virus antigenic site, virus receptor binding and cell susceptibility.

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REFERENCES


