Expansion of host-cell tropism of foot-and-mouth disease virus despite replication in a constant environment

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Foot-and-mouth disease virus (FMDV) variants adapted to BHK-21 cells showed an expanded host-cell tropism that extended to primate and human cell lines. Virus replication in human HeLa and Jurkat cells has been documented by titration of virus infectivity, quantification of virus RNA, expression of a virus-specific non-structural antigen, and serial passage of virus in the cells. Parallel serial infections of human Jurkat cells with the same variant FMDVs indicates a strong stochastic component in the progression of infection. Chimeric viruses identified the capsid as a genomic region involved in tropism expansion. These results indicate that, contrary to theoretical predictions, replication of an RNA virus in a constant cellular environment may lead to expansion of cellular tropism, rather than to a more specialized infection of the cellular type to which the virus has been adapted.

INTRODUCTION

Genetic variation of microbes is a major determinant of microbial disease emergence (Smolinski et al., 2003). In the case of RNA viruses, variation is favoured by high mutation rates (Drake & Holland, 1999), and may lead to modifications of cell tropism and host range (Baranowski et al., 2003). One or a few amino acid substitutions at the surface of virus particles may result in alteration of receptor recognition and use, and the same virus may acquire the capacity to enter cells via alternative receptors (for reviews, see Baranowski et al., 2003; Bergelson, 2003; Skehel & Wiley, 2000). This is the case for the important animal pathogen foot-and-mouth disease virus (FMDV), a member of the family Picornaviridae and the cause of the most economically important disease of cloven-hooved animals which has recently re-emerged in several areas of the world (Rowlands, 2003). FMDV uses a number of integrins as receptors in cell culture and in vivo (Jackson et al., 2003; Mason et al., 2003). Adaptation of FMDV to cell culture resulted in the selection of mutants with positively charged amino acids at critical sites of the capsid surface, which were capable of entering cells via an interaction with heparan sulfate (Jackson et al., 1996; Sa-Carvalho et al., 1997). As a result of serial cytolitic infections or prolonged persistence in BHK-21 cells, FMDV biological clone C-S8c1 (a representative of European FMDV subtype C1; Sobrino et al., 1983) acquired amino acid substitutions in the capsid that increased the capacity to kill BHK-21 cells, and permitted infection of Chinese hamster ovary (CHO) and human erythroleukaemia K-562 cells (Baranowski et al., 1998, 2000). In contrast with the behaviour of FMDV of serotype O (Jackson et al., 1996; Sa-Carvalho et al., 1997), the modified FMDV C-S8c1 did not require heparan sulfate to express its enhanced virulence and expanded host-cell tropism, as CHO mutant cells deficient in glycosaminoglycans were infected as efficiently as wild-type CHO cells (Baranowski et al., 1998). The amino acid substitutions in the capsid rendered dispensable the Arg–Gly–Asp (RGD) located in the G–H loop of capsid protein VP1, a loop that mediates integrin recognition and antibody binding (Acharya et al., 1989; Baranowski et al., 2001b; Mateu, 1995; Ruiz-Jarabo et al., 1999). Replacements within the RGD triplet led to viable virus only when occurring in the context of the capsid of the modified FMDV C-S8c1, but not of the parental FMDV C-S8c1 (Baranowski et al., 2000, 2001a). However, when mutants deficient in heparin binding were selected from the quasispecies of multiply passaged FMDV, the selected mutants could again use the
integrin-mediated entry pathway, as evidenced by inhibition of virus entry with synthetic peptides representing the G–H loop of VP1 (Baranowski et al., 2000). Thus, C-S8c1 acquired the capacity to use at least three alternative pathways to enter the same cell type: integrins, heparan sulfate, or a third pathway the nature of which is unknown (Baranowski et al., 2000; review in Baranowski et al., 2003).

It was unexpected that an expansion of host-cell tropism, rather than a more specialized use of a single cell type, occurred as a result of replication in the specific environment provided by BHK-21 cells. This observation, along with the extensive distribution of FMDV in nature and the frequent contact of humans with infected animals, prompted us to examine in greater detail the extent of the expansion of cell tropism of these variant FMDVs highly adapted to BHK-21 cells, with regard to infection of human and primate cell lines. Here we show that, as a result of extensive passage in BHK-21 cells, FMDV acquired the capacity to productively infect several human and primate cell lines. Studies with chimeric viruses have identified the virus capsid as an important region for infectivity in human cells. The results have implications for understanding of the expansion of the host range of viruses.

METHODS

Cells, viruses and construction of chimeric viruses. The origins of BHK-21 cells, human K-562 erythroleukemia cells, CHO cells, and the two glycosaminoglycan-deficient CHO cell lines pgsD-677 (N-acetylgalosaminyl- and glucuronosyltransferase-deficient) and pgsA-745 (xylosyltransferase-deficient) have been described previously (Baranowski et al., 1998, 2000; Domingo et al., 1980; Sobrino et al., 1983). Monkey kidney LLC-MK2 cells were kindly provided by F. Sobrino, human epithelial HeLa cells by P. Mason and human lymphoid Jurkat cells by M. A. Alonso. BHK-21, CHO, LLC-MK2 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with non-essential amino acids (Gibco) and 5% fetal calf serum (FCS; Gibco). K-562 cells were grown as suspension cultures in the same medium but in the presence of 10% FCS. Jurkat cells were grown as suspension cultures in RPMI (Gibco) in the presence of 10% FCS.

FMDV C-S8c1 is a plaque-purified derivative of natural isolate C1 Santa Pau-Spain 70 (Sobrino et al., 1983). FMDV C-S8c1p100c10 is a plaque-purified clone derived from a population obtained after 100 serial cytolytic passages of C-S8c1 in BHK-21 cells (C-S8c1p100), described by Martinez et al. (1997) (Fig. 1). FMDV RGG is a monoclonal antibody (mAb)-resistant mutant derived from C-S8c1p100, with an Asp-143→Gly change at the RGD motif of VP1 (Martinez et al., 1997). This is the only amino acid difference between the FMDV C-S8c1p100c10 and FMDV RGG capsids. The FMDV C-S8c1 population at passage 213, termed C-S8c1p213, was used to select MARLS, a mAb-resistant mutant which includes substitution Leu-144→Ser in VP1 (Charpentier et al., 1996; Mateu et al., 1990). These closely related variants belong to the same evolutionary lineage derived from C-S8c1, and were chosen because they share a prolonged history of serial passages in BHK-21 cells (Fig. 1).

Full-length chimeric cDNAs of FMDV O1K encoding type C capsid proteins have been described (Baranowski et al., 1998, 2000). The region transferred to the O1K genetic background spans C-S8c1 genomic positions 1739–4066 (Ncol–HindIII fragment, which corresponds to Ser-33 of VP4 to Lys-62 of non-structural protein 2B) (the numbering of FMDV C-S8c1 genomic residues is as described by Escarmís et al., 1996). Chimeric viruses with the capsid-coding region of C-S8c1, p100c10, RGG and MARLS (parental viruses described in Fig. 1) are termed O1K/C-S8c1, O1K/p100c10, O1K/RGG and O1K/MARLS, respectively.

Infections with FMDV. Procedures for infections of BHK-21 cell monolayers and plaque assays with FMDV have been described previously (Domingo et al., 1980; Sobrino et al., 1983). The number of cells and m.o.i. are indicated for each experiment. Virus was allowed to adsorb at 37°C for 1 h (BHK-21 cells) or 1 h 15 min (HeLa, LLC-MK2 and CHO cells); then monolayers were washed once with 0.1 M phosphate buffer (pH 6.0) to inactivate unadsorbed virions, twice with DMEM, and further incubated in 2 ml DMEM/2% FCS. At different times after infection, samples were taken for titration of infectivity on BHK-21 cells.

Immunofluorescence assays. HeLa cells were grown on coverslips and infected at m.o.i. 1 p.f.u. per cell. Jurkat cells were grown in suspension and infected at m.o.i. 1 p.f.u. per cell, then added to coverslips that had previously been treated with 1 mg ml−1 polyLys (Sigma). Cells were fixed with 4% paraformaldehyde (Merck) at different times after infection. Fixation was blocked with 10 mM glycine (pH 8.5) in PBS, and membranes were permeabilized with 0.2% Triton X-100 in PBS. Immunofluorescence testing was performed using a 1:500 dilution of mAb 3B2, which recognizes FMDV non-structural protein 3A, as the primary antibody. The secondary antibody was Alexa 488 anti-mouse (Molecular Probes), used at a 1:500 dilution. The percentage of cells positive for 3A expression was calculated after counting cells from four microscope fields.
cDNA synthesis, PCR amplification, nucleotide sequencing and RNA quantification. Viral RNA extraction and RT-PCR amplification were performed as described previously (Escarmís et al., 1996). Consensus nucleotide sequences were determined on PCR-amplified DNA in an automated sequencer (ABI Prism 3730). The oligonucleotides used for RT-PCR and nucleotide sequencing have been described previously (Baranowski et al., 1998). RNA quantification was performed with a LightCycler Instrument (Roche) using the LightCycler-RNA Master SYBR Green I kit (Roche), which allows a one-step RT-PCR amplification with Tth polymerase. A standard calibration curve and the minimal amounts of FMDV RNA that can be reliably quantified were determined for each experiment and are indicated in the corresponding figures. The genomic region amplified for quantification was the VP1-coding region, and the primers used were 5'-GAGCTCCGGCTACCTGTGGA-3' (sense, 5' position 3194) and 5'-GGATTGGTTGTGTTTTAAGTGC-3' (antisense, 5' position 3518).

RESULTS

FMDV C-S8c1 adapted to BHK-21 cells can infect human cells

Virus production of C-S8c1 and the BHK-21-adapted variants C-S8c1p100c10, RGG and MARLS (Fig. 1) was compared in hamster (BHK-21, CHO), monkey (LLC-MK2) and human (HeLa, K-562, Jurkat) cell lines. In all cases virus production of the BHK-21-adapted variants was higher than that of the parental virus C-S8c1. The largest differences were observed with CHO (wt and pgsD-677 or pgsA-745 mutants), K-562 and Jurkat cells (Fig. 2). Human Jurkat cells failed to sustain replication of C-S8c1 (no virus production, with a detection limit of 10^2 p.f.u. ml^-1),

![Graphs showing virus production in different cell lines](http://vir.sgmjournals.org)
whereas MARLS yielded titres of about $10^5$ p.f.u. ml$^{-1}$. Virus production by LLC-MK2 and HeLa cells of C-S8c1 was 10- to 100-fold lower than that of p100c10, RGG and MARLS. No cytopathic effect was detected in HeLa cells after infection with C-S8c1, even though virus production reached $10^7$ p.f.u. ml$^{-1}$.

To document the replication of FMDV in HeLa and Jurkat cells – as opposed to a process of absorption–desorption from the cell surface – the amount of FMDV RNA was quantified in the course of infection (Fig. 3a, c). Increases of up to $10^5$-fold over basal levels at the initiation of infection were measured 24 h post-infection (p.i.) for variant FMDVs, but not for the parental C-S8c1. The number of cells positive for expression of non-structural protein 3A at 7–20 h p.i. ranged from 10–38% for variant FMDVs (Fig. 3b, d). These results indicate that variant FMDVs replicate efficiently in human cells. C-S8c1 could replicate in HeLa cells, albeit at a much lower level than the adapted variants, probably due to the very limited number of cells infected (1-9% measured by immunofluorescence). In Jurkat cells no replication or expression of

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**Fig. 3.** FMDV-specific RNA and non-structural protein antigen in HeLa and Jurkat cells infected with C-S8c1 and variants p100c10, RGG and MARLS. (a) Amount of extracellular and intracellular FMDV-specific RNA in infected HeLa cells. About $2 \times 10^5$ cells were infected with $2 \times 10^6$ p.f.u. (in 200 µl) of the indicated FMDVs or the same volume of culture medium (negative control). FMDV RNA was quantified by real-time PCR as described in Methods. Horizontal, thick line indicates limit of detection of FMDV RNA for reliable quantification, determined for each experiment. (b) Immunofluorescence analysis of non-structural protein 3A in HeLa cells at 20 h p.i. with MARLS. Positive and negative cells are present. The percentage of positive cells for C-S8c1, p100c10, RGG and MARLS was 1.9 ± 0.8, 10.4 ± 6.7, 13.1 ± 7.9 and 17.2 ± 2.8, respectively. (c) Total amount of FMDV-specific RNA in a suspension of Jurkat cells either infected with the FMDVs indicated or mock-infected. Symbols as in (a). (d) Immunofluorescence analysis of non-structural protein 3A in Jurkat cells at 7 h p.i. with MARLS. Positive and negative cells are present. The percentage of positive cells was 38.4 ± 6.0. For C-S8c1, p100c10 and RGG, the percentage of positive cells at 15 h p.i. was <0.8, 21.7 ± 9.1 and 27.4 ± 0.13, respectively. Procedures for immunofluorescence and for RNA preparation and quantification are described in Methods.
3A protein could be detected at any time after addition of C-S8c1.

Serial passage of FMDV variants in different cell lines

To determine whether FMDV replication could be sustained during successive passages, HeLa, LLC-MK2, K-562 and Jurkat cells were serially infected with C-S8c1, C-S8c1p100c10, RGG or MARLS (Fig. 4). In most infections, production of variant FMDVs but not of C-S8c1 was sustained during at least five passages, albeit with considerable variation in virus titres. Production of C-S8c1 was maintained only in LLC-MK2 cells. Serial infections of Jurkat cells were studied in more detail by infecting six replicas with MARLS, p100c10 and RGG. MARLS, the virus with the highest number of passages in BHK-21 cells (Fig. 1), maintained progeny production with a range of titres of $2 \times 10^5$ to $4 \times 10^6$ p.f.u. ml$^{-1}$ at the fifth passage. In contrast, this range was $< 10^2$ (undetectable) to $3 \times 10^5$ p.f.u. ml$^{-1}$ for p100c10 and $< 10^2$ (undetectable) to $9 \times 10^3$ p.f.u. ml$^{-1}$ for RGG. These results show that virus yield in the first passage is not a predictor of sustainment of virus production, and suggest there is a stochastic component in the progress of infection, as there were up to $10^5$-fold differences in virus yield at the fifth passage of infections started with the same amount of a virus variant. This was most clearly seen in the infection of Jurkat cells with FMDV p100c10, as in one replica a titre exceeding $10^5$ p.f.u. ml$^{-1}$ was attained at passage 5 but in another replica the virus was undetectable at passage 4 (Fig. 4).

The capsid is a major determinant of the expansion of host-cell tropism of FMDV

To map the genomic region that determines the capacity of variant FMDVs to infect LLC-MK2 and Jurkat cells, infections by chimeric FMDV C–O viruses were carried out...
The behaviour of the chimeras, which included only the capsid-coding region of the different type C variants, was indistinguishable from that of the corresponding parental type C FMDV. This suggests that the capsid is the main determinant of the tropism of FMDV for monkey and human cells.

Analysis of viruses adapted to LLC-MK2, K-562, HeLa and Jurkat cells

To evaluate the stability of the FMDV capsid on replication in primate and human cells, the capsid-coding region of several FMDVs at passage 5 in LLC-MK2, K-562, HeLa and Jurkat cells was compared to the corresponding sequence of the parental viruses used to initiate the serial infections (Table 1). A number of amino acid substitutions were observed in VP1, VP2 and even VP4 — usually a very conserved protein. Except for the replacements in the internal protein VP4 and Leu 208→Glu in VP1, all amino acid replacements occurred at the capsid surface (Lea et al., 1994; Mateu et al., 1994). However, serial productive infections of HeLa and Jurkat cells by some FMDVs did not necessitate additional amino acid replacements in the virus capsid. Passage of RGG in LLC-MK2 cells resulted in selection of a revertant with a G–H loop containing the RGD integrin-binding motif, suggesting that use of integrin receptors may be favoured in this cell line. Therefore, either the replacements accompanying adaptation of FMDV to BHK-21 cells alone, or together with a minimal number of capsid replacements, were sufficient to establish human cell lines as hosts for FMDV.

DISCUSSION

FMDV is a genetically and antigenically variable virus that causes acute or persistent infections in a number of farm and wild animal species, and which spreads enzootically or epizootically into many areas of the world (for reviews, see Rowlands, 2003; Sobrino & Domingo, 2004). In the present report we have documented that serial passage of a biological clone of FMDV, involving a total of 213 passages with 10^7 p.f.u. per passage (Fig. 1), resulted in variant FMDVs with the capacity to infect human cell lines. Some authors have regarded FMDV as a zoonotic virus (Armstrong et al., 1967; Donaldson & Knowles, 2001; Krauss et al., 2003). However, humans are rarely infected, and a number of suspected human cases during the UK 2001 epidemic proved negative.
Table 1. Nucleotide and amino acid substitutions in the capsid of FMDVs passaged in primate and human cell lines

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Virus population†</th>
<th>VP1</th>
<th>VP2</th>
<th>VP3</th>
<th>VP4</th>
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<td></td>
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<td>Nt substitution‡</td>
<td>Aa substitution§</td>
<td>Nt substitution‡</td>
<td>Aa substitution§</td>
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<tr>
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<td>Glu167Lys</td>
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<tr>
<td></td>
<td>p100c10p5</td>
<td>C3839G + T3830A</td>
<td>Leu222Glu</td>
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<td></td>
<td></td>
<td>G3834C</td>
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<td></td>
<td></td>
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<td>C3135Y</td>
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<td>C2450T</td>
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*Origins of cell lines are described in Methods.
†Virus populations obtained after five serial passages of FMDVs in LLC-MK2, K-562, HeLa or Jurkat cells. Viruses for which the capsid protein-coding regions have been sequenced are indicated by arrows in Fig. 4. Procedures for infections and serial passages are described in Fig. 4 and Methods.
‡First letter corresponds to the nucleotide found in the parental C-S8c1, C-S8c1p100c10, RGG or MARLS, and the number gives the nucleotide position in the genome, according to Escarmis et al. (1996). –, No change detected.
§The first amino acid is the one found in the parental C-S8c1, C-S8c1p100c10, RGG or MARLS; amino acid residues are numbered independently for each protein. –, No change detected.
¶The capsid of two most adapted sextuples of FMDV RGG passage 5 in Jurkat cells was sequenced. Amino acid 65 was Asn in one of the two FMDV RGG p5 that produced higher yield in Jurkat cells, and a mixture of Asn and Lys in the other (arrows indicate titres higher than 10⁵ p.f.u. ml⁻¹ at passage 5 in Fig. 4).
Infection of established human cell lines does not imply that the corresponding related epithelial or lymphoid cells in humans will be permissive to the same viruses. Even if they were infected, the virus may be only mildly pathogenic due to barriers preventing completion of the infectious cycle. The same issues have been amply discussed in connection with transmission of swine viruses to humans associated with xenotransplantation (Matthews, 2001). Even if some natural variants could replicate in human cells, they are likely to remain as a minority in the mutant spectra of infected animals, and humans may never come in contact with them. Despite these barriers, the adaptive potential of FMDV and the results presented here encourage avoidance of human exposure to the virus. As additional evidence of adaptability to cross host and cell barriers, FMDV mutants with amino acid substitutions at or around the RGD in VP1, showing modifications of cell tropism, were selected in cattle that had been immunized only partially with experimental peptidic vaccines (Taboga et al., 1997; Tami et al., 2003). Host-range alterations are not associated exclusively with structural FMDV proteins: a single amino acid substitution in non-structural protein 3A mediated adaptation of an FDMV from swine to guinea-pig (Núñez et al., 2001). Mutant FMDVs have been described with atypical pathogenic manifestations such as myocarditis, pancreatitis, diabetes or neurological symptoms, selected in animals infected in the laboratory, although the underlying molecular basis has not been investigated (for reviews, see Domingo et al., 1990; Mason et al., 2003). Perhaps the most dramatic demonstration of the adaptive potential of FMDV at the epidemiological level has been the recent expansion of the strains referred to as FMDV O PanAsia to three continents in less than a decade, displacing previously dominant FMDVs (Rowlands, 2003; Sobrino & Domingo, 2004). Our results of expansion of the host-cell tropism of FMDV to human cells, associated with amino acid substitutions in the virus capsid, provide further justification for considering genetic variation as a major factor in microbial disease emergence (Smolinski et al., 2003).

In the different replicas of variant FMDVs used in parallel infections of Jurkat cells (10^5–10^6 p.f.u. MARLS in each passage; Fig. 4), the repertoire of variants found in parallel mutant spectra is unlikely to be identical. This may contribute to stochastic effects in the capacity for adaptation of a small population of a genetically heterogeneous virus to a given cell type or host. Interestingly, MARLS, the variant with the most prolonged passage history in BHK-21 cells, always produced infection in Jurkat cells. Thus, the more adapted the variant, the broader the tropism. It is possible that in p100c10 and RGG populations a majority of variants could sustain infection in Jurkat cells, but this trait was not yet imposed in the quasispecies. Also noteworthy is the difference between HeLa and Jurkat cells regarding infection by the parental C-S8c1. While there is no evidence of infection of Jurkat cells, HeLa cells produced C-S8c1 progeny as shown by infectivity measurements (Figs 2 and 4) and a significant increase in extracellular and intracellular virus RNA (Fig. 3a). These results are explained by a small proportion of HeLa cells that can be infected (Fig. 3b), but whether infection is supported by minority subsets of susceptible HeLa cells, by rare C-S8c1 variants, or by other influences is not known. These possibilities deserve further investigation as the initial infection did not proceed and the virus became spontaneously extinct.

From the standpoint of RNA virus evolution, it was most remarkable that the expansion of host-cell tropism occurred as a result of replication in the constant biological environment provided by cultured BHK-21 cells, and the longer the replication in the latter cells, the broader the expansion of host-cell tropism (Fig. 1). Extended replication in the same cells would be expected to render a virus showing an increased specialization to enter the same cell type. In contrast, alterations in the environment would be expected to produce disequilibria in the mutant spectra of virus quasispecies, and to expand the variant repertoire for exploration of new phenotypes (Domingo et al., 1990). Therefore, our results indicate that, contrary to theoretical predictions, replication of an RNA virus in a constant environment may lead to trait alterations that are highly relevant biologically. One possibility is that BHK-21 cells actually express multiple potential receptors for FMDV, and that prolonged passage permitted selection of viruses able to use multiple entry pathways into BHK-21 cells; some of these routes may also be represented in established human cell lines. More work is needed to elucidate the molecular basis of cell tropism modifications, which are being documented for an increasing number of animal viruses (Baranowski et al., 2003).

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