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Coevolution of Cells and Viruses in a Persistent Infection of Foot-and-Mouth Disease Virus in Cell Culture

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Virus and cells evolve during serial passage of cloned BHK-21 cells persistently infected with foot-and-mouth disease virus (FMDV). These carrier cells, termed C_1-BHK-Rc1 (J. C. de la Torre, M. Dávila, F. Sobrino, J. Ortín, and E. Domingo, Virology 145:24–35, 1985), become constitutively resistant to the parental FMDV C-S8c1. Curing of late-passage C_1-BHK-Rc1 cells of FMDV by ribavirin treatment (J. C. de la Torre, B. Alarcón, E. Martínez-Salas, L. Carrasco, and E. Domingo, J. Virol. 61:233–235, 1987) did not restore sensitivity to FMDV C-S8c1. The resistance of C_1-BHK-Rc1 cells to FMDV C-S8c1 was not due to an impairment of attachment, penetration, or uncoating of the particles but to some intracellular block that resulted in a 100-fold decrease in the amount of FMDV RNA in the infected cells. FMDV R59, the virus isolated from late-passage carrier cells, partly overcame the cellular block and was more cytolytic than FMDV C-S8c1 for BHK-21 cells. Sequencing of the VP1 gene from nine viral clones from C_1-BHK-Rc1 cells showed genetic heterogeneity of 5×10^{-4} substitutions per nucleotide. Mutations were sequentially fixed during persistence. In addition to resistance to FMDV C-S8c1, C_1-BHK-Rc1 cells showed a characteristic round cell morphology, and compared with BHK-21 cells, they grew faster in liquid culture, were less subject to contact inhibition of growth, and had an increased ability to form colonies in semisolid agar. Reconstitution of a persistent infection was readily attained with late-passage C_1-BHK-Rc1 cells and FMDV C-S8c1 or FMDV R59. The results suggest that coevolution of BHK-21 cells and FMDV contributes to the maintenance of persistence in cell culture.

Foot-and-mouth disease virus (FMDV) is an aphthovirus that causes an acute infection of cloven-hooved animals that can also occasionally produce a persistent, inapparent infection of ruminants (24, 33), the latter by mechanisms that are largely unknown. We established BHK-21 cell lines persistently infected with FMDV type C_1 (9) to provide a system in which the basis of long-term virus and cell survival can be explored. One such cell line was established with cloned BHK-21(c-13) cells and plaque-purified FMDV C-S8c1 (28). This line was termed C_1-BHK-Rc1 to indicate the FMDV serotype (C_1), the resistance (R) of the cells to superinfection by FMDV, and the initial BHK-21 cell clone (c1). Upon serial passage of C_1-BHK-Rc1, two distinct stages were identified in the cultures. During the first 60 to 100 passages, infectious FMDV is always detected in the culture medium at titers that decrease from 10^5 to 10^7 to about 10^2 PFU/ml. At later passages, a second stage is reached in which no infectious virus is detected, but FMDV-specific RNA and antigens may be present at a low level and the cells maintain their specific immunity to FMDV (9).

For other virus-cell systems, the establishment and maintenance of a persistent infection in cell culture have been attributed to various genetic changes in the virus including the presence of defective interfering particles (15), or small-plaque virus variants often exhibiting an RNA^- phenotype (36). In other cases, evidence for a role of the host cell or of both cell and virus has been provided (1, 25, 26), indicating that multiple, complex mechanisms may lead to viral persistence.

FMDV RNA from C_1-BHK-Rc1 cultures showed nucleotide sequence variations at an average of 0.3% genomic residues, as quantitated by T1-oligonucleotide mapping of selected FMDV RNA segments (9). At least some of the changes affected viral behavior, since FMDV from carrier cultures were temperature sensitive (*ts*) and formed small plaques on cell monolayers (9). We assumed that such phenotypic alterations reflected defects in the virus replication cycle or in virion stability that resulted in limited cytopathology, permitting increased cell survival. However, when monolayers of BHK-21 cells were infected in liquid culture medium with FMDV isolated from carrier cultures at passage 50 to 80, the viral replication cycle was completed in a shorter time than in a standard infection with FMDV C-S8c1 and the virus yield per cell was normal. Also, the number of cells that survived the infection was 10^2- to 10^3-fold lower than the number in infections with FMDV C-S8c1 (9; unpublished experiments). The increased cytolytic nature of those viruses was not the anticipated result of selective pressures acting during a persistent infection. This prompted us to search for cell alterations during serial passage of C_1-BHK-Rc1 cells. In the present report, we show that evolution of both FMDV and the host BHK-21 cells occurs during persistence in vitro. Cell evolution is documented by several inheritable, stable changes that include increased resistance to FMDV. The analyses suggest that the FMDV carrier state is maintained by a combination of virus and cell genetic variations.

MATERIALS AND METHODS

Cells and viruses. BHK-21(c-13) cells were obtained from L. Carrasco and cloned by serial dilution in microtiter plates.

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Wells containing a single cell were identified by visual inspection, allowed to form a monolayer, passaged, and frozen as usual. The procedures for cell culture have been described (9, 10, 28). FMDV C-S8c1 is a three-times-plaque-purified FMDV C-S8 strain (28), a field isolate from diseased swine (Spain, 1970) (10, 28, 29). Cloned BHK-21c1 cells and FMDV C-S8c1 were used to establish the persistently infected cell lines as described (9).

The cell and virus nomenclature used in the present paper is as follows. The persistently infected BHK-21 cells, also referred to as carrier cultures, are termed C_1-BHK-Rc1, as in previous reports (8, 9), followed by the passage number (examples: C_1-BHK-Rc1p60 and BHK-21c1p60 are persistently infected and standard cloned BHK-21 cells at passage 60, respectively). Cells were cured of any detectable FMDV by treatment with the antiviral agent ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) for 72 h with 150 μg/ml in the culture medium (8). Ribavirin treatment is indicated by the suffix -Rbv; example, C_1-BHK-Rc1p60-Rbv are carrier cells at passage 60 treated with ribavirin. The present study involves C_1-BHK-Rc1 cultures at the stage of infectious FMDV production. Passages 1 to 25 and above 50 are referred to as early and late passages, respectively. Viruses produced endogenously by carrier cultures are designated with R followed by the cell passage number (example: FMDV R58 is the virus shed by culture C_1-BHK-Rc1-p58).

Cell passage involved washing of the monolayer, detachment of the cells by trypsin treatment, homogenization of the cell suspension, and plating of about 10% of the number of cells required to reach confluence after 3 to 4 days of incubation. To measure cell growth in liquid culture, a cell suspension was diluted in Dulbecco modified Eagle medium (DMEM) with 5% fetal calf serum (FCS); 10^6 cells were plated per 60-mm petri dish (in 5 ml of DMEM with 5% FCS) or per 100-mm petri dish (in 15 ml of DMEM with 5% FCS). At the desired times, cells from the culture medium and from the monolayer were treated with trypsin, collected by centrifugation, washed and diluted in DMEM–5% FCS. Viable cells (trypan blue staining) were counted in the hemacytometer. Cell growth in semisolid agar was tested essentially as described by Macpherson and colleagues (20, 21), with an agar base layer of 5 ml in 60-mm petri dishes, composed of 0.5% agar, 0.4× DMEM, yeast hydrolysate (29 mg/ml), and 10% FCS. The upper agar layer was 1.5 ml of a mixture of 1 volume of the cell suspension to be tested (5×10^1 to 5×10^3 cells in DMEM–10% FCS) and 2 volumes of agar medium (final agar concentration, 0.3%). The agar mixtures were prepared at 37°C, added to the plates, allowed to solidify at room temperature, and then left undisturbed for 7 to 10 days at 37°C in a CO_2 incubator until the counting of cell colonies. Values are the result of three platings with different initial cell numbers, each one carried out in duplicate.

Infections. Procedures for infection with FMDV, plaque assays, labeling of virus with $^{32}P_i$, FMDV purification, and RNA transfection have been described (8–10, 28). To prepare ^{35}S-labeled virus, BHK-21 cell monolayers were infected at a multiplicity of infection of 10 to 20 PFU/cell in medium without methionine. At 4 h postinfection (p.i.), [^{35}S]methionine (>800 Ci/mmol) was added to the medium at 500 μCi/ml. Viruses were harvested and purified by sucrose gradient sedimentation (10, 28).

Attachment of FMDV to cells was measured by the kinetics of association of PFU or of [^{35}S]methionine-labeled virus to cells, following published procedures (5, 19). Internalization of ^{32}P-labeled FMDV was quantitated as described by Joklik and Darnell (17).

DNA probes and molecular hybridization. Restriction enzyme fragments from the FMDV cDNA inserts of plasmids pBR-VFAC$_1$-18.5 and -B75 (22, 34) were labeled by nick translation and used to quantitate FMDV-specific sequences in total-cell RNA by molecular hybridization (2, 9, 31). A terminally 5′-labeled oligodeoxynucleotide, 5′-GGGG TGAGACCCTAG(T,C)GCCCCCTTTCAA-3′, complementary to residues 1 to 28 of the 5′ terminus of FMDV RNA O_1 and A_{10} (23), and the complementary 5′-TTGAAAGGGG GC(A,G)CTAGGGTCTCACCCC-3′ were used as probes to quantitate plus- and minus-strand FMDV RNA, respectively. RNA was heat-denatured in 2.2 M formaldehyde before application to a nylon-based membrane (Byodyne).

RNA sequencing. Purified FMDV RNA was sequenced by oligodeoxynucleotide primer extension and dideoxy chain termination (29, 37). The primers used were complementary to positions 111 to 126, 264 to 281, and 471 to 490 of the VP1 gene and to positions 34 to 54 of the P14 gene (sequences and numbering are from reference 29). Some of the band ambiguities seen in the autoradiograms were eliminated by incubating the reaction mixtures with terminal deoxynucleotidyl transferase (7). Ambiguous positions not resolved by this procedure are indicated on the sequences, and they have been treated as invariant in the computation of mutations.

RESULTS

BHK-21 cells persistently infected with FMDV become increasingly resistant to the virus. Cell line C_1-BHK-Rc1, persistently infected with FMDV C-S8c1, is resistant to superinfection by FMDV types C_1 (C-S8), O_1, and A_5 but not by encephalomyocarditis virus (EMCV), vesicular stomatitis virus, or Semliki Forest virus (9). To test whether this specific immunity to FMDV was dependent on the presence of the virus in the carrier cultures, the cells were cured of any detectable FMDV-specific sequences or antigens by treatment with ribavirin (8). Monolayers of BHK-21c1, C_1-BHK-Rc1p21, and C_1-BHK-Rc1p58 treated or untreated with ribavirin were infected with FMDV C-S8c1 or with the viruses from the persistently infected cultures, FMDV R10 or R59. The kinetics of viral production (Fig. 1) indicated that (i) C_1-BHK-Rc1p21 but not -p58 regained full sensitivity to FMDV C-S8c1 after ribavirin treatment; (ii) C_1-BHK-Rc1p58-Rbv cells produced about 10^4-fold fewer PFU than BHK-21 or C_1-BHK-Rc1p21-Rbv cells upon infection with FMDV C-S8c1; this decreased titer was paralleled by absence of cytopathic effect; (iii) FMDV R59 partly overcame the resistance of C_1-BHK-Rc1p58-Rbv cells observed with FMDV C-S8c1; and (iv) the maximum virus titers quantitated intracellularly and in the culture medium were attained at earlier times in infections of BHK-21c1 by FMDV R59 than by FMDV C-S8c1. The results suggest that genetically altered BHK-21 cells, constitutively more resistant to FMDV C-S8c1 than the parental BHK-21c1 cells, were selected during serial passage of the carrier cultures. The shortened replication cycle of FMDV R59 in BHK-21c1 cells and its increased capacity to infect C_1-BHK-Rc1-Rbv cells identifies a phenotypic alteration of FMDV R59, additional to the *ts* and small-plaque characters observed previously (9).

To test whether the increased resistance of late-passage carrier cells to FMDV was due to some block at the early steps of infection or during intracellular viral replication, we measured cell attachment and internalization of virus and quantitated intracellular levels of FMDV RNA.

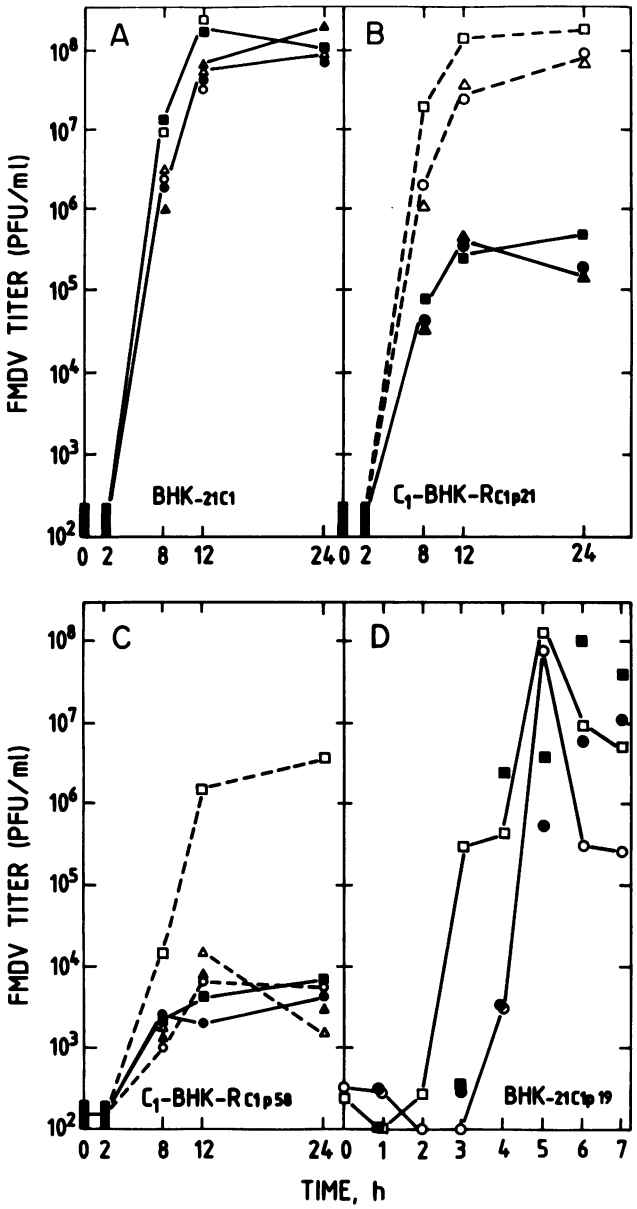

FIG. 1. Infection of BHK-21c1 and C_1-BHK-Rc1 cells with FMDV. (A to C) Virus was added at 3 to 5 PFU/cell. After 1 h of adsorption at 37°C, monolayers were washed with PBS, pH 6.0, and then with medium. The virus titer in the culture medium was determined at the indicated times p.i. Symbols: ●, FMDV C-S8c1; ▲, FMDV R10; ■, FMDV R59. In panel A, solid and open symbols correspond to infection of BHK-21c1p4 and -p60, respectively. In panels B and C, open symbols correspond to infection of the indicated ribavirin-treated cells. (D) Determination of intracellular FMDV. Monolayers (about 10^6 cells) were infected at 5 to 10 PFU/cell with FMDV C-S8c1 (○) or FMDV R59 (□). At the indicated times the cells were washed, frozen and thawed three times, sonicated, clarified (volume, 1 ml), and titrated. Solid symbols indicate virus titers in the culture medium. In each panel, the cells used are indicated. No differences in viral yield were obtained in infections with EMCV (not shown).

Unaltered binding and penetration of FMDV into C_1-BHK-Rc1 cells. The kinetics of attachment of FMDV C-S8c1 and R59 to BHK-21c1, C_1-BHK-Rc1p21, and -p59 cells, treated or untreated with ribavirin, as measured by the association of PFU to cell monolayers, was indistinguishable for all cell-virus combinations tested (Fig. 2). The same conclusion was reached from the kinetics of binding of purified [^{35}S]FMDV to cells. In the latter experiments, plateau values of 40% to 50% cell-attached radioactivity were attained at 20 to 40 min of attachment in all cases, except for HeLa cells, which gave plateau values of about 10% (data not shown).

To determine whether the internalization of FMDV into C_1-BHK-Rc1 cells was impaired, the fate of [^{32}P]FMDV C-S8c1 or R59 in BHK-21c1p6, C_1-BHK-Rc1p58, and -p58-Rbv was followed (17) (Table 1). All cells yielded a similar progression of cell-bound radioactivity with the time of incubation at 37°C. Likewise, the distribution of radioactivity between acid-soluble, RNase-resistant and -sensitive fractions—the latter indicative of the extent of uncoating (17)—was indistinguishable for all infections tested. If the cellular block is not exerted at the attachment or penetration steps, it should not be overcome by FMDV RNA transfection. The number of productively transfected, late-passage C_1-BHK-Rc1-Rbv cells is expected to be severalfold lower than the corresponding number for BHK-21c1 cells, considering the viral yields of the two cells: FMDV-infected BHK-21 cells produce 43 to 134 PFU/cell (28), while the viral production by C_1-BHK-Rc1p58-Rbv was 10^4-fold lower (compare Fig. 1A and C). Thus, about 10^2 infected C_1-BHK-Rc1p58-Rbv cells did not yield infectious FMDV. Determinations of the number of productively transfected BHK-21c1 and C_1-BHK-Rc1p62-Rbv cells, as well as the kinetics of viral production by transfected cells (Table 2), are in agreement with the above prediction. The results suggest that the decreased FMDV production by late-passage C_1-BHK-Rc1 cells is not caused by inhibition of an early event (attachment, penetration, or uncoating) in infection.

Decreased intracellular FMDV RNA in infections of late-passage C_1-BHK-Rc1-Rbv cells. The amount of FMDV-specific RNA present in uninfected and infected BHK-21c1 and C_1-BHK-Rc1 cells was quantitated by dot-blot hybridization to FMDV cDNA probes (Fig. 3). The amount of FMDV RNA in C_1-BHK-Rp56-Rbv cells infected with FMDV C-S8c1 was about 100-fold lower than in infected BHK-21c1 cells (Fig. 3B, D, and L). For cells not treated with ribavirin, the endogenous FMDV RNA obscured a possible amplification of viral RNA due to the added FMDV (Fig. 3E and F, and Fig. 3I and J). C_1-BHK-Rc1p17-Rbv regained full capacity to support FMDV C-S8c1 RNA synthesis (Fig. 3B, D, and H). Infection of BHK-21 cells with FMDV Rp58 resulted in a maximum amount of intracellular FMDV-specific RNA at 2 h p.i. rather than at 5 to 8 h p.i. as observed with FMDV C-S8c1 (Fig. 3M and N). Thus, the limitation in FMDV production by late-passage C_1-BHK-Rc1 cells (Fig. 1) was paralleled by a decrease in the amount of FMDV RNA in the infected cells (Fig. 3). The proportion of minus-strand FMDV RNA, measured by dot-blot hybridization to complementary oligodeoxynucleotides, in FMDV C-S8c1-infected BHK-21c1 or C_1-BHK Rc1p58-Rbv cells was very similar, and it amounted to 2 to 10% of the total FMDV RNA (results not shown). We conclude that cells that are increasingly resistant to FMDV by some specific mechanism acting during intracellular FMDV replication dominate the carrier cultures at late passages. In turn, FMDV variants able to overcome in part the intracellular block(s) were selected.

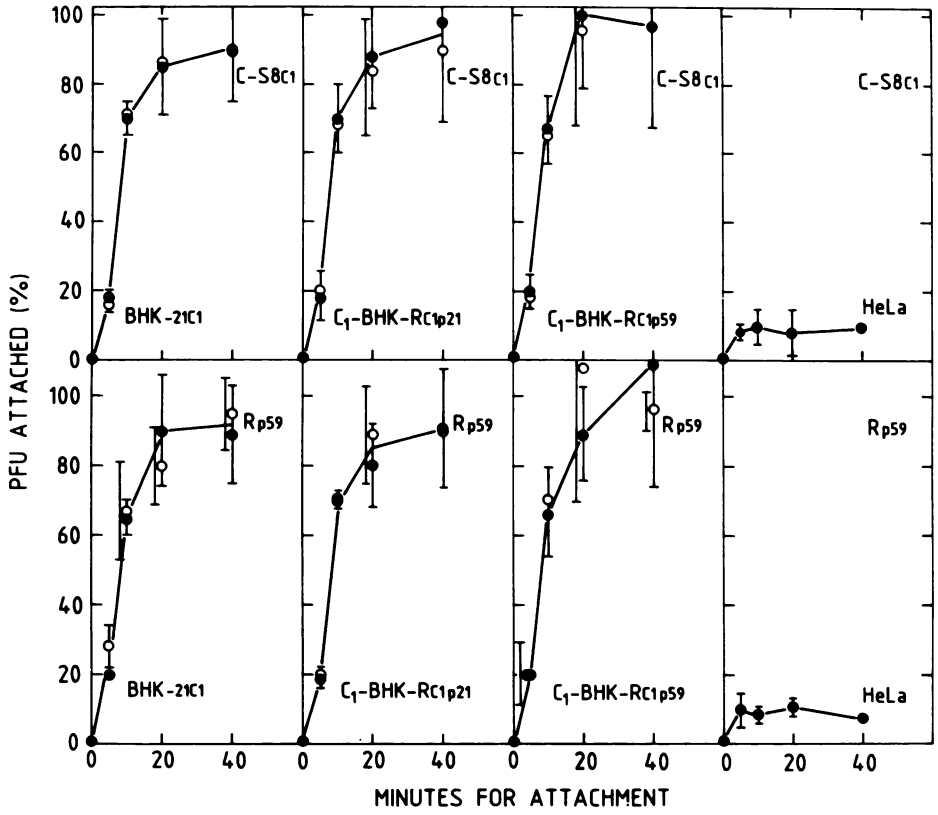

FIG. 2. Attachment of FMDV to cell monolayers. For each assay, 2×10^6 PFU of FMDV (volume, 200 μl) were added to a monolayer of 10^6 cells in a 35-mm petri dish and maintained at 0 to 4°C. At the indicated times, the monolayers were washed with cold PBS and resuspended in 0.5 ml of 10 mM Tris hydrochloride (pH 8.0)–0.5% SDS. The cell suspensions were subjected to three cycles of freezing and thawing and centrifuged for 5 min at 12,000 × *g*, 4°C. The supernatants were diluted in DMEM–2% FCS and titrated by plaque formation on BHK-21 cell monolayers. The amount of virus attached is given as the percentage of the input PFU. In each panel, the virus under analysis (top right) and the cell line (bottom) are indicated. In the two left panels, solid and open circles correspond to values for BHK-21c1p4 and -p60 cells, respectively. In the other panels, solid and open circles correspond to values obtained with cells untreated and treated with ribavirin (8), respectively. All assays were carried out in duplicate plates, and each sample was tested in duplicate. The value at 0 min was less than 1% in all cases. When significant, the standard deviation is indicated.

Fixation of mutations in the FMDV genome. The assignment of specific mutations to a phenotypic behavior is particularly difficult in the case of RNA viruses because of their genetic heterogeneity and variation (10a–12, 16). The sequencing of the VP1 gene of FMDV R19, R58, R100, and nine clones derived from population R58 was aimed at estimating extents of genome variation, not at relating mutations to phenotypes. Three sequential mutations, each one leading to an amino acid substitution, were fixed in the VP1 gene during persistence (Fig. 4): C(443)→A (T-148→K); G(581)→A (G-194→D), and A(247)→G (T-83→A). RNA from eight viral clones, derived by plaque isolation from population R58, yielded sequences indistinguishable from that of the average sequence in R58. However, clone 6 included three substitutions: T(180)→C, silent; C(410)→T, T-136→I; and G(474)→A, silent. Both the estimated rate of fixation of mutations and the level of genetic heterogeneity of FMDV R58 were comparable to the corresponding values derived previously for serial passage of FMDV in lytic infections (28).

Thus, genetic changes occurred in both the cells and the resident FMDV in the course of passaging C_1-BHK-Rc1 cultures.

Altered morphology and growth characteristics of late-passage C_1-BHK-Rc1 cells. Microscopic examination of monolayers of C_1-BHK-Rc1 revealed a gradual change towards a round cell morphology (R-morphology) with passage number (Fig. 5). Ribavirin treatment of monolayers at passages 2 to 20 (early passages) resulted in restoration of the fusiform, BHK-like morphology (B-morphology). In contrast, treatment of late-passage C_1-BHK-Rc1 cells with ribavirin did not substantially alter their characteristic R-morphology (Fig. 5). The latter was always observed after freezing, thawing, and extensive passaging of the cells.

Comparison of the growth characteristics of BHK-21c1 and C_1-BHK-Rc1 cultures indicated (Fig. 6) that (i) ribavirin-treated and untreated cells showed the same growth rate, (ii) late-passage carrier cells grew with a doubling time of about 12 h, whereas the value for BHK-21c1p4, -p60, or early-passage carrier cells was about 21 h, and (iii) when confluency was reached, the number of viable C_1-BHK-Rp58 cells still increased, while the number for BHK-21c1 or C_1-BHK-Rp19 decreased slightly (compare 72 and 96 h in Fig. 6). Similar conclusions were obtained in experiments with cells after freezing, storage, or thawing or with different passage histories. In addition to increased growth rate, C_1-BHK-Rc1 cells at late passages tended to pile up on the monolayers, suggesting decreased contact inhibition of growth. Since these properties are compatible with a transformed phenotype, we tested the growth of cells in semisolid

TABLE 1. Internalization of ^{32}P-FMDV[a]

Cell line	FMDV	Time at 37°C (min)	Mean radioactivity (%) ± SD					
			Culture medium		Cell associated			
			Acid soluble	Acid insoluble	EDTA-washed, acid insoluble	Acid soluble	RNase resistant	RNase sensitive
BHK-21c1p6	C-S8c1	0	2.9 ± 0.6	0.8 ± 0.1	53.7 ± 1.6	0.7 ± 0.2	35.0 ± 0.3	1.1 ± 0.1
			(2.8 ± 0.1)	(0.9 ± 0.1)	(55.2 ± 0.6)	(0.9 ± 0.0)	(36.4 ± 0.5)	(1.0 ± 0.0)
		10	3.2 ± 0.0	34.9 ± 0.3	18.1 ± 0.8	4.7 ± 0.2	27.8 ± 0.5	3.0 ± 0.0
			(4.9 ± 0.2)	(33.3 ± 0.2)	(16.6 ± 0.7)	(4.9 ± 0.1)	(28.2 ± 0.5)	(2.9 ± 0.2)
		20	4.0 ± 0.3	45.5 ± 2.9	10.0 ± 0.3	8.9 ± 0.1	9.9 ± 3.1	5.8 ± 0.0
			(4.0 ± 0.1)	(45.0 ± 0.5)	(10.0 ± 0.3)	(9.2 ± 0.3)	(11.6 ± 0.0)	(6.2 ± 0.7)
		40	6.5 ± 0.7	46.5 ± 0.7	0.9 ± 0.0	16.6 ± 1.2	10.1 ± 0.3	7.0 ± 0.2
			(7.0 ± 0.3)	(47.1 ± 0.5)	(1.1 ± 0.1)	(18.1 ± 0.4)	(9.9 ± 0.0)	(7.2 ± 0.2)
		60	8.1 ± 0.2	54.6 ± 2.5	0.7 ± 0.1	20.3 ± 0.5	8.1 ± 0.1	5.7 ± 0.3
			(7.9 ± 0.1)	(54.0 ± 3.1)	(0.8 ± 0.0)	(19.1 ± 0.9)	(8.1 ± 0.1)	(6.2 ± 0.4)
C_1-BHK-Rc1p58	C-S8c1	0	3.1 ± 0.2	0.9 ± 0.1	54.0 ± 0.3	0.7 ± 0.9	34.3 ± 0.4	1.2 ± 0.1
			(3.0 ± 0.0)	(1.1 ± 0.1)	(48.5 ± 1.3)	(0.7 ± 0.1)	(35.1 ± 0.2)	(0.9 ± 0.1)
		10	4.7 ± 0.2	35.0 ± 0.2	17.3 ± 0.5	7.1 ± 0.1	27.1 ± 0.4	4.05 ± 0.14
			(5.4 ± 0.1)	(34.8 ± 0.3)	(16.4 ± 1.0)	(6.9 ± 0.3)	(28.0 ± 0.2)	(2.8 ± 0.25)
		20	3.9 ± 0.1	44.9 ± 0.7	10.8 ± 0.5	8.8 ± 0.4	12.2 ± 0.4	6.1 ± 0.0
			(4.1 ± 0.2)	(38.8 ± 1.1)	(9.9 ± 0.2)	(7.6 ± 0.2)	(11.9 ± 0.4)	(5.9 ± 0.3)
		40	6.9 ± 0.3	47.1 ± 0.1	1.7 ± 0.1	18.1 ± 0.1	10.0 ± 0.4	6.8 ± 0.0
			(7.0 ± 0.0)	(46.6 ± 1.2)	(1.0 ± 0.0)	(18.1 ± 0.0)	(10.2 ± 0.9)	(5.6 ± 0.3)
		60	8.8 ± 1.0	54.4 ± 1.3	0.9 ± 0.0	19.7 ± 0.1	6.8 ± 0.3	6.2 ± 0.4
			(7.8 ± 0.1)	(55.7 ± 1.2)	(0.9 ± 0.0)	(23.9 ± 0.2)	(7.9 ± 0.0)	(5.9 ± 0.1)
C_1-BHK-Rc1p58-Rbv	C-S8c1	0	3.0 ± 0.0	1.0 ± 0.0	46.7 ± 1.9	1.0 ± 0.1	35.2 ± 0.1	1.2 ± 0.0
			(2.6 ± 0.2)	(0.7 ± 0.0)	(55.2 ± 0.4)	(0.8 ± 0.2)	(34.1 ± 1.5)	(0.9 ± 0.2)
		10	5.0 ± 0.2	35.1 ± 0.2	16.9 ± 0.4	5.1 ± 0.2	27.9 ± 0.2	3.0 ± 0.0
			(4.9 ± 0.2)	(33.8 ± 1.7)	(17.1 ± 0.2)	(4.9 ± 0.2)	(28.0 ± 0.3)	(5.0 ± 0.3)
		20	3.9 ± 0.2	45.0 ± 0.4	9.6 ± 0.5	8.9 ± 0.2	12.0 ± 0.2	5.2 ± 0.3
			(5.0 ± 0.0)	(45.2 ± 0.2)	(8.1 ± 0.9)	(9.1 ± 0.2)	(11.8 ± 0.2)	(6.0 ± 0.0)
		40	7.2 ± 0.3	47.1 ± 0.1	1.7 ± 0.0	17.9 ± 0.2	9.9 ± 0.2	7.0 ± 0.4
			(6.8 ± 0.3)	(46.6 ± 0.6)	(0.9 ± 0.2)	(18.1 ± 0.4)	(10.1 ± 0.2)	(6.9 ± 0.1)
		60	7.7 ± 0.3	54.8 ± 0.1	1.0 ± 0.0	19.5 ± 0.5	7.9 ± 0.2	6.0 ± 0.1
			(7.9 ± 0.3)	(56.6 ± 3.4)	(0.8 ± 0.1)	(20.9 ± 0.5)	(8.1 ± 0.5)	(6.0 ± 0.1)

[a] For each assay (17) ^{32}P-labeled FMDV (10^5 cpm, 2×10^{-3} cpm/PFU, volume of 200 μl) was added to 10^6 cells (50 PFU/cell) in a 35-mm petri dish and allowed to attach for 45 min at 0 to 4°C. Then the inoculum was withdrawn and the monolayer was washed three times with cold phosphate-buffered saline (PBS)–1% bovine serum albumin. At this point the total amount of radioactivity bound to cells was measured on parallel monolayers whose cells were suspended in 10 mM Tris hydrochloride (pH 8.0)–0.5% sodium dodecyl sulfate (SDS). Subsequent radioactivity values are given as percentage of that total amount. To measure internalization, 1 ml of DMEM–2% FCS, warmed to 37°C, was added to each monolayer. At the indicated times, the culture medium was withdrawn and the amount of acid-soluble and acid-insoluble radioactivity was measured. The cell-associated radioactivity was determined in several subfractions as follows: immediately after medium was removed each monolayer was kept for 10 min with 0.5 ml of 10 mM EDTA at 4°C, and the eluted insoluble radioactivity was measured. The cells were collected in 1 ml of PBS, subjected to three cycles of freezing and thawing, and sonicated for 15 min. Then chloroform (50 μl) was added, mixed vigorously, and centrifuged briefly. Total, insoluble, and RNase-resistant radioactivity (200 μg of RNase A per ml, 15 min, 37°C) was measured. The values for FMDV R59 are given in parentheses for comparison.

agar. The colony-forming ability of C_1-HBK-Rc1p19 and -p70 cells was two- and five- to sevenfold, respectively, more efficient than that of BHK-21c1 cells. The actual values (percentage of initial cell number ± standard deviation) were 3.6 ± 0.5, 2.9 ± 0.7, 6.7 ± 0.7, 6.7 ± 0.3, 21.4 ± 5.0, and 19.4 ± 2.2% for BHK-21c1-p4, -p60, and C_1BHK-Rc1-p19, -p19-Rbv, -p70, and -p70-Rbv cells, respectively. Thus, late-passage C_1-BHK-Rc1 cells show genetic alterations that result in altered morphology and growth characteristics.

The replication cycle of FMDV, like that of other picornaviruses, is not known to include a DNA intermediate. However, we considered the unlikely possibility that a

TABLE 2. Transfections with viral RNA

Cells	RNA	No. of productively transfected cells[a]	Virus production (PFU/ml)[b]			
			0 h	2 h	12 h	24 h
BHK-21c1p8	FMDV	4.8×10^4	<5	<5	2.5×10^7	4.4×10^7
	EMCV	6.1×10^4	<5	<5	5.5×10^7	9.5×10^7
C_1-BHK-Rc1p62-Rbv	FMDV	2.0×10^2	<5	<5	2.5×10^3	5.0×10^4
	EMCV	4.5×10^4	<5	<5	4.5×10^7	1.1×10^8

[a] Monolayers (about 10^6 cells) were treated with DEAE-dextran, and viral RNA (1 μg) was added as described (8). Cells were washed and treated with trypsin, and serial dilutions were plated with about 10^6 BHK-21c1 cells under an agar overlay. Plaques were visualized as usual (9).

[b] Transfected cell monolayers were washed and incubated with DMEM–2% FCS. At the indicated times, the virus titer was determined.

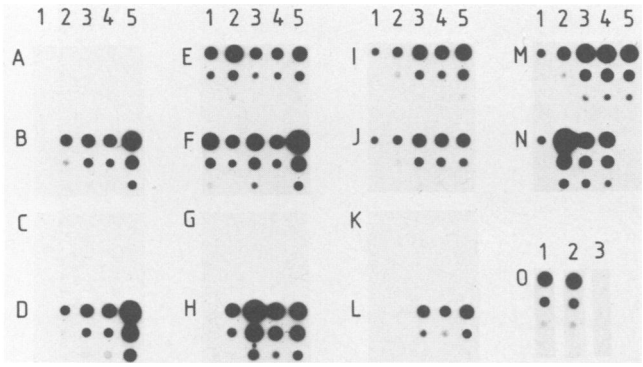

FIG. 3. Autoradiograms of dot-blot hybridizations of cellular RNAs to ^{32}P-labeled FMDV cDNA. Total RNA was extracted from cells (2), applied to nitrocellulose, and hybridized to FMDV cDNA, as described (9). Panels A to N: Columns 1 to 5 correspond to 0, 2, 5, 8, and 24 h p.i., respectively. In each panel, the three rows include 10-fold serial dilutions of the RNA preparation. The type and number of cells from which the RNA applied to the first row was extracted were: A, BHK-21c1p4 (10^5 cells); B, BHK-21c1p4 infected with FMDV C-S8c1 (10^5); C, BHK-21c1p60 (10^5); D, BHK-21c1p60, infected with FMDV C-S8c1 (10^5); E, C_1-BHK-Rc1p17 (10^5); F, C_1-BHK-Rc1p17, infected with FMDV C-S8c1 (10^5); G, C_1-BHK-Rc1p17-Rbv (10^5); H, C_1-BHK-Rc1p17-Rbv, infected with FMDV C-S8c1 (10^5); I, C_1-BHK-Rc1p56 (5×10^5); J, C_1-BHK-Rc1p56 infected with FMDV C-S8c1 (5×10^5); K, C_1-BHK-Rc1p56-Rbv (8×10^5); L, C_1-BHK-Rc1p56-Rbv infected with FMDV C-S8c1 (8×10^5); M, BHK-21c1p4 infected with FMDV C-S8c1 (2×10^5); N, BHK-21c1p4 infected with FMDV R58 (2×10^5). Infections in B, D, F, H, J, and L were at 1 to 2 PFU/cell and in M and N at 10 PFU/cell. Panel O: Lanes 1 and 2, 10 ng of purified FMDV RNA mixed with 5 μg of BHK-21c1 RNA; lane 3, 10 μg of yeast RNA.

cDNA copy of the FMDV genome could be made in the persistently infected cells (perhaps via endogenous reverse transcriptase activities) and integrated in the chromosome, causing inheritable cell alterations. Southern blot analyses were carried out with *Eco*RI-*Pst*I-digested DNA from BHK-21c1 or C_1-BHK-Rc1p59 DNA mixed with known amounts of plasmid pBR-VFAC$_1$-16.8 (34). Under conditions in which 1 FMDV genome equivalent per cell was detected, no evidence for the presence of FMDV DNA in chromosomal DNA was obtained (results not shown).

Since C_1-BHK-21c1 cultures were established with cloned BHK-21c1 cells, the results suggest that mutant cells arose in the persistently infected cultures. The variant cells selected had an advantage over the parental BHK-21c1 cells because of their increased growth rate and resistance to FMDV.

Reconstitution of persistent infections from cured cells and FMDV C-S8c1 or R59. Infections of C_1-BHK-Rc1p59-Rbv with FMDV C-S8c1 resulted in no detectable cytopathology, and yet viral RNA replicated in the cells (Fig. 3). Subculturing of such infected cells showed that a persistent infection was reestablished, since the cultures produced the expected levels of FMDV at those late passages (10^4 to 10^2 PFU/ml), and this production was maintained during at least nine serial passages tested. After the first infectious cycle, the progeny FMDV yielded large plaques, typical of FMDV C-S8c1. After two to four cell passages, the viral population consisted of a mixture of normal-sized- and small-plaque-producing FMDV. Finally, the latter dominated the population at passages 4 to 6. Thus, C_1-BHK-Rc1p59-Rbv cells rapidly

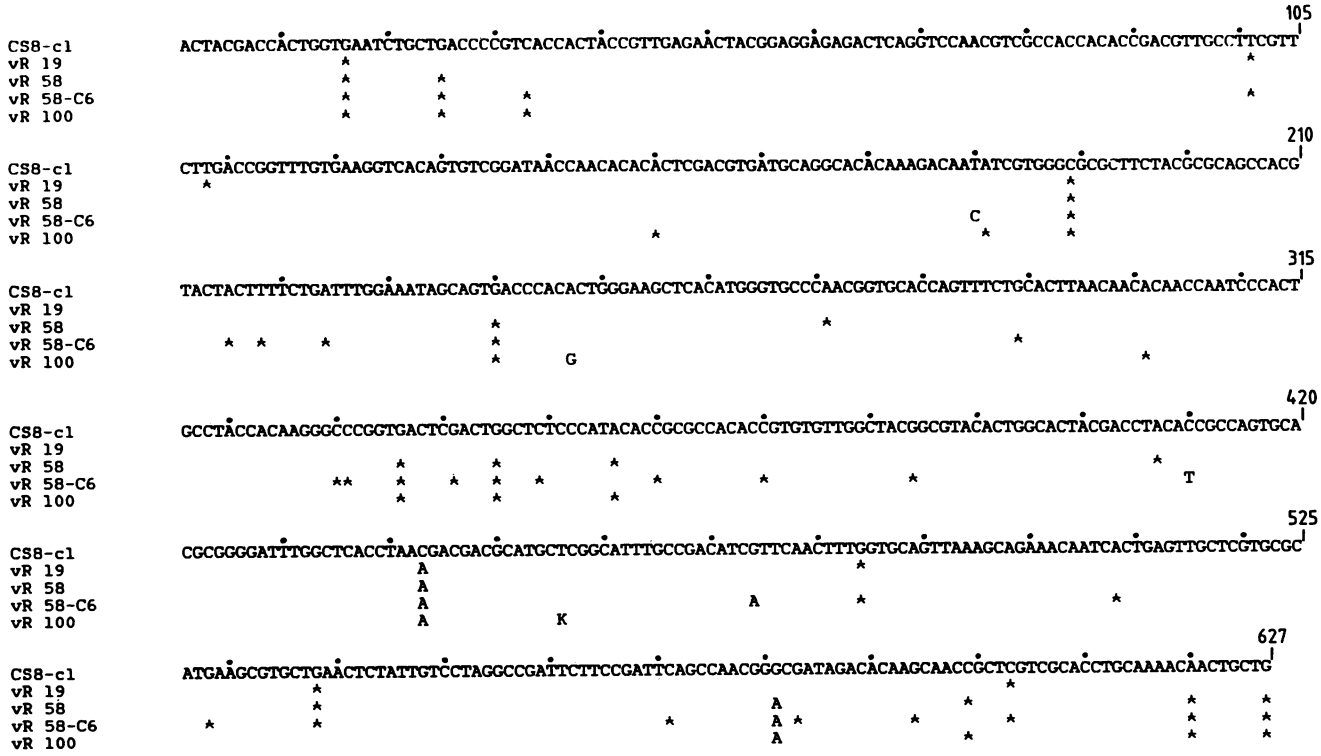

FIG. 4. Nucleotide sequence of the VP1 coding region of FMDV isolated from C_1-BHK-Rc1 cultures. Only variations with respect to the FMDV C-S8c1 sequence (29, 34) are indicated. Symbols: *, undefined reading (four bands) on the autoradiograms; K, T, and G. In addition, the following double bands were seen in individual clones: clone 4, A(576)→A/T; clone 7, C(305)→A/C; and clone 8, C(324)→A/C. Their origin was not investigated.

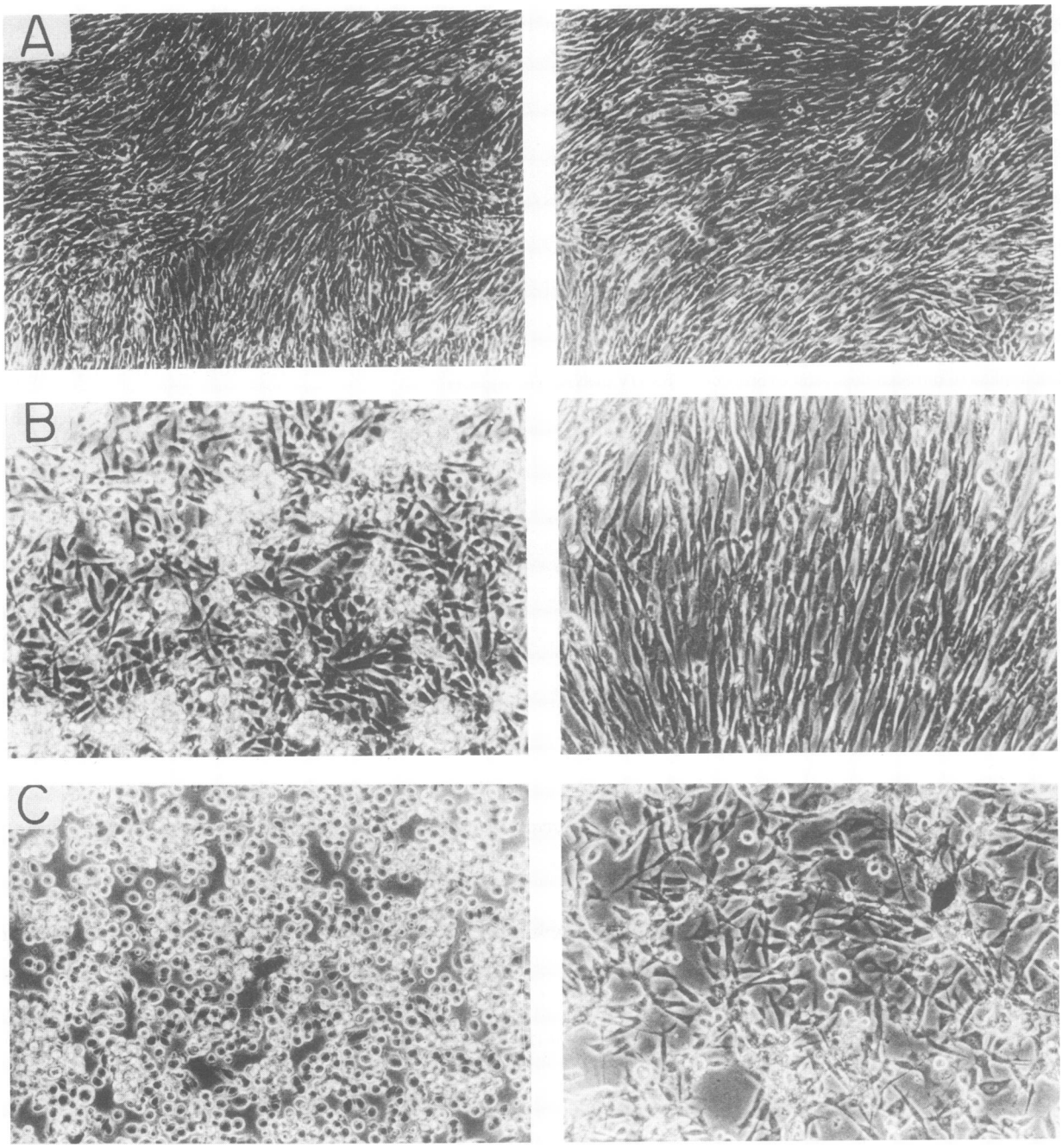

FIG. 5. Phase-contrast micrographs of cell monolayers. (A) BHK-21c1p4; (B) C_1-BHK-Rc1p22; (C) C_1-BHK-Rc1p52. Right panels show the corresponding ribavirin-treated cells. Magnification, ×200.

selected small-plaque variant FMDVs that either were present at an undetectable level in the plaque-purified FMDV C-S8c1 preparation or arose in the first infectious cycles in C_1-BHK-Rc1p59-Rbv cells.

Infection of C_1-BHK-Rc1p59-Rbv with FMDV R59 resulted in cytopathology with about 10^{-1} to 10^{-2} surviving cells. Again, growth and subculturing of these cells resulted in an FMDV carrier culture that behaved like the standard C_1-BHK-Rc1p59 strain.

DISCUSSION

C_1-BHK-Rc1 cell are an in vitro model system for the study of FMDV persistence (9), a frequent type of FMDV infection in nature (24, 33). The studies with C_1-BHK-Rc1 cells were greatly facilitated by the observation that the antiviral agent ribavirin (27) eliminates FMDV from the cultures (8). Assigning the term coevolution to a biological system requires interdependence of the mode of evolution of the two entities (13, 18). Some of the observations reported for the C_1-BHK-Rc1 system support such a designation. (i) In the initial establishment of C_1-BHK-Rc1 cells, the small-plaque morphology FMDV was predominant only at cell passages 50 to 60 (9), while in the reconstitution of a persistent infection with C_1-BHK-Rc1p59-Rbv, the appearance of the small-plaque variant occurred within cell passages 2 to 4. (ii) FMDVs with increased virulence for

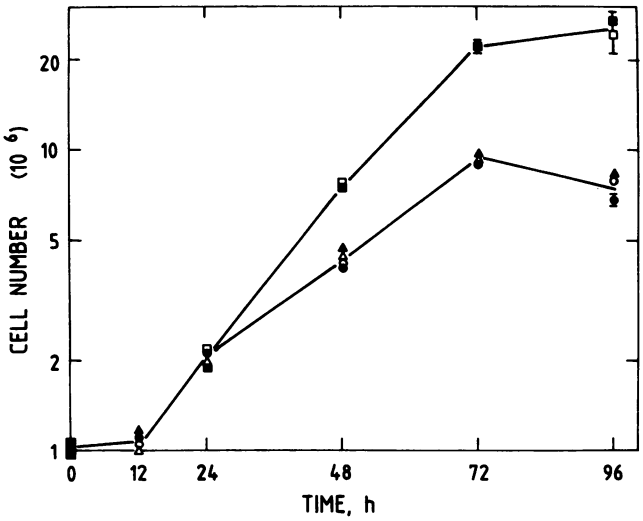

FIG. 6. Cell growth in liquid culture. For each cell line, 10^6 cells were plated on each of several 50-mm and 100-mm petri dishes. At the indicated times, the total number of viable cells was counted as detailed in Materials and Methods. The number of cells plated was such that confluency was never reached before 72 h. Symbols: ●, BHK-21c1p4; ○, BHK-21c1p60; ▲, C_1-BHK-Rc1p19; △, C_1-BHK-Rc1p19-Rbv; ■, C_1-BHK-Rc1p58; □, C_1-BHK-Rc1p58-Rbv. Values are the average of two determinations. Significant standard deviations are indicated.

BHK-21c1 cells were not selected after serial passage of FMDV C-S8c1 in lytic infections (28), suggesting that their selection in C_1-BHK-Rc1 cells was dependent on the concomitant cell evolution towards FMDV resistance. (iii) Conversely, the increased virulence of FMDV R59 rendered coexistence with BHK-21c1 difficult: fewer than 10^{-6} cells survived the infection by FMDV R59. Contrary to the case of lytic infections of BHK-21c1 with FMDV C-S8c1, which led to 10^{-3} to 10^{-4} survivors that permitted establishment of persistence (9), it has been extremely difficult to derive carrier cultures from BHK-21c1 and FMDV R59 (J.C.T., unpublished experiments). Thus, it appears that during persistence FMDV C-S8 evolved as a response to the changes in BHK-21c1 cells and the latter, in turn, evolved in response to FMDV C-S8c1 variation. Cell evolution also played an important role in a persistent infection of L cells with reovirus (1) and with the minute virus of mice (25, 26).

In addition to virus-cell coevolution, other observations point to an as yet unidentified interfering activity in C_1-BHK-Rc1 cells. (i) Early-passage C_1-BHK-Rc1 cells cured of FMDV by ribavirin treatment (8) regained susceptibility to FMDV, suggesting that FMDV-related interfering activity conferred the specific immunity. (ii) Likewise, C_1-BHK-Rc1p59-Rbv cells responded to an infection by FMDV R59—the virus they harbored prior to curing—with considerable cytopathology, yet no interfering activity has been detected in the culture medium of C_1-BHK-Rc1 cells, and attempts to endow BHK-21 cells with specific resistance to FMDV by transfection with RNA fractionated from carrier cells have been inconclusive. Measurement of interferon (IFN) levels by a cytopathic effect inhibition assay indicated <2 U of IFN per ml in the C_1-BHK-Rc1 cultures (J. C. de la Torre and M. Fresno, unpublished experiments), supporting our previous suggestion that IFN does not play a role in this system (9).

Comparison with carrier cell systems established with other picornaviruses suggests that a remarkable variety of mechanisms mediate persistence of these viruses in cell cultures. In an early study, morphologically altered HeLa cells with increased resistance to poliovirus were obtained (35). In a coxsackie A9-HeLa carrier cell system, cells with increased resistance to the virus and viruses with higher virulence for HeLa cells were selected (30). Coxsackie B1 virus failed to attach to HeLa-coxsackie B3 carrier cell lines (6); coxsackie B5 was impaired in penetration and/or eclipse in those cells, and both blocks could be overcome by infecting with viral RNA (6). More recently, persistent infections with hepatitis A virus (32) and echovirus 6 (14) have been described. In the latter study, cloned human WISH cells and plaque-purified echovirus 6 were used to establish a persistent infection in which defective viruses, unable to attach to the parental WISH cells, were selected (14).

Viral variation via generation of defective-interfering RNAs or particles (15) or of other variant genomes (36) is a feature of many virus-cell carrier systems. In C_1-BHK-Rc1 cultures, FMDV variation occurred by serial dominance of heterogeneous genome populations (Fig. 4), as expected from previous work (10–12, 16, 28). One of nine clones analyzed from population FMDV R59 included three mutations in the VP1 gene (Fig. 4). This level of heterogeneity (three mutations in a total of 5,647 residues sequenced, 5×10^{-4} substitutions per nucleotide), although it points to an important characteristic of a viral population, namely its genetic heterogeneity, cannot be taken as an estimate of the mutation rate since it is the combined result of mutation rate and competitive growth of all variant genomes in the population (10a, 11, 12, 16, 28; Domingo et al., in press).

The intracellular block that limits the amount of FMDV RNA in late-passage carrier cells (Fig. 3) could be the direct result of diminished activity of the RNA synthesis machinery (i.e., limitation of a host factor or presence of an inhibitor) or the indirect result of other primary defects (i.e., impairment of translation or increased FMDV RNA degradation) in those cells. Whatever the mechanism, it is specific for FMDV since no restrictions occur in infections or transfections with the picornavirus EMCV.

The FMDV-resistant phenotype was coselected with other traits—decreased doubling time (Fig. 6), decreased contact inhibition of growth, and enhanced ability to form colonies in semisolid agar—that are indicative of cellular transformation. The last trait endows cells with a selective growth advantage in carrier cultures, and thus it could be argued that transformation, in itself, is not relevant to FMDV resistance. While there are no facts to exclude this possibility, it is noteworthy that in a previous study (3, 4), transformed BHK-21 clones were shown to be resistant to some FMDV strains in lytic infections. In particular, the increased resistance to FMDV Asia 1 Iran 1/73 was due to some intracellular restriction in cell line BHK AA7 that showed transformed behavior (3, 4).

It is difficult to assess the relevance that coevolution of cells and viruses might have in vivo. Rapidly dividing cells in tissues of organisms could respond to persistent viral infection by genetic variation and differential survival of cell subpopulations. The process would be enhanced by the rapid evolution of the infecting RNA genome (16). Among other metabolic effects, cellular antigens normally kept at very low levels could become a significant stimulus for the immune system. They would become neoantigens in the absence of virus in the cells.

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LITERATURE CITED

1. **Ahmed, R., W. M. Canning, R. S. Kauffman, A. H. Sharpe, J. V. Hallum, and B. N. Fields.** 1981. Role of the host cell in persistent viral infection: coevolution of L cells and reovirus during persistent infection. Cell **25**:325–332.
2. **Cheley, S., and R. Anderson.** 1984. A reproducible microanalytical method for the detection of specific RNA sequences by dot-blot hybridization. Anal. Biochem. **137**:15–19.
3. **Clarke, J. B.** 1983. Transformation and foot-and-mouth disease virus (FMDV) productivity of some BHK-cell lines. Acta Virol. **27**:534.
4. **Clarke, J. B., and R. E. Spier.** 1983. An investigation into causes of resistance of a cloned line of BHK cells to a strain of foot-and-mouth disease virus. Vet. Microbiol. **8**:259–270.
5. **Crowell, R. L., and B. J. Landau.** 1983. Receptors in the initiation of picornavirus infections, p. 1–42. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 18. Plenum Publishing Corp., New York.
6. **Crowell, R. L., and J. T. Syverton.** 1961. The mammalian cell-virus relationship. VI. Sustained infection of HeLa cells by coxsackie B3 virus and effect of superinfection. J. Exp. Med. **113**:419–435.
7. **DeBorde, D. C., C. W. Naeve, M. L. Herlocher, and H. F. Maassab.** 1986. Resolution of a common RNA sequencing ambiguity by terminal deoxynucleotidyl transferase. Anal. Biochem. **157**:275–282.
8. **de la Torre, J. C., B. Alarcón, E. Martínez-Salas, L. Carrasco, and E. Domingo.** 1987. Ribavirin cures cells of a persistent infection with foot-and-mouth disease virus in vitro. J. Virol. **61**:233–235.
9. **de la Torre, J C., M. Dávila, F. Sobrino, J. Ortín, and E. Domingo.** 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. Virology **145**:24–35.
10. **Domingo, E., M. Dávila, and J. Ortín.** 1980. Nucleotide sequence heterogeneity of the RNA from a natural population of foot-and-mouth disease virus. Gene **11**:333–346.

10a. **Domingo, E., and J. J. Holland.** 1988. High error rates, population equilibrium and evolution of RNA replication systems, p. 3–36. *In* E. Domingo, J. J. Holland, and P. Ahlquist, (ed.), RNA Genetics, vol. 3. CRC Press Inc., Boca Raton, Fla.

11. **Domingo, E., E. Martínez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortín, C. López-Galindez, P. Pérez-Breña, N. Villanueva, R. Nájera, S. VandePol, D. Steinhauer, N. DePolo, and J. Holland.** 1985. The quasi-species (extremely heterogeneous) nature of viral RNA genome populations: biological relevance—a review. Gene **40**:1–8.
12. **Domingo, E., D. Sabo, T. Taniguchi, and C. Weissmann.** 1978. Nucleotide sequence heterogeneity of an RNA phage population. Cell **13**:735–744.
13. **Futuyma, D. J., and M. Slatkin.** 1983. Coevolution. Sinauer Associates Inc., Sunderland, Mass.
14. **Gibson, J. P., and V. F. Righthand.** 1985. Persistence of echovirus 6 in cloned human cells. J. Virol. **54**:219–223.
15. **Holland, J. J., S. I. T. Kennedy, B. L. Semler, C. L. Jones, L. Roux, and E. A. Grabau.** 1980. Defective interfering RNA viruses and the host-cell response, p. 137–192. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 16. Plenum Publishing Corp., New York.
16. **Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
17. **Joklik, W. K., and J. E. Darnell.** 1961. The adsorption and early fate of purified poliovirus in HeLa cells. Virology **13**:439–447.
18. **Levin, B. R., and R. E. Lenski.** 1983. Coevolution in bacteria and their viruses and plasmids, p. 99–127. *In* D. J. Futuyma and M. Slatkin (ed.), Coevolution. Sinauer Associates Inc., Sunderland, Mass.
19. **Lonberg-Holm, K., R. L. Crowell, and L. Philipson.** 1976. Unrelated animal viruses share receptors. Nature (London) **259**:679–681.
20. **Macpherson, I., and M. Stoker.** 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**:147–151.
21. **Macpherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology **23**:291–294.
22. **Martínez-Salas, E., J. Ortín, and E. Domingo.** 1985. Sequence of the viral replicase gene from foot-and-mouth disease virus C_1-Santa Pau (C-S8). Gene **35**:55–61.
23. **Newton, S. E., A. R. Carroll, R. D. Campbell, B. E. Clarke, and D. J. Rowlands.** 1985. The sequence of foot-and-mouth disease virus RNA to the 5′ side of the poly(C) tract. Gene **40**:331–336.
24. **Pereira, H. G.** 1981. Foot-and-mouth disease, p. 333–363. *In* E. P. G. Gibbs (ed.), Virus diseases of food animals, vol. 2. Academic Press, Inc., New York.
25. **Ron, D., and J. Tal.** 1985. Coevolution of cells and virus as a mechanism for the persistence of lymphotropic minute virus of mice in L cells. J. Virol. **55**:424–430.
26. **Ron, D., and J. Tal.** 1986. Spontaneous curing of a minute virus of mice carrier state by selection of cells with an intracellular block of viral replication. J. Virol. **58**:26–30.
27. **Smith, R. A., and W. Kirkpatrick.** 1980. Ribavirin: a broad spectrum antiviral agent. Academic Press, Inc., New York.
28. **Sobrino, F., M. Dávila, J. Ortín, and E. Domingo.** 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell cultures. Virology **128**:310–318.
29. **Sobrino, F., E. L. Palma, E. Beck, M. Dávila, J. C. de la Torre, P. Negro, N. Villanueva, J. Ortín, and E. Domingo.** 1986. Fixation of mutations in the viral genome during an outbreak of foot-and-mouth disease: heterogeneity and rate variations. Gene **50**:149–159.
30. **Takemoto, K. K., and K. Habel.** 1959. Virus-cell relationship in a carrier culture of HeLa cells and coxsackie A9 virus. Virology **7**:28–44.
31. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
32. **Vallbracht, A., L. Hofmann, K. G. Wurster, and B. Flehmig.** 1984. Persistent infection of human fibroblasts by hepatitis A virus. J. Gen. Virol. **65**:609–615.
33. **Van Bekkum, J. G., H. S. Frenkel, H. H. J. Frederiks, and S. Frenkel.** 1959. Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. Tijdschr. Diergeneesk **84**:1159–1164.
34. **Villanueva, N., M. Dávila, J. Ortín, and E. Domingo.** 1983. Molecular cloning of cDNA from foot-and-mouth disease virus C_1-Santa Pau (C-S8). Sequence of protein VP1 coding segments. Gene **23**:185–194.
35. **Vogt, M., and R. Dulbecco.** 1958. Properties of a HeLa cell culture with increased resistance to poliomyelitis virus. Virology **5**:425–434.
36. **Youngner, J., and O. T. Preble.** 1980. Viral persistence: evolution of viral populations, p. 73–135. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 16. Plenum Publishing Corp., New York.
37. **Zimmern, D., and P. Kaesberg.** 1978. 3′-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **75**:4257–4261.