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Response of Foot-and-Mouth Disease Virus to Increased Mutagenesis: Influence of Viral Load and Fitness in Loss of Infectivity

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Passage of foot-and-mouth disease virus (FMDV) in cell culture in the presence of the mutagenic base analog 5-fluorouracil or 5-azacytidine resulted in decreases of infectivity and occasional extinction of the virus. Low viral loads and low viral fitness enhanced the frequency of extinction events; this finding was shown with a number of closely related FMDV clones and populations differing by up to 10^6-fold in relative fitness in infections involving either single or multiple passages in the absence or presence of the chemical mutagens. The mutagenic treatments resulted in increases of 2- to 6.4-fold in mutation frequency and up to 3-fold in mutant spectrum complexity. The largest increase observed corresponded to the 3D (polymerase)-coding region, which is highly conserved in nonmutagenized FMDV populations. As a result, nucleotide sequence heterogeneity for the 3D-coding region became very similar to that for the variable VP1-coding region in FMDVs multiply passaged in the presence of chemical mutagens. The results suggest that strategies to combine reductions of viral load and viral fitness could be effectively associated with extinction mutagenesis as a potential new antiviral strategy.

RNA viruses replicate and evolve as complex mutant distributions termed viral quasispecies as a result of limited copying fidelity of viral replicases and retrotranscriptases (2, 11, 13, 16, 28, 49). Error-prone replication predicts the existence of an error threshold for the maintenance of genetic information (11, 16, 55, 58). The stability of a quasispecies is determined by the selective superiority of the most fit and most abundant genome—termed the master sequence (15, 17)—and by the copying fidelity during genome replication (11, 55, 58). An increase in the average error rate above a critical threshold during template copying should result in the loss of genetic information in a process that has been referred to as violation of the error threshold or entry into error catastrophe (11, 15, 55, 58). Such a critical transition has been equated with "melting" of meaningful information through randomization of nucleotide sequences (15, 55, 58). If applicable to viral infections, violation of the error threshold should result in a loss of viral infectivity.

In line with theoretical predictions, a number of studies have documented decreases in viral infectivity concomitant with increased levels of mutagenesis during RNA genome replication. Ethyl methanesulfonate, nitrous acid, 5-fluorouracil (FU), or 5-azacytidine (AZC) increased no more than 1.1- to 2.8-fold the mutation frequencies at defined single base sites of poliovirus and vesicular stomatitis virus, even with levels of virus survival below 1% of the yield in the absence of chemical mutagenesis (29). The high rate of mutation during a single round of retroviral replication was increased up to 13-fold by AZC (46). Although vesicular stomatitis virus readily gained fitness upon large population passages in cell cultures (43), such a gain was severely limited by the presence of mutagenic agents (31). More recently, it was shown that mutagenic nucleoside analogs produced a loss of replicative potential of human immunodeficiency virus type (HIV-1) upon serial passage in human cells, in a process that has been termed lethal mutagenesis of HIV-1 (33, 34). These observations, together with the generally high mutation rates during RNA genome replication (2, 11, 13, 15, 28), have suggested that replication fidelity of RNA viruses may already be close to the error threshold and that such proximity may maximize virus adaptability (13, 15, 28, 29, 31).

There is little information on the effects of viral load, viral fitness, and the types and numbers of mutations associated with a loss of viral infectivity of RNA viruses associated with increased mutagenesis. Such information is needed to assess the possible development of antiviral strategies based on virus entry into error catastrophe. In the present report, we have analyzed the effect of two mutagenic base analogs, FU and AZC, on the mutation frequency, mutant spectrum complexity, and survival of the important picornavirus pathogen foot-and-mouth disease virus (FMDV) (48, 51). Multiple virus passages in the presence of mutagens resulted in increases of up to 6.4-fold in the mutation frequency at some genome segments. Additionally, we document important effects of viral load and viral fitness on virus extinction driven by increased mutagenesis.

MATERIALS AND METHODS

Cells, viruses, and infections. The origin of baby hamster kidney 21 (BHK-21) cells has been described previously (12, 57). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with nonessential amino acids (Gibco) and 5% fetal calf serum (Gibco). A number of FMDV clones have been used in studies with chemical mutagens. (i) FMDV C-S8c1 is a plaque-purified derivative of natural isolate C_1-Sta Pau-Spain 70, a representative of the European subtype C_1 FMDVs (57). (ii) MARLS is a monoclonal antibody escape mutant obtained from FMDV C-S8c1p213 (C-S8c1 passaged 213 times in BHK-21 cells [1, 4]); MARLS includes substitution Leu-144 → Ser at antigenic site A located within the G-H loop of capsid protein VP1 (40). The replicative fitness of MARLS in BHK-21 cells is about 130-fold higher than that of C-S8c1 (4; S. Sierra and C. Escarmís, unpublished results). (iii) C^9_{22} is a highly debilitated clone derived from multiple plaque-to-plaque transfers of FMDV C-S8c1 (19). The replicative fitness of C^9_{22} in BHK-21 cells was about 10% that of C-S8c1 (19, 20). (iv) A number of low-fitness subclones were obtained from clone H5 (derived from C-S8c1p113 [19]) which was passaged 91 times in plaque-to-plaque transfers (termed H^5_{91} [19; Sierra and Escarmís, unpublished]). For these subclones, relative fitness values were estimated on the basis of the number

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of infectious progeny produced per plaque; this estimate indicated that the fitness of H^5_{91} subclones was about 10^3-fold lower than the fitness of C^9_{22} (19; Sierra and Escarmís, unpublished). A good correspondence between relative fitness values obtained in growth competition experiments and those estimated on the basis of infectious progeny production has been previously documented (19).

Procedures for infection of BHK-21 cell monolayers with FMDV in liquid medium and for plaque assays in semisolid agar medium have been described previously (1, 12, 57). The standard viral production assay consisted of the infection of 4×10^6 BHK-21 cells with FMDV at a multiplicity of infection ranging from 10^{-5} to 10 PFU per cell. DMEM used for FMDV infections contained 2% fetal calf serum. Infections were allowed to proceed until cytopathology (cell detachment) was nearly complete (6 to 24 h postinfection). To control for the absence of contamination, parallel passages of supernatants of mock-infected cells were carried out throughout the experiments, with no signs of infectivity or cytopathology in the cultures.

Mutagenic agents and mutagenesis treatments. FU (Sigma) and AZC (Sigma) were used as mutagenic base analogs. Both can be incorporated into DNA and RNA, and both interfere with RNA processing and translation (5, 21, 44, 45). They have been shown to be mutagenic for a number of RNA viruses (14, 23, 26, 29, 32, 46, 50). To prepare DMEM containing FU or AZC, the appropriate amount of analog was dissolved in the medium and sterilized by filtration to yield solutions of 2.5 mg/ml, which were diluted as needed. Medium with FU was stored at 4°C for a maximum of 15 days; medium with AZC was freshly prepared for each experiment.

The effects of a number of doses and times of exposure to mutagens on BHK-21 cells and FMDV production were tested in a series of preliminary experiments. In the standard assay for mutagenesis (used for all experiments, except when indicated otherwise), confluent cell monolayers were pretreated for 13 h with 200 μg of FU per ml or 6 h with 10 μg of AZC per ml; then, the cells were washed with DMEM, infected with FMDV (adsorption for 1 h at 37°C), washed for 1 min with 0.1 M phosphate buffer (pH 6.0) (to inactivate unadsorbed virions), and washed again extensively with DMEM. The infection was allowed to proceed in the presence of the same concentration of either FU or AZC for 20 to 24 h.

For serial passages in the presence of a mutagen, viral progeny were used either undiluted or diluted 10-fold before the next infection. Virus was titrated after each passage. When no cytopathology was observed, at least three serial blind passages were carried out in the absence of a mutagen prior to viral detection tests. We define extinction as the situation in which no infectivity and no reverse transcription (RT)-PCR-amplifiable material are detected in the culture supernatant after these three blind passages. Populations passaged in the presence of a mutagen are indicated with the abbreviation of the mutagen and passage number (e.g., C-S8c1FUp15 is C-S8c1 passaged 15 times in the presence of FU).

The toxicity of mutagenic agents for confluent BHK-21 cell monolayers was monitored by determining cell viability either by fluorescence-activated cell sorter (FACS) analysis using propidium iodide (61) or by trypan blue exclusion after exposure to the base analogs. Cells were detached by trypsin treatment and added to the culture medium (which contained cells detached during culturing) prior to centrifugation and viability measurements. Viable cells comprised at least 75% of the total under the doses and times of exposure to mutagens used prior to infection.

cDNA synthesis, PCR amplification, and nucleotide sequencing. Viral RNA was extracted by mixing 150 μl of medium containing virus with 300 μl of Triazol reagent (Gibco) and incubating the mixture for 5 min at room temperature. Then, 100 μl of chloroform was added and mixed vigorously, and the mixture was incubated for 10 min at room temperature. The RNA was recovered from the aqueous phase by ethanol precipitation. cDNA synthesis and PCR amplification (RT-PCR) were performed as previously described (19). Prior to RT-PCR amplification, the amount of FMDV-specific RNA was quantitated by dot blot hybridization as previously described (8, 9) using purified FMDV C-S8c1 RNA as a standard. The same amount of viral RNA (0.5 to 2 ng) was used for each RT-PCR amplification to ensure an excess of template molecules for copying by avian myeloblastosis virus reverse transcriptase and amplification by *Pfu* DNA polymerase (Promega) (6). For each RNA sample, one or several independent amplification reactions were carried out, including a negative control RT-PCR (excluding viral RNA). Two FMDV genomic regions were subjected to RT-PCR amplification: residues 3193 to 3869 (spanning the VP1-coding region) and residues 6609 to 8035 (spanning the entire 3D [polymerase]-coding region). Numbering of residues is that used in reference 20. The oligonucleotide primers used for PCR amplification and nucleotide sequencing are based on primers which have been previously described (1, 19) and which included restriction enzyme sites for molecular cloning (*Bam*HI and *Sac*I for the VP1-coding region and *Bam*HI and *Eco*RI for the 3D-coding region). Amplified DNAs were digested with the appropriate restriction enzymes, ligated to plasmid pGEM-4Z (Promega) digested with the same restriction enzymes, and cloned in *Escherichia coli* DH5α. White colonies were grown in Luria-Bertani medium, and plasmid DNA was obtained using a Wizard SV Minipreps kit (Promega). DNA manipulations and cloning techniques were carried out using standard procedures (53). Nucleotide sequences were determined with PCR-amplified DNA or plasmid DNA using a Big Dye Terminator Cycle Sequencing kit (Abi Prism; Perkin-Elmer) and an automated sequencer (ABI373). Sequences were analyzed with a DNA Star 4.0 pack.

The heterogeneity of the mutant spectrum of viral quasispecies was quantitated by use of the mutation frequency, which is the number of mutations found relative to the number of nucleotides sequenced. The mutation frequency is calculated by dividing the number of different mutations found in a set of genomes (when compared with the consensus nucleotide sequence) by the total number of nucleotides sequenced (11, 15). Another parameter used to quantitate heterogeneity is the normalized Shannon entropy, which is a measure of the proportion of identical sequences in a distribution. The possible values of the normalized Shannon entropy range from zero to one. For example, a set of 20 identical genomic sequences is defined by the minimum normalized Shannon entropy of zero. A set of 20 genomes, each one differing in nucleotide sequence from any other genome in the set, is defined by the maximum normalized Shannon entropy of one, irrespective of the number of mutations distinguishing each sequence from the others (reviewed in reference 60).

RESULTS

Serial passage of FMDV in the presence of FU or AZC may lead to viral extinction. In initial experiments, we monitored cell viability and production of FMDV in a single round of infection of BHK-21 cells with FMDV C-S8c1 in the presence of increasing concentrations of FU or AZC (Fig. 1). Concentrations of FU of 100 to 1,000 μg/ml resulted in a reduction in virus progeny production of 50- to 100-fold (Fig. 1B), with a BHK-21 cell viability of 75% after 36 h of exposure to the base analog (Fig. 1A). A similar decrease in FMDV production was obtained in the presence of 10 μg of AZC per ml (Fig. 1D), with a BHK-21 cell viability of 25% after 24 h of exposure to the drug (Fig. 1C). To investigate whether additional losses of infectivity could be induced by extended mutagenic treatments, FMDV C-S8c1 was serially passaged in the presence or absence of FU or AZC and with or without a 1/10 dilution of viral infectivity prior to each infection. The results (Fig. 2) show a decline in virus titer and occasional episodes of viral extinction when virus progeny were diluted to titers in the range of 10^3 to 10^4 PFU/ml. Viral extinction was dependent on the presence of a mutagen, since the same preextinction populations (Fig. 2) survived and produced titers of 10^4 to 10^6 PFU/ml in parallel infections in the absence of a mutagen. These results suggest that a small population size contributes to FMDV extinction in the presence of FU or AZC.

Low fitness favors extinction by increased mutagenesis. To investigate the effect of viral fitness on FMDV production in the presence of mutagens, clones C^9_{22} and MARLS (both derived from C-S8c1 and with fitnesses of 0.1 and 130 relative to C-S8c1, respectively [19; Sierra and Escarmís, unpublished]) were compared with C-S8c1 with regard to virus production in the presence or absence of FU or AZC. For the high-fitness FMDV MARLS, the decrease in virus production attributable to the mutagenic treatment was 2- to 11-fold, whereas for the low-fitness C^9_{22} clone, the decrease was 87- to 446-fold (Fig. 3A). The effect of FU also was tested on preextinction population C-S8c1FUp15 (described in Fig. 2) and on six highly debilitated subclones of H^5_{91} (Fig. 3B). Decreases in virus yield of 10^2- to 10^4-fold were observed for four subclones, and an irreversible loss of infectivity was seen for C-S8c1FUp15 (Fig. 3B).

In serial passages in the absence or presence of mutagens (Fig. 4), extinction of low-fitness C^9_{22} was more frequent than extinction of high-fitness MARLS. In particular, extinction of C^9_{22} in FU was rapid even with undiluted virus. Again, when a preextinction population was used to infect BHK-21 cells in the absence of a mutagen, 10^3 to 10^6 (depending on fitness values) PFU of progeny virus were produced. Thus, low fitness contributes to FMDV extinction in the presence of FU or AZC.

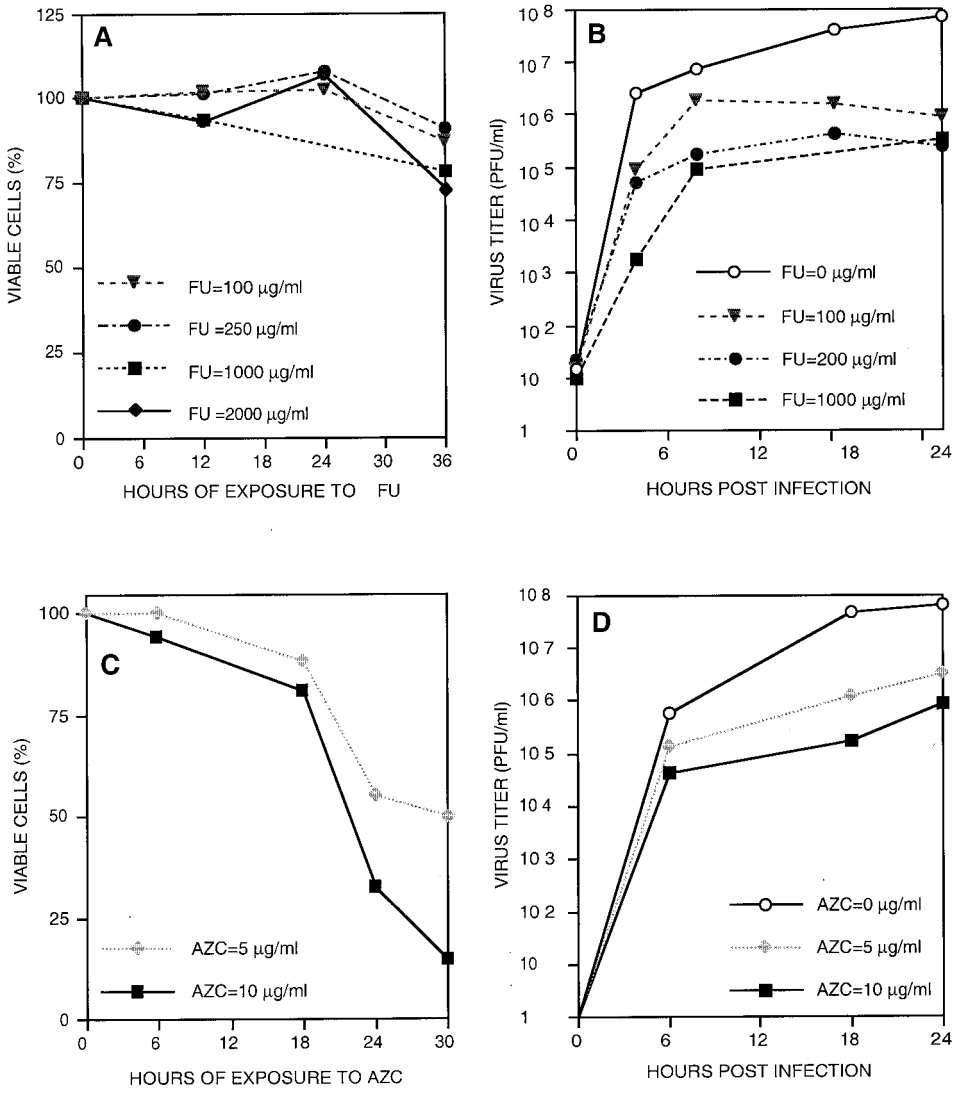

FIG. 1. BHK-21 cell viability and FMDV C-S8c1 production in the presence of FU (A and B) and AZC (C and D). The origin of BHK-21 cells and FMDV C-S8c1 and procedures for cell growth, mutagenesis treatment, quantification of cell viability, and infection with FMDV are detailed in Materials and Methods. Percent viabilities are calculated relative to those in parallel untreated BHK-21 cell cultures. Absolute viabilities for untreated BHK-21 cells were at least 95%. Time in the cell viability panels (A and C) refers to the time elapsed between the addition of a mutagen to cells and the determination of cell viability. In the experiments with FU, cell viability was quantitated by FACS analysis (5,000 events per sample); in the experiments with AZC, trypan blue exclusion (200 to 500 cells per sample) was used. Control assays showed that differences in percentages of viable cells after exposure to the same doses of mutagens in independent experiments and with the two procedures to determine cell viability did not exceed 20%.

Treatment of FMDV with FU and AZC results in nonuniform increases in mutation frequency. Consensus nucleotide sequences and sequences from molecular clones (mutant spectrum) were determined for FMDV C-S8c1 at passages 1 and 25 in the absence or presence of FU and at passages 1 and 10 (a preextinction population) in the presence of AZC. The maximum increase in the mutation frequency (Table 1) was 6.4-fold, observed for the 3D-coding region of virus passaged in FU relative to the population passaged in the absence of the mutagen; for other populations and genomic regions, the increase in the mutation frequency ranged between 2- and 4-fold. Shannon entropies of 1 or near 1 were attained in populations subjected to multiple mutagenesis passages, indicating an increased algorithmic complexity of the mutant spectrum (see Discussion). The most abundant types of mutations after multiple passages in FU were base transitions (mainly U → C, C → U, and G → A). With AZC treatment, preextinction population C-S8c1AZCp10 preferentially showed transitions C → U, G → A, and A → G (Table 2). Thus, increases in mutation frequency and in mutant spectrum complexity which were nonuniformly distributed along the FMDV genome were observed in FMDV populations that were passaged in the presence of chemical mutagens and that underwent occasional extinction.

DISCUSSION

Nature and location of chemically induced mutations in the FMDV genome. In the present study, we have reported the effect of two mutagenic base analogs, FU and AZC, previously used to generate mutants of other RNA viruses (14, 23, 29, 32, 46, 50), on genomic sequences and infectivity of FMDV. With FU, the most abundant mutation type found in the mutant

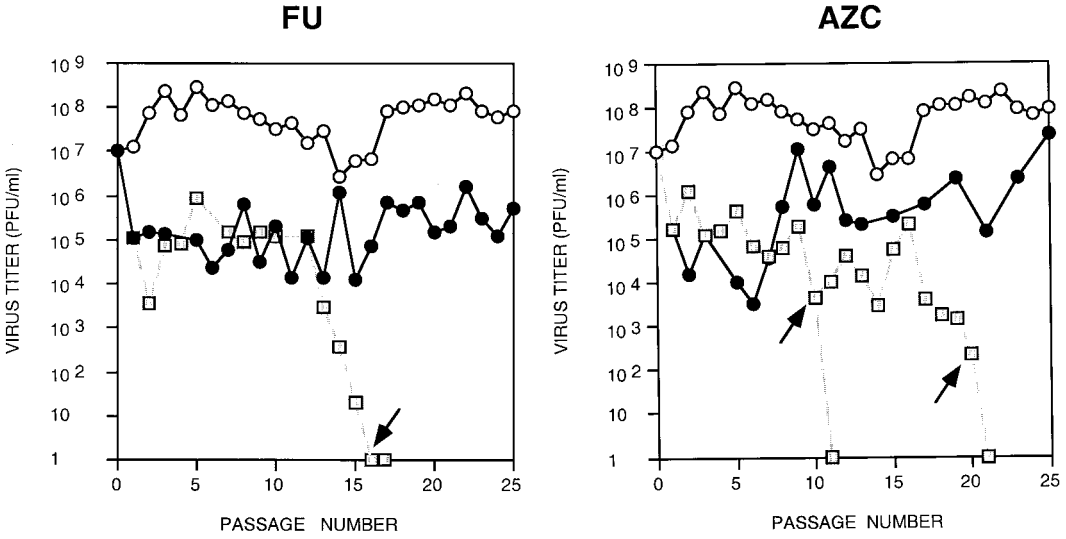

FIG. 2. Infectivity values upon passage of FMDV C-S8c1 in the absence or presence of FU or AZC. Conditions for mutagenic treatment and for determination of FMDV infectivity are detailed in Materials and Methods. Filled circles indicate passages of undiluted virus in the presence of mutagens, and squares correspond to passages in which the virus was diluted 10-fold prior to each infection (to a titer 1/10 the one indicated on the ordinate), irrespective of the virus titer obtained. Empty circles indicate serial passages of undiluted virus in the absence of mutagens (the same experimental series plotted on the two panels). No extinction of FMDV C-S8c1 has ever been observed in serial passages of viruses, even with 100- or 1,000-fold dilutions intervening between passages, in the absence of mutagens (56). Preextinction populations are indicated by arrows. The preextinction population C-S8c1AZCp10 was split into two sublineages; one was extinguished at passage 11, and the other could be further passaged and was extinguished at passage 21. Loss of infectivity was ascertained by three additional blind passages, with no evidence of infectivity or RT-PCR-amplifiable FMDV RNA in the culture supernatant from the third passage. Preextinction populations were tested in infections of BHK-21 cells in the absence of a mutagen; in all instances, titers produced were 10^4 to 10^6 PFU/ml. All titrations were done in triplicate. Standard deviations (not included in the plots) never exceeded 50%.

spectrum of treated populations was transition U → C, which affected a total of 11 sites; only 1 site in untreated populations included this mutation (Table 2). This mutation is expected from the ambiguous reading of fluorouridine (as complementary to adenosine) once incorporated into plus-strand RNA molecules acting as replicative intermediates (52, 63). The second most abundant transitions (C → U and G → A) are expected to occur when fluorouridine takes the place of cytidine, often when concentrations of cytidine are low (63). In AZC-treated populations, the most frequent mutations were A → G, G → A, and C → U transitions. Transition G → A was also reported as an abundant type of AZC-induced mutation in a retroviral vector (47). However, in the latter study, as well as in bacteria (7, 10), C → G and G → C transversions were the

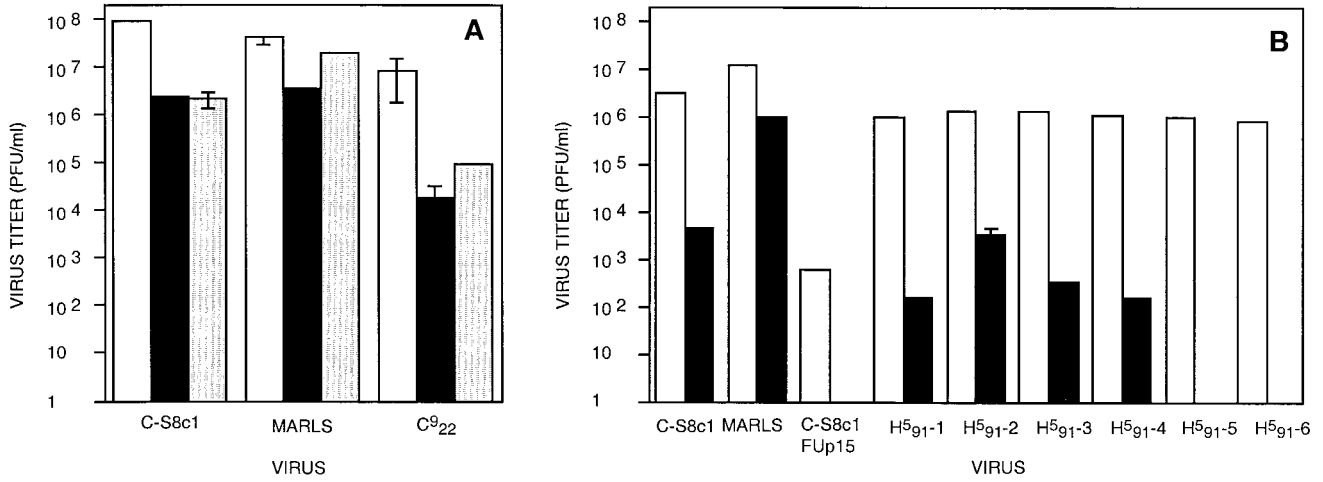

FIG. 3. Effect of viral fitness on the decrease in infectivity in a single round of infection in the absence or presence of FU or AZC. Empty columns indicate viral yield in the absence of a mutagen; black columns indicate yield in the presence of FU (200 μg/ml); grey columns indicate yield in the presence of AZC (10 μg/ml). (A) FMDV populations MARLS, CS8c1, and C^9_{22} (with relative fitness values of 10, 1, and 0.1, respectively; described in Materials and Methods) were tested by infecting 4×10^6 BHK-21 cells with 5×10^5 PFU of virus. (B) In this experiment, 3×10^5 BHK-21 cells were infected with 2×10^2 PFU of the indicated virus. C-S8c1 and MARLS were the same preparations as those used in panel A, diluted in DMEM; C-S8c1FUp15 is the preextinction population shown in Fig. 2; H^5_{91}-1, H^5_{91}-2, H^5_{91}-3, H^5_{91}-4, H^5_{91}-5, and H^5_{91}-6 are highly debilitated clones derived from C-S8c1 (19; Sierra and Escarmís, unpublished; see Materials and Methods). Empty columns indicate viral yield in the absence of a mutagen; filled columns indicate yield in the presence of FU (200 μg/ml). Yields of H^5_{91}-5 and H^5_{91}-6 in the presence of FU were undetectable, but virus reemerged after one blind passage in the absence of the mutagen. Extinction of C-S8C1FUp15 was confirmed by blind passages, as detailed in the legend to Fig. 2. Titrations were done in triplicate, and standard deviations are indicated. Procedures for chemical mutagenesis and determination of viral infectivity are described in Materials and Methods.

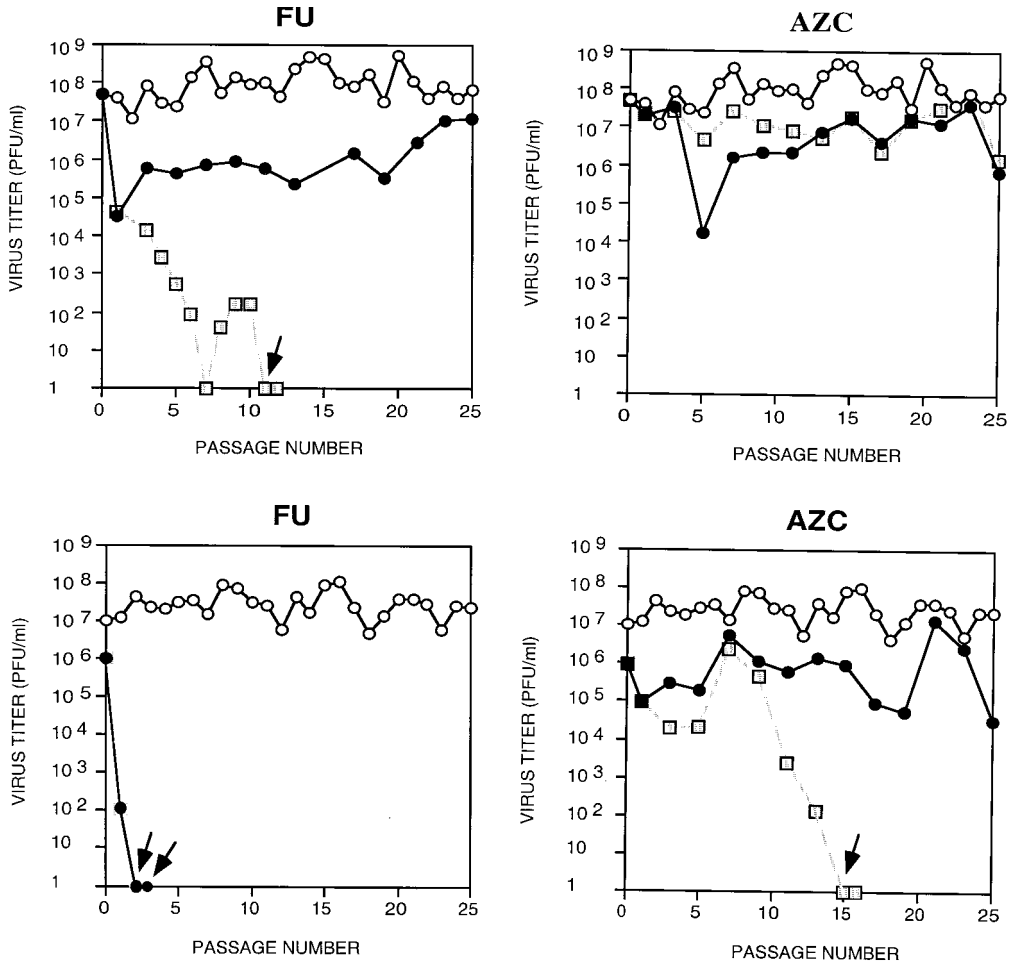

FIG. 4. Serial passage of MARLS (top panels) and C^9_{22} (bottom panels) in the absence or presence of FU or AZC. FMDV MARLS and C^9_{22} are described in Materials and Methods. MARLS has a 1,300-fold-higher relative fitness than C^9_{22} (19; Sierra and Escarmís, unpublished). The experimental design is identical to that described in the legend to Fig. 2. Empty circles correspond to serial passages of undiluted virus in the absence of a mutagen. Filled circles correspond to passages of undiluted virus, and squares correspond to passages in which the virus was diluted 10-fold prior to infection, irrespective of the virus titer obtained. Preextinction populations are indicated by arrows. Loss of infectivity was ascertained by absence of infectivity and RT-PCR-amplifiable material after three blind passages, as detailed in Materials and Methods and the legend to Fig. 2.

most frequent AZC-induced mutations, while in our analyses, these transversions were infrequent (Table 2). The *Pfu* polymerase used for the RT-PCR procedure is about 10 times more accurate than the *Taq* polymerase, an enzyme which yielded a basal error rate of $<10^{-4}$ substitution per nucleotide in a similar number of amplification cycles with FMDV RNA as a template (41). Therefore, although the possibility cannot be excluded that one particular mutation could be due to misincorporation during the in vitro RT-PCR amplification rather than to spontaneous or chemical mutagenesis during FMDV replication, the mutation frequency values observed (Table 1) ensure that the vast majority of mutations were present in FMDV RNA.

Two complementary types of measurements have been carried out to characterize the extent of heterogeneity in the mutant spectra of FMDV quasispecies: mutation frequencies and normalized Shannon entropies (60). The former describes the numbers of mutations present in the mutant spectrum, without attending to their distribution among individual genomes. The latter is a measure of algorithmic complexity in that it reflects the number of genomes with a different nucleotide sequence in a given mutant spectrum. We chose to compare two regions of the FMDV genome: the capsid protein VP1- and the 3D-coding regions (Table 1). In the VP1- and 3D-coding regions, 59% (excluding two point deletions; Table 2) and 36%, respectively, of mutations found in populations treated with FU or AZC were nonsynonymous (led to an amino acid replacement; Table 3). A comparison of these nonsynonymous replacements with those found among natural FMDV type C isolates and laboratory variants indicated that most replacements found in populations treated with FU or AZC were unique to mutagenized populations (Table 3). Except for 3D substitution I-334 → M, which affected β-strand 2 (located within motif C, three residues before the catalytic triad GDD [25]), all other 3D substitutions were located in loops linking α helices and β strands, assuming that amino acids assigned to structural motifs in poliovirus 3D (25) are located in the equivalent amino acids of aphthovirus 3D (37).

The 3D-coding region is very conserved among aphthoviruses and did not show any increase in mutation frequency after FMDV C-S8c1 was subjected to 25 passages in the absence of mutagens (Table 1). Treatment with FU resulted in an interesting equalization of mutation frequencies for the VP1-coding region (2.4×10^{-3} substitutions per nucleotide) and

TABLE 1. Quantification of genetic heterogeneity in the mutant spectrum of FMDV progeny in the absence or presence of the mutagenic base analog FU or AZC[a]

Protein[b]	Mutagen	Passage	No. of		Mutation frequency[e]	Shannon entropy[f]
			Clones[c]	Nucleotides[d]		
VP1	None	1	25	16,925	4.7×10^{-4}	0.38
		25	5	3,385	5.9×10^{-4}	0.42
	FU	1	25	16,925	8.3×10^{-4}	0.65
		25	5	3,385	2.4×10^{-3}	1.00
	AZC	1	5	3,385	8.9×10^{-4}	0.83
		10	5	3,385	2.1×10^{-3}	0.83
3D	None	1	5	7,025	2.8×10^{-4}	0.59
		25	5	7,025	2.8×10^{-4}	0.59
	FU	1	5	7,025	2.8×10^{-4}	0.59
		25	5	7,025	1.8×10^{-3}	1.00
	AZC	1	5	7,025	1.4×10^{-4}	0.31
		10	5	7,025	8.5×10^{-4}	1.00

[a] Populations analyzed and conditions of chemical mutagenesis are described in Materials and Methods and in the legend to Fig. 2. FU passage 25 is the undiluted population shown in Fig. 2. AZC passage 10 is the preextinction population indicated with an arrow in Fig. 2 (right panel). Changes in the consensus nucleotide sequences (relative to the sequence of C-S8c1) were observed in passage 25 with no mutagen (one replacement—C-3656 →A—which leads to T-150 → K in VP1) and in passage 25 with FU (six replacements—A-3328 → G and K-41 → E [VP1], A-3530 → G and H-108 → R [VP1], G-3706 → A and E-167 → K [VP1], A-6734 → G and K-42 → R [3D], T-7275 → C [synonymous], and T-6777 → C and T [mixture, synonymous]). The list of mutations observed in the different populations is given in Table 2, and nonsynonymous replacements are given in Table 3.

[b] VP1 and 3D, products of genomic regions at positions 3193 to 3869 and 6609 to 8035, respectively.

[c] Number of cDNA clones in *E. coli* (see Materials and Methods) analyzed.

[d] Total number of nucleotides sequenced for each genomic region.

[e] Number of different mutations found divided by the number of nucleotides sequenced (given for each population and genomic region), expressed as substitutions per nucleotide. Mutations were counted by comparing sequence of genomes of a mutant spectrum with the consensus sequence of the corresponding population.

[f] The normalized Shannon entropy is calculated as $-\Sigma_i[(p_i \times \ln p_i)/\ln N]$ in which p_i is the frequency of each sequence in the quasispecies and N is the total number of sequences compared (60).

the 3D-coding region (1.8×10^{-3} substitutions per nucleotide). These comparisons (summarized in Table 1) led to similar conclusions when mutation frequencies were calculated relative to the consensus sequence of each particular population or to the sequence of the initial C-S8c1 clone. Therefore, rather than large increases in average mutation frequencies, the mutagenic treatment was noted as inducing increases in mutation frequencies in a genomic region (encoding 3D) which generally undergoes little variation and which maintains a mutant spectrum of limited complexity. Modest increases in mutational load in otherwise conserved regions may be critical to drive the virus to extinction. Mutagenic treatments may override mechanisms that normally tend to reduce the heterogeneity of particular genomic regions. This point is currently under further investigation.

Prospects for viral extinction as a therapeutic tool. The results of increased mutagenesis and loss of infectivity reported here for FMDV indicate that extinctions were stochastic, not systematic. This conclusion is illustrated by both extinction and survival after identical passages of C-S8c1AZCp10 (Fig. 2). Low viral loads favored a loss of infectivity of FMDV populations which had already accumulated mutations due to a history of passage in the presence of a mutagen (Fig. 2). Extinctions were not due exclusively to small population sizes, since passage of small FMDV C-S8c1 populations in the absence of a mutagen did not lead to extinction (56) and preextinction populations always yielded large numbers of progeny virus in the absence of a mutagen. Furthermore, extinction was very unlikely even after many serial plaque-to-plaque transfers of C-S8c1 (16; C. Escarmís et al., unpublished results).

A second, important influence on FMDV extinction was viral fitness. Low fitness favored viral extinction by increased mutagenesis (Fig. 3 and 4). This information on the effects of viral load and viral fitness is relevant to a potential application of extinction mutagenesis as an antiviral strategy in vivo. Administration of antiviral inhibitors often results in a decrease in viral load that may be sustained in the case of combination therapy, as documented in many studies on HIV-1 (18, 22, 24, 54). Also, selection of inhibitor-resistant variants may involve transient decreases in viral fitness (42). Viruses would appear to be vulnerable to extinction mutagenesis at these low viral loads and low fitness intervals. Our results with a number of FMDV clones and populations suggest that FU was more efficient than AZC in driving viral extinction (Fig. 4), in agreement with its increased mutagenic potential, with respect to AZC, reported for other viruses (14, 50). Although FU is used in anticancer therapy (45), it would be desirable to design new drugs to target the fidelity properties of RNA replicases or retrotranscriptases, a notion supported by mounting evidence that structural alterations of these enzymes may increase or decrease their copying fidelity properties (e.g., references 38 and 62). In spite of obvious difficulties in the implementation of extinction mutagenesis as a valid antiviral strategy (11), nonretroviral riboviruses would seem to be the most vulnerable to enhanced mutation rates, since their replication and survival depend on an RNA-dependent RNA replication process in infected organisms, with no insertion of DNA copies of the viral genome into cellular DNA. In this respect, studies done with additional RNA viruses to find parallels or differences

TABLE 2. Types of mutations in the mutant spectrum of FMDV C-S8c1 progeny in the absence or presence of the mutagenic base analog FU or AZC[a]

Mutation type[b]	No. of mutations in the FMDV C-S8c1 population at the indicated passage in the presence of the following mutagen:					
	None		FU		AZC	
	1	25	1	25	1	10
A → C	0	1	1	0	0	0
A → G	1	0	3	3	2	3
A → U	1	0	0	0	0	0
C → A	3	1	3	1	1	1
C → G	0	0	0	0	1	1
C → U	3	0	1	5	0	3
G → A	1	1	0	5	0	3
G → C	0	0	0	0	0	0
G → U	1	0	1	0	0	0
U → A	0	0	1	0	0	0
U → C	0	1	4	7	0	2
U → G	0	0	0	0	0	0
Deletions[c]	0	0	2	0	0	0
Total	10	4	16	21	4	13
No. of mutations per nucleotide	0.00042	0.00038	0.00068	0.0020	0.00038	0.0012

[a] Populations, numbers of clones and nucleotides analyzed, and conditions of chemical mutagenesis are described in Materials and Methods and in Table 1, footnotes *a* and *c*, and the legend to Fig. 2.

[b] Mutations are those scored in the VP1- and 3D-coding regions.

[c] Deletion of a G (corresponding to the last triplet of VP1), observed in two clones, which should correspond to defective genomes. Only one accompanying mutation was found in these clones; exclusion of these mutations does not alter the calculations and conclusions reported in this table, in Table 1, and in the text.

TABLE 3. Amino acid replacements found in VP1 and 3D of the mutant spectrum of FMDV populations subjected to chemical mutagenesis

Protein	Amino acid replacement[a]	Structural element[b]	Presence (+) or absence (−) of replacements in the following population[c]:		
			FU	AZC	Other
VP1	K-41 → E[d]	βB	−	+	+
	T-50 → A	βC	+	−	−
	I-61 → V	αA	+	−	−
	Y-72 → C	βD	+	−	−
	P-94 → L	l	+	−	−
	H-108 → R[d]	βF	−	+	−
	G-142 → W	l	+	−	−
	T-148 → K	l	+	−	+
	T-149 → K	l	+	+	+
	T-150 → K	l	−	+	+
	H-151 → R	l	−	+	+
	E-167 → D	l	+	−	−
	E-167 → K[d]	l	−	+	−
3D	A-116 → T	l	+	−	−
	V-181 → I	l	+	−	−
	I-196 → T	l	+	−	−
	F-230 → S	l	+	−	−
	R-289 → C	l	−	+	−
	I-334 → M	β2	−	+	−
	D-349 → E	l	−	+	−

[a] The single-letter amino acid code is used; amino acids are numbered independently for each protein.

[b] Structural elements (α, α helix; β, β strand; l, loop) of VP1 are assigned according to the three-dimensional structure of FMDV C-S8c1 (30, 39); structural elements of 3D are based on the three-dimensional structure of poliovirus 3D (25), assuming that the equivalent positions in FMDV 3D in an alignment of the two sequences (37) have the same structure.

[c] FU and AZC, FMDV populations derived from C-S8c1 after one or multiple passages in the presence of FU and AZC, respectively; they correspond to the populations described in Tables 1 and 2. Other, possible presence of the replacements in populations of FMDV type C natural isolates (35, 36, 39) or laboratory variants of FMDV C-S8c1 (3, 19, 20, 27, 40, 59).

[d] These three replacements have been found in the mutant spectrum of some populations and are dominant in some consensus sequences: K-41 → E and H-108 → R in three out of five clones of C-S8c1FUp25 and E-167 → K in two out of five clones of C-S8c1FUp25. Minority amino acids which coincide with the amino acid in the corresponding position of C-S8c1 are not included.

with the conclusions drawn from the FMDV results would be of great interest.

Given the pertinacious adaptability of RNA viruses and the high-frequency isolation of escape mutants (resistant to antibodies, cytotoxic lymphocytes, and antiviral agents) whenever an effective selective constraint is in operation (11, 28, 41, 42), it is important to explore whether entry into error catastrophy (lethal mutagenesis) could be developed into a new antiviral strategy, as has been suggested by several authors (11, 28, 29, 33, 34). In the present report, we have shown, using chemical mutagens as model drugs, that such an approach can also be effective with the important animal pathogen FMDV (48, 51). The observations on the influence of viral load and viral fitness encourage the design of virus-specific, fidelity-reducing drugs that could be applied in combination with immunotherapy and inhibitors of viral replication. Research in this direction is currently in progress.

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