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Evolution

Rapid cell variation can determine the establishment of a persistent viral infection

(quasispecies/mutant frequency/foot-and-mouth disease virus/DNA evolution/viral virulence)

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ABSTRACT **Evidence for a mechanism of initiation of viral persistence in which the cell, and not the virus, plays a critical role has been obtained using the important animal pathogen foot-and-mouth disease virus (FMDV). We have developed a virulence assay consisting of quantification of the ability of virus to kill cells and of cells to divide in the presence of virus and to initiate a carrier state. Cells were cured of FMDV at early times following a cytolytic infection of BHK-21 monolayers with FMDV. When cured cells were subjected to the virulence assay they showed an increased ability to survive a second infection by FMDV but not by other RNA viruses. This altered phenotype was maintained as a stable genetic trait. When the virus present in such early surviving cells was used to infect BHK-21 cells, it proved to be as virulent as the initial cytolytic FMDV and, furthermore, its ability to kill BHK-21 cells increased upon replication in the surviving cells. Both the level of genetic heterogeneity and the rate of evolution of FMDV were similar to those previously documented during acute and persistent FMDV infections. The results suggest that, in contrast to most other viral systems, the critical element in the establishment of a persistent infection of BHK-21 cells with FMDV is the ability of the host cells to vary genetically and phenotypically, which promotes selection of cells with increased resistance to virus. The possible relevance of this mechanism to viral persistence *in vivo* is discussed.**

Viruses that are normally lytic for cells can occasionally diverge from this behavior and establish persistent infections in their host organisms or in cultured cells. Persistence of viruses cannot be attributed to a single mechanism since several genetic features of both the virus and the host cells or organisms have been implicated in long-term survival of infected cells (1–5).

Picornaviruses are no exception to such a dual behavior of either killing cells or surviving with them (6–8). Persistent infections in cell culture have been described for most picornaviruses, including Theiler's murine encephalomyelitis virus (TMEV) (9), poliovirus (10–12), rhinovirus (13), hepatitis A virus (14), echovirus (15), and foot-and-mouth disease virus (FMDV) (16, 17). Persistent infections of cloned BHK-21 cells with cloned FMDV C-S8c1 were established by growing the cells that survived a cytolytic infection (16). Following the establishment of persistence, and upon serial passage of the carrier cells, the resident FMDV became progressively more virulent for BHK-21 cells than its parental FMDV C-S8c1. The carrier cells, in turn, evolved to become increasingly resistant to FMDV but not to other RNA viruses, suggesting a coevolution of the cells and of their resident virus (17, 18).

An important role of the host cell in maintenance of a persistent infection in cell culture has been previously documented for reovirus (19, 20) and poliovirus (21, 22), but a role of cell variation in the initiation of persistence has not been established. Here we report experiments aimed at defining the critical element in the initiation of a viral persistent infection. The results indicate that BHK-21 cells, and, more specifically, their ability to vary genetically, are the determinant element in initiating FMDV persistence. This was surprising in view of the well-documented genetic heterogeneity and phenotypic flexibility of RNA virus quasispecies (23–25) and the accumulated evidence that in most other systems either the down-regulation of viral functions or the expression of specific viral genes is the main contributor to limiting cell killing (3). We show that FMDV genomic sequences fluctuate at the onset of persistence but that the virus adapts to the first surviving cells by selecting more virulent—rather than attenuated—variants. These results provide evidence of a mechanism of initiation of viral persistence in which the cell, and not the virus, plays the critical role.

MATERIALS AND METHODS

Cells, Viruses, and Infections. The origin of cloned BHK-21c1 cells, of plaque-purified FMDV C-S8c1, and procedures for cell growth, subcloning of cells by end-point dilution, infection of BHK-21 cell monolayers, release of intracellular virus by freeze-thawing of a cell suspension followed by sonication, and plaque assays with FMDV have been described (16, 17).

Treatment of Cells with Ribavirin and Aphidicolin. Cells were cured of FMDV by treatment with the cytostatic nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4 triazole-3-carboxamide) (26) as described (27). Curing of cells was routinely monitored by the absence of infectious FMDV, by negative hybridization to a labeled oligonucleotide probe (17), or by reverse transcription and PCR amplification using primers corresponding to essential regions of the genome (limit of detection, 0.02 FMDV genome per cell; A.M.M.H., unpublished results). To inhibit cell division during infection of BHK-21 cells with FMDV, the cells were pretreated for 6 hr and throughout the infection with aphidicolin (tetradecahydro-3,9-dihydroxy-4,11b-dimethyl-8,11a-methano-11a-*H*-cyclohepta[*a*]naphthalene-4,9-dimethanol), a specific inhibitor of cellular DNA polymerase α (28). The cytostatic and cytotoxic actions of ribavirin, aphidicolin, or mixtures of the two drugs were analyzed under our culture conditions of BHK-21 cells by determining the number of viable and nonviable cells by trypan blue staining (data not shown). At the concentration used (5 μg/ml), aphidicolin caused a 50% inhibition of FMDV production by BHK-21 cells.

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Abbreviations: ATCC, American Type Culture Collection; p.i., postinfection; FMDV, foot-and-mouth disease virus; IRES, internal ribosome entry site; pfu, plaque-forming unit(s); TMEV, Theiler's murine encephalomyelitis virus.

Table 1. Frequency of cell survivors upon infection of cloned and uncloned BHK-21 cell populations with FMDV

Cells	Frequency of survivors
BHK-21c1	$(1.6 \pm 1.1) \times 10^{-3}$
Subclone B12	$(8.1 \pm 0.5) \times 10^{-4}$
Subclone A5	$(1.2 \pm 0.2) \times 10^{-3}$
Subclone H4	$(1.8 \pm 0.5) \times 10^{-3}$
BHK-21 ATCC	$(1.0 \pm 0.3) \times 10^{-3}$
C_1-BHK-Rc1p86-Rbv (cured)	$(3.3 \pm 0.4) \times 10^{-1}$

Monolayers of 2–4 × 10^6 cells were subjected to the virulence assay by infecting with FMDV C-S8c1. The frequency of cell survivors (mean ± SD) was determined as detailed in the text. Subclones B12, A5, and H4 were derived from our cloned stock BHK-21c1 (17) by isolation of single cells by end-point dilution and growth of single cells to the minimum extent required for freezing and for use in the virulence assay (about 10^7 cells). BHK-21 ATCC were received from the American Type Culture Collection and used without cloning. C_1-BHK-Rc1p86-Rbv are BHK-21 cells persistently infected with FMDV C-S8c1 passaged 86 times and then cured of FMDV by ribavirin treatment (17).

Virulence Assay. The susceptibility of cells to killing by FMDV and the ability of FMDV to kill cells were quantitated by a virulence assay as follows. The cells to be tested were grown until they formed a complete cell monolayer and then were infected with FMDV C-S8c1 at a multiplicity of infection of 0.1 plaque-forming unit (pfu) per cell. This multiplicity of infection was chosen to minimize the possible accumulation of defective interfering RNAs in infected cells. Under these conditions, overt cytopathic effect was seen at 12–24 hr postinfection (p.i.). Then, at 48 hr p.i. the medium was withdrawn and the surviving cells were washed and either fixed (10% formaldehyde), stained (2% crystal violet in 10% formaldehyde), and counted directly under a grid or maintained in culture medium for 3–4 days until surviving cells formed small colonies. Then, the colonies were fixed, stained, and compared with dishes containing known numbers of cell colonies. Standard calibration plates consisted of eight triplicate series of plates with 10^2 up to 10^6 cells per dish grown for 4 days. To test the virus shed by cells without the virus undergoing any amplification, virulence assays had to be frequently performed at multiplicities of infection as low as 10^{-4} pfu per cell, including the reference virus C-S8c1. No significant effect of the multiplicity of infection on the frequency of cell survivors has been seen over the range of 10^{-4} to 10 pfu per cell (ref. 16; unpublished results). All assays were carried out at least in triplicate, always including BHK-21c1 cell monolayers as reference cells. To test the virulence of FMDV the assay was as described above using BHK-21c1 cell monolayers as indicator cells and FMDV C-S8c1 as virulence standard.

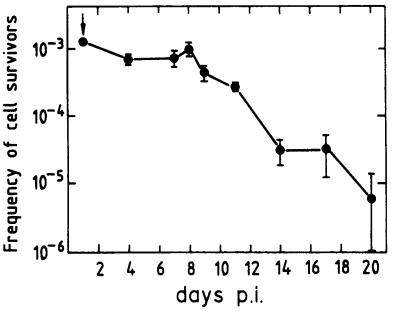

FIG. 1. Virulence of FMDV replicating in cells that survived a cytolytic infection. Monolayers of BHK-21c1 cells were infected with FMDV C-S8c1 as indicated in the legend to Table 1. When the cytopathic effect was complete, the surviving cells were washed extensively and maintained in culture. Virus from the culture medium was isolated at the indicated times p.i. and subjected to the virulence assay. The results (mean ± SD) expressed as frequency of surviving cells are the average of at least three determinations. The virulence of the progeny FMDV C-S8c1 from the initial cytolytic infection was $(1.6 \pm 1.1) \times 10^{-3}$ (average of 22 determinations, arrow).

cDNA Nucleotide Sequencing. The RNA corresponding to the internal ribosome entry site (IRES) and the leader (L) protease was amplified by reverse transcription and PCR using primers 5′-TCGCTTTCGAGTTC-3′ (complementary to FMDV genomic positions 1322–1307) and 5′-CACGAGCTAAGCAGGTTTCC-3′ (corresponding to nucleotides 246–265 of the genomic RNA) as described (29).

RESULTS

Cells That Survive a Cytolytic Infection by FMDV Show a Rapid Increase of Resistance to the Virus. The frequency of cells that survived a cytolytic infection with FMDV C-S8c1 varied from 1×10^{-3} to 3×10^{-4} in different experiments, independently of using cloned or uncloned BHK-21 cell populations (Table 1). Carrier cells cured of FMDV after

Table 2. Survival of BHK-21 cells upon infection with FMDV

Experiment	Cells	Treatment	Frequency of cell survivors upon reinfection with FMDV
1	BHK-21c1	None	$(3.1 \pm 0.3) \times 10^{-3}$
		Ribavirin	$(2.7 \pm 0.8) \times 10^{-3}$
	Infected BHK-21c1	Ribavirin at 12 hr p.i.	$(6.9 \pm 0.4) \times 10^{-3}$
		Ribavirin at 24 hr p.i.	$(9.9 \pm 0.2) \times 10^{-3}$
		Ribavirin at 48 hr p.i.	$(9.5 \pm 0.4) \times 10^{-3}$
		Ribavirin at 4 days p.i.	$(1.0 \pm 0.1) \times 10^{-2}$
2	BHK-21 ATCC	None	$(1.1 \pm 0.1) \times 10^{-3}$
		Ribavirin	$(1.0 \pm 0.3) \times 10^{-3}$
	Infected BHK-21 ATCC	Ribavirin at 48 hr p.i.	$(2.0 \pm 0.1) \times 10^{-3}$
		Ribavirin at 60 days p.i.	$(2.2 \pm 0.3) \times 10^{-1}$
3	BHK-21c1	None	$(3.7 \pm 1.0) \times 10^{-4}$
	BHK-21c1 infected in the presence of aphidicolin	Aphidicolin ribavirin at 36 hr p.i.	$(8.3 \pm 4.9) \times 10^{-4}$
		Aphidicolin ribavirin at 48 hr p.i.	$(2.7 \pm 0.5) \times 10^{-4}$

Monolayers of 2–4 × 10^6 BHK-21 cells were either mock-infected or infected with FMDV C-S8c1 at a multiplicity of infection of 0.1 pfu per cell in the absence (experiments 1 and 2) or the presence (experiment 3) of aphidicolin. The infection was allowed to proceed for the indicated times after infection (p.i.) and then the surviving cells were extensively washed and treated with ribavirin (2 mg/ml for 72 hr) as described (27). In experiment 3 the cells were not maintained in the presence of aphidicolin for longer than 48 hr p.i. to avoid loss of cell viability. The resulting cells, cured of any detectable FMDV (see text), were grown to the minimal extent needed for freezing and to form a monolayer (1×10^7 cells). These monolayers, along with untreated or ribavirin-treated mock-infected cells, were reinfected with FMDV C-S8c1 (0.1 pfu per cell) for 48 hr until the cytopathology was complete. Each value (mean ± SD) is the average of at least four determinations.

prolonged persistence with FMDV showed a 200-fold increase in survival frequency upon infection with FMDV C-S8c1 (Table 1). To establish whether any increase of cell resistance took place at the time of establishment of persistence, we cured cells at 12 hr to several days p.i. and subjected the cured cells to the virulence assay (Table 2). Cells cured as early as 12–48 hr p.i. were 2- to 3-fold more resistant to FMDV C-S8c1 than mock-infected cells subjected to parallel ribavirin treatment.

Basal Cell Resistance Does Not Require Cell Division in the Presence of Virus. Cells with increased resistance to FMDV C-S8c1 either could preexist in the population or be the result of the infection and selection by FMDV. To distinguish between these two possibilities we carried out infections in the presence of the cytostatic agent aphidicolin (see *Materials and Methods*). The results (experiment 3, Table 2) suggest that when cell division was prevented by maintaining aphidicolin prior to and during the cytolytic infection, the resistance of cured BHK-21c1 cells to FMDV C-S8c1 was not significantly different from the resistance manifested by the parental BHK-21c1 cells. The same result was obtained at multiplicities of 10 pfu per cell in infections carried out in the presence of aphidicolin (results not shown). Thus, the basal level of cell resistance seen in any cytolytic infection by FMDV (16) (Table 1) does not require cell division in the presence of FMDV.

The basal level and the enhanced resistance of BHK-21 appear to be specific for FMDV since no significant survival of cells was seen when encephalomyocarditis virus—a picornavirus closely related to FMDV—or vesicular stomatitis virus was used as challenge virus in the virulence assay (results not shown). Increased resistance of BHK-21 cells to FMDV appears to be a stable genetic trait since 10 serial cell passages did not result in any detectable alteration in the degree of resistance of the cells to the virus.

The Virus That Replicates in the Initial Surviving Cells Is Not Attenuated and It Rapidly Becomes Hypervirulent for BHK-21 Cells. To test whether the virus that replicated within the surviving cells at the beginning of persistence was attenuated for BHK-21 cells, we subjected virus shed by carrier cells—at 1–20 days after the initial cytolytic infection, without any amplification—to the virulence assay. The results (Fig. 1) indicate that the virulence of the virus isolated during the first 10 days was either indistinguishable from or higher than the virulence of the parental FMDV C-S8c1. After day 10, sequential viral samples revealed a dramatic increase in virulence, with $<10^{-5}$ frequency of surviving cells in infections with the virus rescued at day 20 (Fig. 1).

This result did not exclude that the virus that remained inside the surviving cells (rather than being shed from the cells) could constitute a distinct subpopulation with decreased ability to kill cells. To test this possibility, the cells that had survived the cytolytic infection were lysed and the liberated virus, without any further amplification, was subjected to the virulence assay. The intracellular virus and the virus shed into the medium during the first 3 hr immediately following cytopathology were indistinguishable from their parental FMDV C-S8c1 with regard to virulence for BHK-21 cells (Table 3). It was still possible that, in spite of the virus being fully virulent, some defective interfering viral RNA could be present that contributed to limiting cell killing. No interference activities were present in the culture medium or in the RNA from cells that showed increased resistance to FMDV (results not shown). We conclude that the virus able to survive a cytolytic infection of BHK-21 cells is not attenuated for these cells and that it rapidly becomes hypervirulent relative to its parental FMDV C-S8c1.

The FMDV Quasispecies During the Establishment of Persistence Shows Rapid Evolution and a High Level of Genetic Heterogeneity. The above results suggest that the determinant element in the initiation of a persistent FMDV infection is not the virus but the ability of BHK-21 cells to vary upon growth from a single cell to provide phenotypes with a slightly increased resistance to FMDV. This was surprising in view of the extensive genetic and phenotypic heterogeneity of FMDV populations documented *in vivo* as well as in cell culture (30). It was possible, however, that the virus that survived in early cells might be unusually homogeneous, thus explaining its inability to provide components of the mutant spectrum with reduced ability to kill cells. To sample the extent of genetic heterogeneity and variation of FMDV during the establishment of persistence we sequenced the IRES (an essential element for initiation of translation) and the RNA region encoding the L protease (an essential protein for polyprotein processing and for cleavage of the p220 component of the cap-binding complex) of sequential virus samples from the early surviving cells. The rate of fixation of mutations was 4×10^{-5} to 6×10^{-5} substitutions per nucleotide per day and the mutant frequency ranged between 1.6×10^{-3} and 1.6×10^{-4} substitutions per nucleotide (Fig. 2). None of these values is lower than previously determined for other evolving FMDV populations (30). Remarkably, in each of the four viral populations analyzed, a silent mutation in the L-coding region (A-1159 → G) became dominant. Repeated silent mutations in coding regions have been recently described during poliovirus persistence (12). The possible significance of this silent replacement in early stages of FMDV persistence is not known.

DISCUSSION

Mechanism of Initiation of Viral Persistence. A role of the host cell in maintenance of viral persistence has been documented for several systems (17, 19–22, 31, 32). In the present report we have provided evidence of an essential role of the host cell at the crucial step of initiation of viral persistence.

Table 3. Virulence of intracellular and extracellular virus that replicated in surviving cells at the onset of FMDV persistence

Virus	Frequency of cell survivors upon infection of BHK-21c1 cells
FMDV C-S8c1	$(2.6 \pm 0.2) \times 10^{-3}$
Extracellular, collected within 3 hr after the peak of cytopathology	$(2.1 \pm 0.3) \times 10^{-3}$
Intracellular, from surviving cells lysed at 3 hr after the peak of cytopathology	$(1.5 \pm 0.2) \times 10^{-3}$

BHK-21c2 cell monolayers ($1–2 \times 10^7$ cells in each of twenty 100-mm Petri dishes) were infected with FMDV C-S8c1. At 36 hr p.i. the cytopathic effect was complete and the surviving cells were washed 20 times with 5 ml of culture medium. Aliquots of the medium from the last washing were titrated. The surviving cells (about 10^4 per infected monolayer) were incubated with culture medium (500 μl per Petri dish) for 3 hr at 37°C. The total number of pfu shed by the cells during this period was 3×10^4 pfu (the last washing of cells included 5% of this amount). Then the cells were washed, collected, resuspended in 0.5 ml of phosphate-buffered saline, and lysed by three cycles of freezing and thawing and sonication. The pfu recovered from lysed cells was 2×10^4. Extracellular and intracellular viruses were used in the virulence assay without prior amplification, as detailed in the text. The results (mean ± SD) are the average of four determinations.

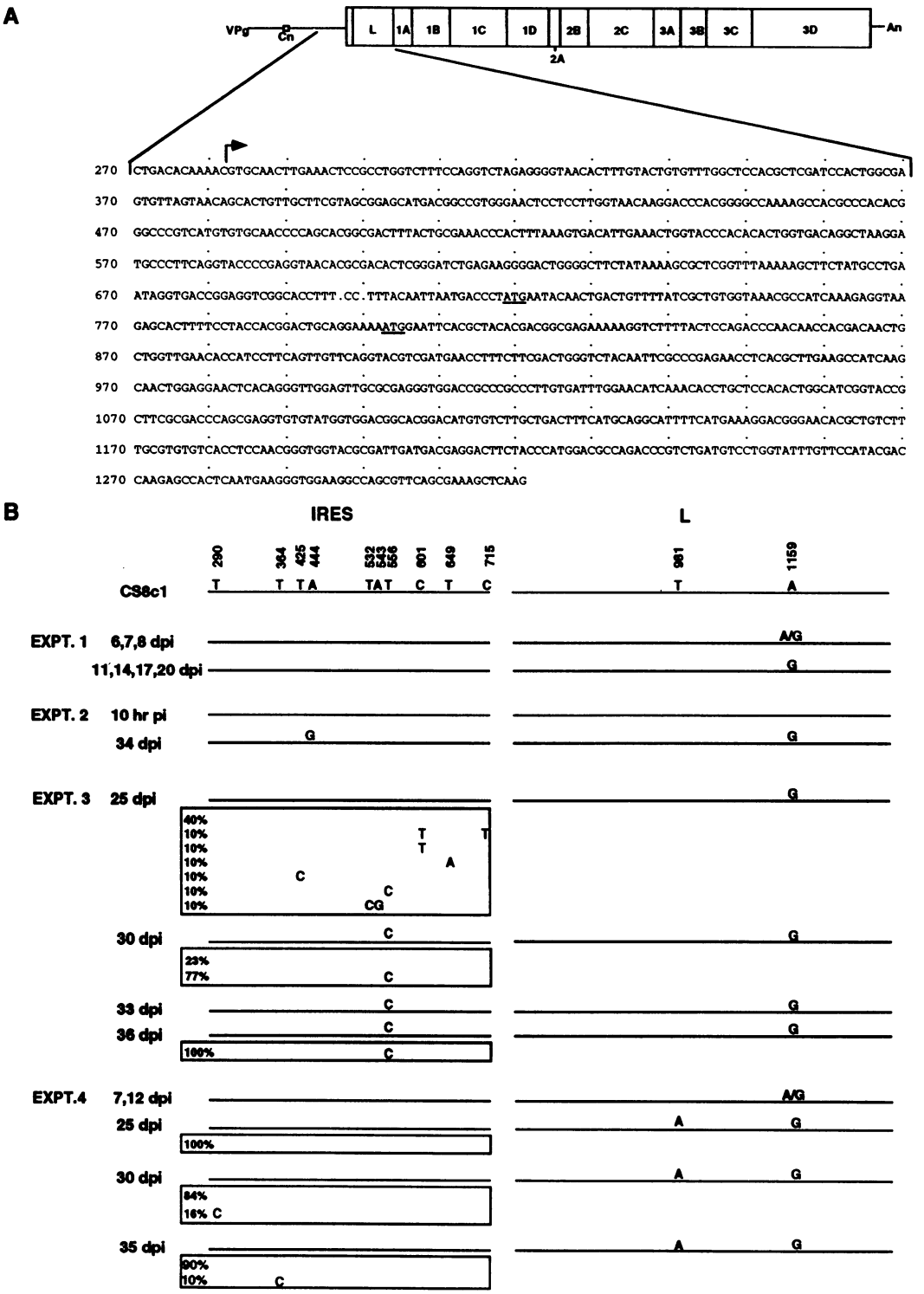

FIG. 2. Temporal evolution of the FMDV quasispecies during the establishment of a persistent infection. (*A*) Scheme of the FMDV genome and location of the 5′ untranslated region and L protease-coding region sequenced in the present study. The sequence for FMDV C-S8c1 has been previously reported by Escarmís *et al.* (ref. 29; unpublished data). Numbering of residues is according to ref. 29, counting as number 1 the first nucleotide to the 3′ side of the polyribocytidylate tract (Cn). The arrow indicates the beginning of the IRES element. The dots at positions 696 and 699 were introduced to align the FMDV C-S8c1 sequence with that of other FMDV strains (29). The two initiation codons that direct synthesis of two forms of L are underlined. (*B*) Summary of nucleotide sequence comparisons of the IRES and L-coding region of FMDV during four independent events of establishment of persistence of BHK-21c1 cells with cloned FMDV C-S8c1 (experiments 1–4). In experiments 1 and 2, only average sequences at the indicated days p.i. (dpi) were analyzed. Average sequences are depicted as lines on which only those residues that vary relative to the C-S8c1 sequence (top line) are indicated. In experiments 3 and 4, in addition to average sequences, individual molecular clones were also sequenced from pUC19 recombinants (box below the corresponding average sequence); in each case 10 clones were sequenced, except for the samples at 30 and 36 dpi, experiment 3, for which 13 and 14 clones, respectively, were analyzed; the percentage of clones with a nucleotide sequence is given in each box. The total number of residues screened was 46,490. It cannot be excluded that the six substitutions found only once in the clonal analyses could result from misincorporations during reverse transcription or *Taq* amplification; elimination of these unique replacements from the calculations decreased only the estimated levels of heterogeneity of population 25 dpi, experiment 3, by a factor 3.5 (see text). The silent replacement A-1159 → G was not found in any of six samples of FMDV C-S8c1 passaged 2, 6, and 10 times in lytic infections of BHK-21 cells (results not shown).

The small increase in resistance seen in cells cured at 12 hr to 4 days p.i. (Table 2, experiment 1) could result from the clonal expansion of the variant cells present in any clonal BHK-21 population (Table 1) freed from fully susceptible cells in the first infection. However, the small increase could also be the result of additional cell variation and further selection by FMDV, a process that becomes apparent when cells that survive cytolysis are allowed to grow for prolonged periods of time prior to curing (Table 1, C_1-BHK-RC1p86-Rbv cells, and Table 2, experiment 2). Irrespective of the selection by FMDV being exerted during or immediately following a cytolytic infection, the essential feature that determined cell survival was the ability of the cells to vary genetically in the course of growth from a single cell to a monolayer (Tables 1 and 2). Generation of cell diversity may be favored by a certain degree of cellular transformation—with its inherent general genetic instability (33)—documented for established cell lines, thus providing BHK-21 cells with an expanded range of phenotypes. Additional genetic lesions in the cells could also be induced by the replication of FMDV by an as yet unidentified mechanism.

The possibility that cell variation could contribute to initiation of viral persistence in multicellular organisms must be considered since cell heterogeneity has been described for normal tissues (34, 35). Even though observations in cell culture may not necessarily apply to entire organisms, it must be considered that epithelial cells—the target of FMDV infection—divide and are replaced at considerable rates. Thus, cell variation could also play a role in the initiation of viral persistence in rapidly dividing cells *in vivo*.

Were Previously Observed Phenotypic Alterations of Viruses Essential for Initiation of Persistence? The FMDV replicating in the initially surviving cells was not only fully virulent, but it showed neither a *ts* character nor a small-plaque phenotype (results not shown), traits that become dominant at later stages of persistence (16). These results with FMDV pose the question of whether in other host-virus systems the genetic lesions frequently observed in the persistent virus were essential for initiation of persistence (3) or were a consequence of the optimization of virus to survive in carrier cells once persistence had already been initiated by other mechanisms. It is clear from our results that the quasispecies structure and consequent adaptability of RNA genomes (23–25, 30) do not justify any generalization on the participation of viral quasispecies, rather than the seemingly more static cellular DNA genomes, in initiation of viral persistence.

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