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Virulence as a Positive Trait in Viral Persistence

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A population replacement experiment has been devised to test the ability of a challenge virus to replace the resident virus in a persistently infected cell culture. BHK-21 cells persistently infected with foot-and-mouth disease virus of serotype C (clone C-S8c1) were challenged with a large excess of either the parental foot-and-mouth disease virus C-S8c1, genetically marked variants differing in their degree of virulence, or a mutant rescued after prolonged persistence in BHK-21 cells. After challenge, the composition of the resident virus population in the carrier culture was analyzed by reverse transcription-PCR amplification and nucleotide sequencing. The dominance of the initial persisting virus was seen in all cases, except when virulent viruses were used in the challenge. The experiments document that, paradoxically, virulence can be a positive factor in the reestablishment of a virus population in a persistently infected cell culture. A model based on the selection of virus-resistant cell variants during persistence is proposed to interpret these observations. Implications about the persistence of viruses in their host cells and organisms are discussed.

The molecular basis of viral persistence is largely unknown, and a variety of mechanisms seem to operate in different cell-virus systems (reviewed in references 2, 23, and 30). Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family with a single-stranded positive-sense RNA genome (3, 34), can establish long-term persistent infections both in cell culture (13, 16–18, 20) and in ruminants (8, 9, 22, 39, 41). Carrier animals represent an important natural reservoir of FMDV and a source of virus capable of occasionally causing acute disease (11, 37).

BHK-21–FMDV carrier cultures were established by growing the cells which survived a cytolytic infection of cloned BHK-21 cells with FMDV (clone C-S8c1) (13). In the course of persistence in cell culture, a coevolution of the host cells and the resident FMDV was observed (15, 16). The carrier cells—which could be freed of FMDV by ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) treatment (12)—became increasingly resistant specifically to FMDV C-S8c1, and the resident virus, in turn, became increasingly virulent for the parental BHK-21 cells (15, 16). A host cell-virus coevolution is not unique to FMDV. A similar observation had been previously documented for reovirus (1), lymphotropic minute virus of mice (33), poliovirus in neural cells (7), and, more recently, murine hepatitis virus in mouse DBT cells (5, 6).

R100, the FMDV rescued after 100 passages of the carrier BHK-21 cells, differs from the parental C-S8c1 virus in about 1% of the nucleotides in coding and in noncoding regions, as well as in the length of the poly(C) tract (17, 21). It has not been possible to unequivocally assign the virulence phenotype of R100 to one or to a group of the genetic differences between R100 and C-S8c1, although a pyrimidine transition in the internal ribosome entry site may contribute to this trait (25, 26). Recent experiments intended to identify possible components of the FMDV C-S8c1 quasispecies capable of initiating persistence led to the unexpected conclusion that rapid cell variation was critical for the establishment of FMDV persistence in BHK-21 cells (27). The latter, when cured of any detectable FMDV within days or weeks after the initial cytolytic infection, showed a slight but measurable increase in resistance specifically to FMDV, thus facilitating cell survival (27). Moreover, the FMDV rescued from the cells at such an early stage of persistence was more virulent for BHK-21 cells than the parental C-S8c1 virus. Thus, the paradox that a persistent virus was more virulent than its parental cytolytic counterpart (16) was fully confirmed by extending the same observation to early stages of persistence (27). To document the contribution of the hypervirulence trait to viral persistence, we have devised a population replacement experiment which examines the ability of a virus population to replace the resident virus in a carrier culture. We compared viral populations with a history of persistence and others with a history of cytolytic infections but differing in their degree of virulence for BHK-21 cells.

The origins of the cloned BHK-21 cells, the three times plaque-purified FMDV C-S8c1, and the persistently infected BHK-21 cells, as well as the procedures for cell growth, infections with FMDV, and plaque assays have been previously described (13, 19, 36, 38). Persistently infected cells at passages 45 and 100 are termed Rp45 and Rp100, respectively. The resident viruses rescued from these cells are termed FMDV R45 and FMDV R100, respectively. (They correspond to C1-BHK-Rp45 and C1-BHK-Rp100 in reference 16.) FMDV HR is a monoclonal antibody-resistant mutant of C-S8c1 with the replacement H→R at VP1 position 146 (29). FMDV HR100 was derived from HR after 100 cytolytic passages in BHK-21 cells. These viruses differ in several point mutations in their genomic RNAs.

Procedures for extraction of FMDV RNA from the culture medium of carrier cells, cDNA synthesis with reverse transcriptase, PCR amplification with *Taq* polymerase, and sequencing of PCR products by the fmol method (Promega) have been described previously (4, 13, 21, 24, 32, 35). The oligonucleotides used for cDNA synthesis and for sequencing (from Isogen Bioscience bv, Amsterdam, The Netherlands) and their positions in the C-S8c1 genome were 5′GAAGGG CCCAGGGTTGGACT (complementary to positions 35 to 54 of the 2AB coding region [35]), 5′CCTGTGGACGCTAG ACA (spanning positions 1546 to 1562 of the VP3 coding

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region; numbering according to reference 17), 5′CGTACGC CACCATGTACCG (complementary to positions 1264 to 1282 of the VP3 coding region [17]), and 5′CAGGTGCCCAACA GATC (spanning positions 830 to 846 of the VP2 coding region [17]). In addition, oligonucleotides 5′GCACGCTTCAT GCGCAC (complementary to positions 2086 to 2102) and 5′GCCGGCCAAGTAGGTGTTTGA (complementary to positions 1165 to 1185 [17]) were used for sequencing.

Variant FMDVs with increased virulence for BHK-21 cells. Carrier R cells are partially immune to superinfection by FMDV but not by other RNA viruses (13). Immunity is not due to lack of receptor molecules on R cells, since FMDV binds, penetrates, and uncoats both in R cells and in BHK-21 cells with similar levels of efficiency (16). Furthermore, cell fusion experiments indicated that the immunity of R cells to FMDV infection was due to intracellular *trans*-acting products which have not been identified (14). Thus, it should be possible to reinfect carrier Rp45 cells with variant FMDVs and to establish an intracellular competition between the resident and the externally added virus. First, we determined the virulence of a number of FMDV variants by two procedures: (i) kinetics of viral production in infection of BHK-21 or Rp45 cell monolayers and (ii) quantification of the number of cells that survived the infection.

The kinetics of virus production by BHK-21 cells have extended previous results with FMDV R59 (16), documenting a shorter replication cycle (maximum viral yield at about 10 h postinfection for R100 and HR100 than for C-S8c1, HR, and R45 (maximum yield at about 24 h postinfection) (Fig. 1A and B). Infection of Rp45 cells by R100 and HR100 led to a yield of about 10^2 PFU over the endogenous production by the Rp45 cells; in contrast, infection by C-S8c1, R45, and HR did not yield virus at levels significantly above the endogenous production by Rp45 cells (Fig. 1C and D).

The ability of the variant FMDVs to kill cells was quantitated by direct cell counting of the cells that survived a cytolytic infection. The live cells present 24, 48, 96, and 168 h after infection (Table 1) indicated an increased ability of FMDV HR100 and R100 to kill BHK-21 cells (hypervirulence trait as defined in reference 27). R100 showed remarkable cell killing ability even for Rp45 cells. Thus, variant FMDVs with increased virulence for BHK-21 cells, including HR100, which had not been selected in the course of a persistent infection, were capable of overcoming to some extent the resistance to superinfection of Rp45 cells. The frequency of BHK-21 cells that survived infection by FMDV C-S8c1 was very similar to the frequency previously determined by Martín-Hernández et al. (27).

A population replacement experiment shows that hypervirulent FMDVs manifest a selective advantage in reestablishing a persistent infection. The main objective of the population replacement experiment was to test whether a challenge virus can replace the resident virus in a persistently infected cell culture. Rp45 cells were chosen for this experiment because (i) the FMDV R45 rescued from them showed a degree of virulence intermediate between that of C-S8c1 and R100 (16, 17), and (ii) FMDV R45 RNA differs from C-S8c1, HR, HR100, and R100 RNA at two to seven positions within residues 924 to 953 and 1984 to 2014 of the P1-coding region (numbering according to reference 17). Also, several point mutations allow the different challenge viruses to be readily distinguishable (17, 22a, 29). Rp45 cell monolayers (about 2×10^6 cells) were extensively washed with culture medium (Dulbecco's modified minimal essential medium), and then infected with either virus C-S8c1, HR, HR100, R100, or a mixture of HR and HR100 at a multiplicity of infection of 5 to 10 PFU per cell. After a 1-h adsorption period at 37°C in 5% CO_2, the cell monolayers were extensively washed with Dulbecco's modified minimal essential medium and kept at 37°C in Dulbecco's modified minimal essential medium with 1% fetal calf serum for 24 h. This procedure was repeated two times prior to passage of the cells. An alternative procedure consisted in passaging the cells after each superinfection to allow a new monolayer to form prior to the following superinfection. Again, the procedure was carried out a total of three times prior to cell passage in the absence of any added virus. Each cell line was established in quadruplicate. At passage 5, viral RNA was extracted from the culture medium, subjected to reverse transcription-PCR, and sequenced at the P1-coding region, which included diagnostic mutations. Rp45 cells superinfected by FMDV C-S8c1 or HR shed the initial, resident virus, R45. In contrast, Rp45 cells superinfected with the hypervirulent viruses HR100 and R100 produced only HR100 or R100, respectively. When Rp45 cell monolayers were superinfected with a mixture of the two cytolytic viruses HR and HR100 (1:1), the virus that was established was HR100 in all cases. Since at passage 1 after reestablishment of persistence the resident viruses were always identical to those found at passage 5, the replacement by HR100 and R100 was very rapid. Thus, viruses with different passage histories and sharing hypervirulence as a common phenotypic trait showed a selective advantage in displacing the resident virus from FMDV carrier cell cultures.

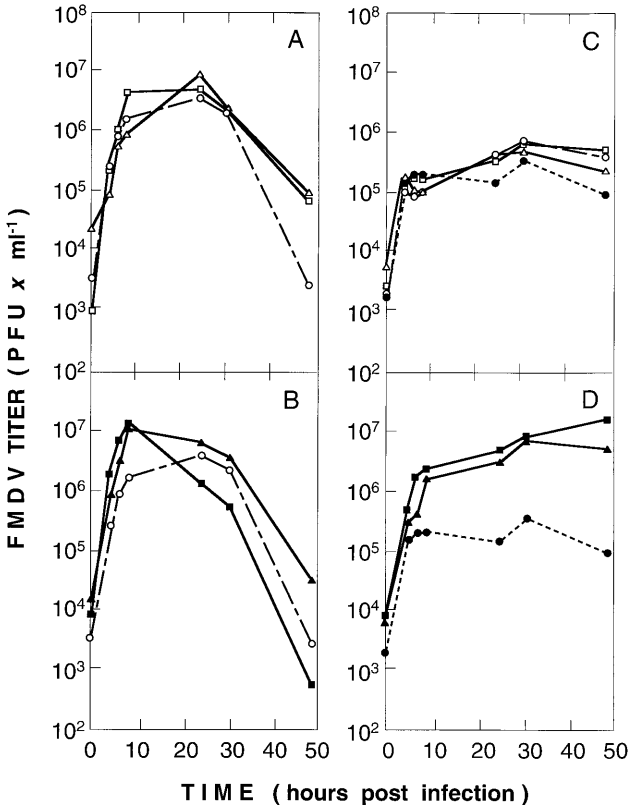

FIG. 1. Virus production upon infection of BHK-21 and Rp45 cell monolayers with variant FMDVs. Procedures are described in Materials and Methods. (A) Infection of BHK-21 cells with R45 (○), C-S8c1 (□), and HR (△). (B) Infection of BHK-21 cells with HR100 (▲) and R100 (■). Infection with R45 (○) is indicated in panels A and B for comparison. (C) Infection of Rp45 cells with R45 (○), C-S8c1 (□), and HR (△). (D) Infection of Rp45 cells with HR100 (▲) and R100 (■). The endogenous production of virus by the carrier Rp45 cells (●) is indicated in panels C and D for comparison.

TABLE 1. BHK-21 or Rp45 cells that survived infection by variant FMDVs[a]

Cell	FMDV	Proportion of surviving cells at (h postinfection):			
		24	48	96	168
BHK-21	None	1	$(5.0 \pm 1.7) \times 10^{-1}$	$(8.6 \pm 1.7) \times 10^{-4}$	$<1 \times 10^{-4}$
	C-S8c1	$(3.3 \pm 1.6) \times 10^{-3}$	$(1.4 \pm 1.0) \times 10^{-3}$	$(1.4 \pm 0.1) \times 10^{-3}$	$(2.7 \pm 1.4) \times 10^{-2}$
	R45	$(3.2 \pm 1.3) \times 10^{-3}$	$(1.5 \pm 1.5) \times 10^{-3}$	$(6.6 \pm 5.0) \times 10^{-4}$	$<1 \times 10^{-4}$
	HR	$(1.7 \pm 1.5) \times 10^{-3}$	$(3.9 \pm 4.3) \times 10^{-3}$	$(4.6 \pm 2.0) \times 10^{-3}$	$(1.2 \pm 0.4) \times 10^{-2}$
	HR100	$(1.8 \pm 0.9) \times 10^{-3}$	$(1.4 \pm 0.4) \times 10^{-3}$	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$
	HR/HR100	$(2.7 \pm 1.6) \times 10^{-3}$	$(1.0 \pm 0.7) \times 10^{-3}$	$(2.7 \pm 0.9) \times 10^{-4}$	$<1 \times 10^{-4}$
	R100	$(1.7 \pm 0.9) \times 10^{-3}$	$(8.4 \pm 3.4) \times 10^{-4}$	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$
Rp45	None	1	1.0 ± 0.3	$(3.2 \pm 0.7) \times 10^{-1}$	$(1.0 \pm 0.3) \times 10^{-1}$
	C-S8c1	$(5.2 \pm 2.4) \times 10^{-1}$	$(6.8 \pm 2.9) \times 10^{-1}$	$(4.0 \pm 0.5) \times 10^{-2}$	$(1.9 \pm 0.1) \times 10^{-2}$
	R45	$(7.0 \pm 2.4) \times 10^{-1}$	$(5.7 \pm 1.8) \times 10^{-1}$	$(3.9 \pm 0.5) \times 10^{-1}$	$(7.5 \pm 0.1) \times 10^{-2}$
	HR	$(7.2 \pm 1.8) \times 10^{-1}$	$(3.5 \pm 1.9) \times 10^{-1}$	$(2.1 \pm 0.1) \times 10^{-1}$	$(1.3 \pm 0.1) \times 10^{-1}$
	HR100	$(5.1 \pm 4.5) \times 10^{-2}$	$(7.4 \pm 1.0) \times 10^{-3}$	$(1.2 \pm 0.6) \times 10^{-3}$	$(4.1 \pm 1.6) \times 10^{-1}$
	HR/HR100	$(7.1 \pm 2.3) \times 10^{-2}$	$(5.1 \pm 2.3) \times 10^{-3}$	$(4.7 \pm 1.0) \times 10^{-2}$	$(4.6 \pm 1.6) \times 10^{-1}$
	R100	$(1.5 \pm 1.8) \times 10^{-2}$	$(9.2 \pm 1.0) \times 10^{-4}$	$<1 \times 10^{-4}$	$(1.0 \pm 0.7) \times 10^{-3}$

[a] BHK-21 and Rp45 cell monolayers (about 2×10^6 cells) were infected with the FMDV variants indicated in the second column. The origin of the cells and of the FMDVs is given in the text. The proportion of surviving cells was determined at the indicated hours postinfection by direct cell counting with trypan blue as a vital stain. The results are the average of at least three independent experiments. The increased life span of Rp45 relative to that of uninfected BHK-21 cells agrees with the higher degree of transformation of the persistently infected cells (15, 16).

The effectiveness of the displacement was statistically solid, since it occurred in each of the 24 independent experiments with HR100, R100, or a mixture of HR and HR100 as the challenge viruses and in none of the 16 experiments with C-S8c1 or HR as the challenge viruses. It is very unlikely that phenotypic traits other than virulence were responsible for the selective advantage of HR100 and R100 in the competition for population dominance in carrier cells. HR100 and R100 have widely different passage histories, HR100 having evolved in serial cytolytic infections and R100 having evolved in persistently infected BHK-21 cells (17). Furthermore, HR100 manifested a selective advantage for residency in carrier cells in direct competition with its HR ancestor. In addition to the diagnostic mutations, the virus found in carrier cells after challenge retained the cell killing ability and, in the case of R100, the small-plaque morphology of the challenge virus. Even if it cannot be excluded that recombinant viruses could be generated during challenge, it is unlikely that in a single passage after challenge, a massive replacement by recombinant viruses could take place.

Two mechanisms may be considered to explain the displacement of R45 by R100 or HR100 from the persistently infected cells. One is that the challenge virus penetrated the carrier cells and established an intracellular competition with the resident virus. The virus capable of completing replication cycles in a shorter time then won over the resident virus in cells kept alive by their increased resistance to FMDV and decreased duplication time (15, 16). An alternative mechanism is that the hypervirulent virus caused extensive but incomplete cell killing, and then it infected a few surviving, uninfected cells. Both mechanisms are consistent with the presence of FMDV receptors in the carrier cells, previously documented by penetration (16) and cell fusion (14) experiments. We favor the intracellular competition model, since it is unlikely that hypervirulent viruses capable of causing extensive cytopathology would selectively spare uninfected cells from death.

Implications for viral persistence. The results presented here, together with the previous observations of a tendency of the FMDV residing in BHK-21 cells to evolve toward increased virulence (15, 16, 27), have established virulence as a potentially advantageous trait for viral persistence. This may occur when cells express functional receptors for a virus but display limited permissiveness for intracellular viral replication. It may also occur when cells do not express the standard receptor for the virus but the latter can use an alternative, secondary receptor with low efficiency (6). A hypervirulent virus capable of partially overcoming such blocks may create a new cell-virus interaction that results in a level of viral replication compatible with cell division and survival.

Picornaviruses have long been suspected to be involved in a number of chronic diseases, such as acquired inflammatory muscle diseases (10), motor neuron syndromes (10), diabetes mellitus (31), and cardiopathies (28). The mechanisms for such diseases are largely unknown, and the multiple hit concept of several contributing environmental, immunological, and genetic factors seems the most plausible at present. However, it is generally thought that attenuated forms of a virus must be involved to limit the extent of cytopathology and to ensure persistence. This need not be the case. Recently, a single mutation at the 5′-untranslated region of coxsackievirus B3 was associated with its cardiovirulent phenotype, and this was linked to increased RNA synthesis in cultured cardiac mouse cells (40). The results with the FMDV-carrier cells model point to an interesting possibility: that from the point of view of long-term virus and cell survival, an attenuated virus in interaction with a susceptible cell may lead to the same outcome as that with a hypervirulent virus interacting with a partially resistant cell.

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