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Extensive Cell Heterogeneity during Persistent Infection with Foot-and-Mouth Disease Virus

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Coevolution of viruses and the host cells occurred in BHK-21 cell cultures persistently infected with foot-and-mouth disease virus (FMDV) (J. C. de la Torre, E. Martínez-Salas, J. Diez, A. Villaverde, F. Gebauer, E. Rocha, M. Dávila, and E. Domingo, J. Virol. 62:2050–2058, 1988). In the present report we provide evidence of an extreme phenotypic heterogeneity of the cells, which was generated in the course of persistence. A total of 248 stable cell clones isolated from FMDV carrier cultures at early or late passages were analyzed. At least six distinct cell phenotypes were distinguished with regard to cell morphology, resistance to FMDV strain C-S8c1, and cell growth characteristics. No infectious FMDV or viral RNA was detected in variant cell clones, suggesting that the altered phenotypes were caused by inheritable cell modifications, selected in the course of persistence. Thus, the FMDV–BHK-21 carrier cell system must be described as a dynamic interaction between an evolving heterogeneous population of virus and multiple cell variants. We suggest that cell heterogeneity confers a selective advantage for long-term virus and cell survival by providing the cell population with a range of responses toward FMDV.

Foot-and-mouth disease virus (FMDV) is an aphthovirus of the family *Picornaviridae* that causes the economically most important disease of cattle (2, 3, 18). The mechanisms of FMDV pathogenesis are poorly understood. In addition to causing acute disease, the virus may persist in ruminants for extended time periods (11, 13, 25) by unknown mechanisms. Carrier, asymptomatic animals pose an important practical problem since: (i) they are a reservoir of virus (13) and promote its antigenic drift (11); (ii) they cannot be cured of FMDV by vaccination, and persistence may be established in vaccinated cattle (24). To provide a model system for the study of FMDV persistence, we established FMDV carrier BHK-21 cell lines termed C_1-BHK-Rc1 (8, 9). They originated from cloned BHK-21 cells and a plaque-purified FMDV (C-S8c1) preparation by growth of cells that survived a standard cytolytic infection (8). Upon serial passage of C_1-BHK-Rc1 cells, multiple genetic and phenotypic changes occurred both in the virus and the host cells (9). The latter, freed of any detectable FMDV, were constitutively more resistant to FMDV; in turn, variant viruses were selected that were able to overcome in part the cellular block (9). Insight into the mode of evolution of the cell population was provided by the isolation and analysis of 248 cell clones derived from C_1-BHK-Rc1 cultures at different passages, reported here. Such clonal analysis documents a novel feature in persistently infected cell cultures, namely, an extremely high cell phenotypic diversity. We suggest that, in conjunction with viral genetic heterogeneity, cell diversity may be an important element for long-term virus and cell survival.

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MATERIALS AND METHODS

Culturing and cloning of cells. The establishment of C_1-BHK-Rc1 cultures and the procedures for their cultivation and passage have been described (8, 9). Uncloned cell populations are termed as in previous reports (8, 9), with a suffix indicating passage number; example, C_1-BHK-Rc1p19 is the FMDV-carrier BHK-21 culture at passage 19 after the establishment of persistence. For cell cloning, cell monolayers were washed, detached with trypsin, treated for 2 min with 0.2 M phosphate (pH 6.1) to inactivate extracellular FMDV, and washed and diluted in Dulbecco modified Eagle medium with 10% fetal calf serum. After counting, appropriate dilutions of the cells were distributed in 96-well microdilution plates. Wells containing a single cell were identified under the microscope, and cell division and clone growth were monitored during 6 to 10 days of incubation at 37°C in 7% CO_2. Stable clones were passaged to provide the number of cells required for the experiments (about 10^7 cells). They were frozen, thawed, and grown as usual (8, 13).

Assays with FMDV. To test for the presence of FMDV, 50 μl from the culture medium of each cell clone was transferred to a microdilution plate with a preformed BHK-21 cell monolayer. Cytopathic effect (CPE) was monitored by visual inspection and crystal violet staining.

To free the cell monolayers of detectable FMDV, they were treated with the antiviral agent ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide) as previously described (7). Other assays with FMDV (binding to cells, internalization, titrations) and with cells (growth in liquid culture, efficiency of colony formation in semisolid agar) were performed as indicated previously (9).

FMDV RNA determination. To quantify intracellular FMDV RNA, total cellular RNA was extracted (4), applied to nitrocellulose, and hybridized to a ^{32}P-labeled cDNA insert of plasmid pBR-VFAC$_1$-18.5 as described previously (9).

TABLE 1. Clonal heterogeneity of BHK-21 cells persistently infected with FMDV

Parental culture[a]	Clone group	n	Morphology[b]	Infectivity of FMDV (C-S8c1)[c]	
				CPE	Titer
C_1-BHK-Rc1p17 (56, 49, 87%)	17B-1	17	B	+++	ND
	17r-1	20	r	+++	ND
	17R-1	12	R or other	+++	ND
C_1-BHK-Rc1p19 (177,[d] 142, 80%)	19B-1	20	B	+++	2×10^7–2×10^8
	19B-2	1	B	+	8×10^5
	19r-1	3	r	+++	3×10^7–2×10^8
	19r-2	4	r	++	5×10^6–1×10^7
	19r-3	5	r	+	8×10^2–2×10^3
	19r-4	5	r	−	1×10^2
	19R-1	1	R	+++	3×10^6
	19R-2	5	R	+	3×10^3–1×10^5
	19R-3	1	R	−	ND
C_1-BHK-Rc1p74 (61,[e] 57, 93%)	74B-1	6	B	−	1×10^3–1×10^5
	74R-1	27	R	−	1×10^3–1×10^5
	74R-2	3	other	−	ND
BHK-21c1p62 (136 clones)	62B-1	133	B	+++	1×10^7–5×10^7
	62r-1	3	r	+++	ND

[a] In brackets are given the number of individual cells isolated, the number of derived stable clones, and the calculated viability (percentage of the isolated cells that yielded a stable clone). Viability was not determined for BHK-21 cells.
[b] Micrographs of cell monolayers representative of B, r, and R morphologies are shown in Fig. 1.
[c] Monolayers (4×10^6 to 8×10^6 cells) were infected with 0.05 to 0.2 PFU of FMDV C-S8c1 per cell. The extent of CPE and virus titer were determined at 20 to 48 h postinfection. Symbols for CPE: +++, >90% detached cells; ++, partial cell detachment; +, <10% detached cells; −, no evidence of detachment. Titers (PFU per milliliter of culture medium) were determined for all the clones of a group except for 19B-1, 19r-3, 74B-1, and 74R-1 for which titers were determined for two clones. When no range is given, variations were <20%. ND, Not determined. The FMDV production by some clones is shown in Fig. 2.
[d] Ninety-seven clones with B, r, R, or other morphology were not tested for infectivity of FMDV C-S8c1.
[e] Twenty-one clones with B or R morphology were not tested for infectivity of FMDV C-S8c1.

RESULTS

Cell heterogeneity in C_1-BHK-Rc1 cultures. C_1-BHK-Rc1 cultures at passages 17, 19, and 74 were actively producing FMDV, which was shed into the culture medium (8). Cells were cloned from such cultures by endpoint dilution as detailed in Materials and Methods. A total of 294 individual cells were isolated, which yielded 248 stable cell clones that were subcultured by standard procedures. None of these stable cell clones produced infectious FMDV (see below). Based on cell and monolayer morphology and on infectivity of FMDV C-S8c1, at least six distinct cell phenotypes were represented in the clonal cell cultures (Table 1): (i) cells with fusiform, BHK-like morphology (B morphology) that produced high FMDV C-S8c1 yield upon infection with FMDV C-S8c1 (group 19B-1 in Table 1); (ii) B morphology, low-FMDV-yield (group 74B-1); (iii) slightly rounded cells (r morphology), high FMDV yield (group 19r-1); (iv) r morphology, low FMDV yield (group 19r-4); (v) cells with rounded morphology (R morphology), high FMDV yield (group 19R-1); (vi) R morphology, low FMDV yield (group 19R-2). Micrographs of monolayers of some clones showing B, r, and R morphologies are shown in Fig. 1.

Resistance to FMDV C-S8c1 is relevant to persistence since it may modulate long-term virus and cell survival. We determined the extent of cytopathologic changes produced in cloned cell monolayers upon infection with FMDV C-S8c1 (Table 1) and measured the viral yield (Table 1 and Fig. 2). Considering those two parameters, the main cell clone phenotypes could be further subdivided, giving the groups shown in Table 1. A remarkable heterogeneity, with nine distinguishable clone groups, was present in culture C_1-BHK-Rc1p19. A predominance of cell clones undergoing no detectable CPE and producing low viral yields upon infection with FMDV C-S8c1 was obtained from C_1-BHK-Rc1p74, albeit with differences among clones (Table 1).

We showed previously (9) that the resistance of uncloned C_1-BHK-Rc1 cell populations to FMDV C-S8c1 was not due to an impediment of viral attachment or a defect during the penetration or uncoating steps but rather to some specific block acting during intracellular FMDV replication. Likewise, the extent of binding, internalization, and uncoating of FMDV C-S8c1 or FMDV R59—the virus from C_1-BHK-Rc1p59 cultures (9)—upon infection of cell clones R1C4, 17C1, 74A12, and 74C12 (origin of these clones and viral yields shown in Fig. 2) was indistinguishable from that observed during infection of BHK-21c1. The results with each of the four clones (data not shown) were similar to those obtained with uncloned C_1-BHK-Rc1 populations (9). The decreased viral yield produced by clones 74C12, 74A12, and R1C4 paralleled a 50- to 200-fold decrease in the amount of intracellular FMDV RNA, measured at 0, 2, 5 and 12 h postinfection, as compared with the amount of FMDV RNA in BHK-21- or 17C1-infected cells at the corresponding times postinfection (Fig. 3). FMDV R59 was able to overcome the intracellular block in clone 74A12 (Fig. 3N and O). Thus, cell clones resistant to FMDV C-S8c1 were isolated from C_1-BHK-Rc1 at early passages (clone R1C4) and at late passages (clone 74A12). In all cases tested, the resistance was due to an intracellular block rather than to an early event in infection, as previously shown for the parental uncloned populations (9). We conclude that in FMDV carrier cell cultures there is an extensive cell phenotypic diversity.

Phenotypic cell heterogeneity is not dependent on the presence of FMDV. The phenotypic alterations shown by cell clones from C_1-BHK-Rc1 cultures (Table 1) could be due either to inheritable cell modifications selected in the carrier

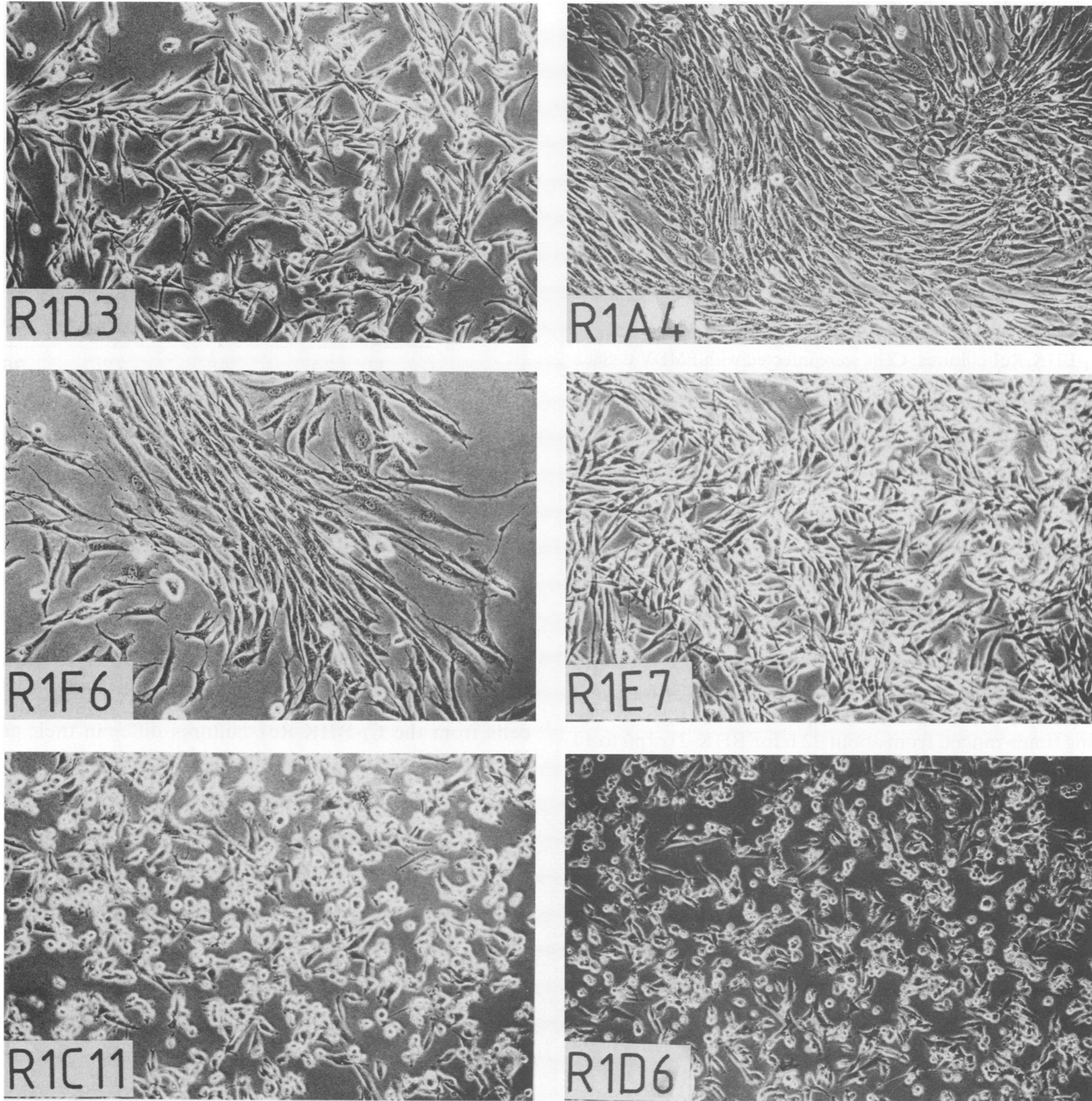

FIG. 1. Phase-contrast micrographs of monolayers of cell clones derived from the C_1-BRK-Rc1p19 culture. The clones were classified as having either B morphology (R1A4, R1F6), r morphology (R1D3, R1E7), or R morphology (R1C11, R1D6). They belong to the 97 ungrouped clones in Table 1. Magnification, ×200.

cultures upon serial subculturing (9) or to the presence of FMDV in the cell clones. To test the latter possibility, FMDV was assayed in the culture medium of the 177 individual cells derived from C_1-BHK-Rc1p19 at 4 h, 2 days, and 6 days after cell isolation. Infectivity was detected in the medium for eight cells only at 4 h after isolation but not at later times. None of those eight cells grew to yield a stable clone. Infectious FMDV was not detected in the culture medium of any stable cell clone. To test whether intracellular FMDV RNA was present, total RNA was extracted (4) from cultures of 9 clones from population C_1-BHK-Rc1p17, 3 clones from C_1-BHK-Rc1p19, and 18 clones from C_1-BHK-Rc1p74 and analyzed by dot blot hybridization to cloned FMDV cDNA (9). Hybridization was negative with RNA from the individual clones (no signal above background was detected) and positive with RNA from the uncloned parental cell populations, as described previously (9) (Fig. 3A, C, E, G, and I) (data not shown). Furthermore, monolayers of two clones from group 17B-1 and five clones from group 74R-1 were treated with the antiviral agent ribavirin, which is known to eliminate FMDV from persistently infected cultures (7). Ribavirin treatment had no effect on cell morphology or resistance to FMDV. Some cell clones have been serially propagated for up to 20 passages, subcloned, and again propagated without change in their characteristic morphology or behavior upon infection with FMDV. The results suggest that the altered phenotypes of individual cell clones (Table 1) are the result of inheritable cell modifications.

Heterogeneity of cell growth characteristics. Late-passage

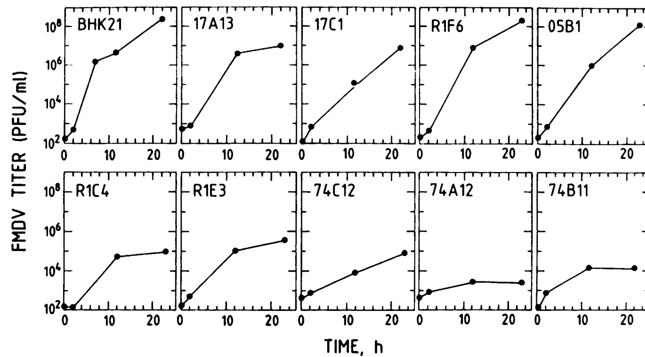

FIG. 2. FMDV production by monolayers of cell clones derived from C_1-BHK-Rcl cultures. Cells were infected with FMDV C-S8cl at 10 PFU per cell. Samples of culture medium were withdrawn at the indicated times, and titers were determined in duplicate. The clones tested were 17A13 and 17C1 (group 17B-1) R1F6 (19B-1), 05B1 (19r-1), R1C4 (19R-2), R1E3 (19R-3), 74C12 (74B-1), and 74A12 and 74B11 (74R-1). The clone groups given within parenthesis are shown in Table 1.

C_1-BHK-Rcl cultures, treated with ribavirin or untreated, grew faster than BHK-21 cells in liquid culture and showed an increased efficiency of colony formation in semisolid agar (9). To test whether the C_1-BHK-Rcl cell population was heterogeneous with regard to growth characteristics, the growth of cell clones R1F6 (group 19B-1, Table 1), O5B1 (19r-1), R1C4 (19R-2), 74C12 (74B-1), 74A12 (74R-1), and 74D12 (74R-2) was measured in liquid culture (Fig. 4). The doubling times ranged from about 22 h for BHK-21clp6 to 14 h for clone 74A12. Faster-growing cell clones showed also a decrease in contact inhibition of growth and an enhanced colony-forming ability in semisolid agar (Table 2). Ribavirin treatment had no effect on the ability of each cell clone tested to form colonies in semisolid agar. Thus, individual cells from the C_1-BHK-Rcl cultures differ in their growth characteristics.

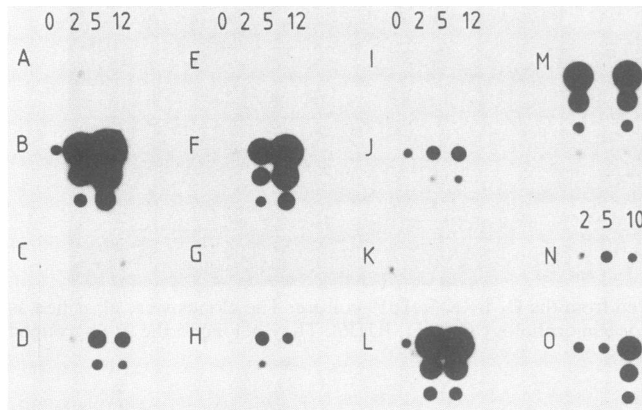

FIG. 3. Autoradiograms of dot blot hybridizations of cellular RNAs to ^{32}P-labeled FMDV cDNA. Procedures were as described previously (9). In panels A to L, N and O, columns indicate the hours postinfection of RNA extraction. The three rows in each panel include 10-fold serial dilutions of the RNA preparation. The types (and numbers) of cells from which the RNA applied to the first row was prepared were as follows: A, R1F6 (10^5); B, R1F6 infected with FMDV C-S8c1 (10^5); C, 74C12 (2×10^5); D, 74C12 infected with FMDV C-S8c1 (2×10^5); E, 17C1 (10^5); F, 17C1 infected with FMDV C-S8c1 (10^5); G, 74A12 (2×10^5); H, 74A12 infected with FMDV C-S8c1 (2×10^5); I, R1C4 (10^5); J, R1C4 infected with FMDV C-S8c1 (10^5); K, BHK-21c1p6 (10^5); L BHK-21c1p6 infected FMDV C-S8c1 (10^5); M, duplicate applications of 10 ng (first row) of FMDV RNA from purified virions and 10-fold serial dilutions; N, 74A12 infected with FMDV C-S8c1 (2×10^5); O, 74A12 infected with FMDV R59 (2×10^5). All infections were at 5 PFU per cell.

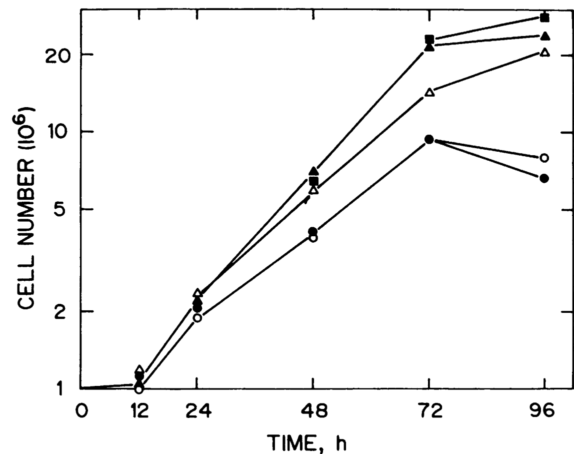

FIG. 4. Clonal variation in rates of cell growth in liquid culture. For each cell clone, 10^6 cells were plated on each of several 5- and 100-mm petri dishes, and at the indicated times the number of viable cells was counted as described previously (9). Symbols: ●, BHK-21clp6 (curves for BHK-21clp4, p6, p30, and p60 were indistinguishable [9]); ○, R1F6; ▲, R1C4; △, 74C12; ■, 74A12. Growth of clones O5B1 and 74D12 was indistinguishable from that of R1F6 and 74A12, respectively. Values are the averages of at least two determinations.

DISCUSSION

The increased resistance to FMDV C-S8c1 and enhanced transformation phenotype of C_1-BHK-Rcl cultures at late passages (9) are in reality the result of an average of different cell behaviors. FMDV was present in the culture medium of only 8 of 177 single cells from C_1-BHK-Rclp19, suggesting that a small proportion (about 4%) of cells in the population is lytically infected. This figure probably represents a lower limit (the upper limit being 20%, according to the cell viability of that population; Table 1), since the half-life of FMDV C-S8c1 infectivity at 37°C is about 50 min (E. Domingo, unpublished result) and the burst size is of 43-134 PFU per cell (22). Thus, unless cloning entailed loss of FMDV from the cells, only a small proportion of cells were detectably infected at the time of cloning. If this were the case, our previous finding that viral antigens were present in most of the cells and the inability of the cultures to be cured of FMDV by prolonged cultivation with anti-FMDV C-S8 antibodies (8) would be unexpected. This point is under investigation.

TABLE 2. Growth of cell clones in semisolid agar[a]

Cell clone[b]	Colonies (% of initial cell number)			
	R1F6	R1C4	74C12	74A12
Ribavirin treated	4.1 ± 1.4	8.9 ± 3.4	13.1 ± 1.6	20.6 ± 2.3
Untreated	3.9 ± 1.6	8.1 ± 1.9	12.9 ± 0.9	21.6 ± 2.3

[a] Cells (50, 5×10^6, and 5×10^3) were added to the upper agar layer in 60-mm petri dishes, and colonies were allowed to form (9); values are the averages of six determinations ± the standard deviations.

[b] The origin of the cell clones is indicated in the legend for Fig. 2.

An interesting question raised by our results is the origin of the cell diversity revealed by the clonal analysis (Table 1). Cell mutants were also selected during persistence of reovirus (1). Normal tissues and cultured cells show some heterogeneity (14) that, at least on occasions, plays a role in functional cellular diversification (15, 20). Some morphological variation in uninfected BHK-21 cultures, originated from a single cell, was also observed in our experiments (clone groups 62B-1 and 62r-1 in Table 1). The extent of cellular heterogeneity is greatly enhanced in tumors (6, 16), where it probably plays an important role in invasiveness and metastatic potential (5, 16). Tumor cell heterogeneity appears to originate from an increased genetic instability of transformed cells (5, 12, 16). C_1-BHK-Rc1 cells manifest a transformed phenotype, more pronounced than that of the parental BHK-21 cells. This is shown by the increased growth rate and colony-forming ability in semisolid agar of the global C_1-BHK-Rc1 populations at late passages (9) and of cell clones from those populations (Fig. 4 and Table 2). It is noteworthy that the C_1-BHK-Rc1 cultures provide a model system to explore the molecular basis of a gradual increase in cellular transformation. Thus, inheritable cell alterations could have been favored by selection of increasingly transformed cells in the cultures. Nonetheless, DNA hypermutability has been described in other systems such as shuttle plasmid vectors during their replication in mammalian cells (19) or in immunoglobulin gene segments (17, 21, 26). Similar mechanisms could be operating in cells persistently infected with FMDV.

In conclusion, the C_1-BHK-Rc1 cultures must be described as a dynamic interaction between an evolving heterogeneous population of FMDV (9, 10, 23) and multiple cell variants. The result is a biologically highly flexible system with many cell types responding to a distribution of viral genomes (10, 23), thus facilitating survival of adequate cell-virus combinations. Cell heterogeneity may be an important element for long-term viral persistence.

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