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Mutagenesis versus Inhibition in the Efficiency of Extinction of Foot-and-Mouth Disease Virus

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RNA viruses replicate near the error threshold for maintenance of genetic information, and an increase in mutation frequency during replication may drive RNA viruses to extinction in a process termed lethal mutagenesis. This report addresses the efficiency of extinction (versus escape from extinction) of foot-and-mouth disease virus (FMDV) by combinations of the mutagenic base analog 5-fluorouracil (FU) and the antiviral inhibitors guanidine hydrochloride (G) and heparin (H). Selection of G- or H-resistant, extinction-escape mutants occurred with low-fitness virus only in the absence of FU and with high-fitness virus with some mutagen-inhibitor combinations tested. The combination of FU, G, and H prevented selection of extinction-escape mutants in all cases examined, and extinction of high-fitness FMDV could not be achieved by equivalent inhibitory activity exerted by the nonmutagenic agents. The G-resistant phenotype was mapped in nonstructural protein 2C by introducing the relevant mutations in infectious cDNA clones. Decreases in FMDV infectivity were accompanied by modest decreases in the intracellular and extracellular levels of FMDV RNA, maximal intracellular concentrations of FU triphosphate, and a decrease in the intracellular concentrations of UTP. In addition to indicating a key participation of mutagenesis in virus extinction, the results suggest that picornaviruses provide versatile experimental systems to approach the problem of extinction failure associated with inhibitor-escape mutants during treatments based on enhanced mutagenesis.

The error rate during template copying by viral RNA-dependent RNA polymerases and reverse transcriptases is close to the maximum tolerable for the maintenance of the genetic information of the virus. This is supported by theoretical predictions (reviewed in references 14 and 15) and experimental results on the adverse effects of enhanced mutagenesis on infectivity during cytolytic or persistent infections of viruses as diverse as picornaviruses (1, 9, 10, 23, 35, 52), retroviruses and retroviral vectors (29, 30, 37), the rhabdovirus vesicular stomatitis virus (VSV) (23), the flavivirus GB virus B (27), the arenavirus lymphocytic choriomeningitis virus (LCMV) (21, 46), and Hantaan virus (50).

Virus extinction by enhanced mutagenesis was accompanied by increases in mutant spectrum complexity, as quantitated by mutation frequency and Shannon entropy (1, 21, 35, 46, 52). LCMV showed a very high sensitivity to extinction by 5-fluorouracil (FU) during persistent infections in BHK-21 cells (21, 46), and recently it has been shown that administration of FU to mice prevented the establishment of a persistent LCMV infection (46), providing the first evidence of feasibility of a lethal mutagenesis approach to control viral infections in vivo.

Studies with the important animal pathogen foot-and-mouth disease virus (FMDV) have documented that low viral relative fitness and low viral load favored FMDV extinction by enhanced mutagenesis (35, 52). The effect of fitness was evidenced using FMDV clones which differed up to 10^6-fold in relative fitness values. In particular, FMDV MARLS (a monoclonal antibody [MAb]-escape mutant of FMDV clone C-S8c1 passaged 213 times in BHK-21 cells [7]) displays high fitness in BHK-21 cells and low fitness in Chinese hamster ovary (CHO) cells. In CHO cells, three serial passages in the presence of FU were sufficient to extinguish FMDV MARLS, while in BHK-21 cells, a combination of FU and the antiviral inhibitors guanidine hydrochloride (G) and heparin (H) was necessary to extinguish FMDV MARLS (35).

Treatment of FMDV of different relative fitness values with FU with or without the inhibitors G and H offered the possibility of studying the effect of viral fitness on the competing forces exerted by the mutagenic action of FU towards viral extinction and the tendency to select G- or H-escape mutants of FMDV that would result in an increase in viral load and avoidance of virus extinction. The results presented here document an advantage of mutagenic versus purely inhibitory treatments with regard to loss of infectivity and point to relative fitness as a key parameter to permit survival of inhibitor-escape mutants.

Effect of FU treatment on intracellular nucleotide pools and FUTP accumulation in BHK-21 cells. The mutagenic effect of FU in RNA viruses (6, 13, 19, 21–23, 35, 42, 46, 52, 57) is the result of incorporation of FU triphosphate (FUTP) into RNA (8, 20, 28, 36, 58). The mutagenic pathway involves the preferential incorporation of FUTP opposite to A and the ensuing misincorporation of G instead of A at the complementary position, presumably because pairing of G with FU in the template RNA is favored (59). This mechanism would result in the generation of transitions A→G and U→C, which are the most frequent mutation types induced by FU in two experimental models, FMDV and LCMV (21, 35, 46, 52). To determine the intracellular concentrations of FUTP, UTP, ATP, CTP, and GTP, extracts of BHK-21 cells were obtained at

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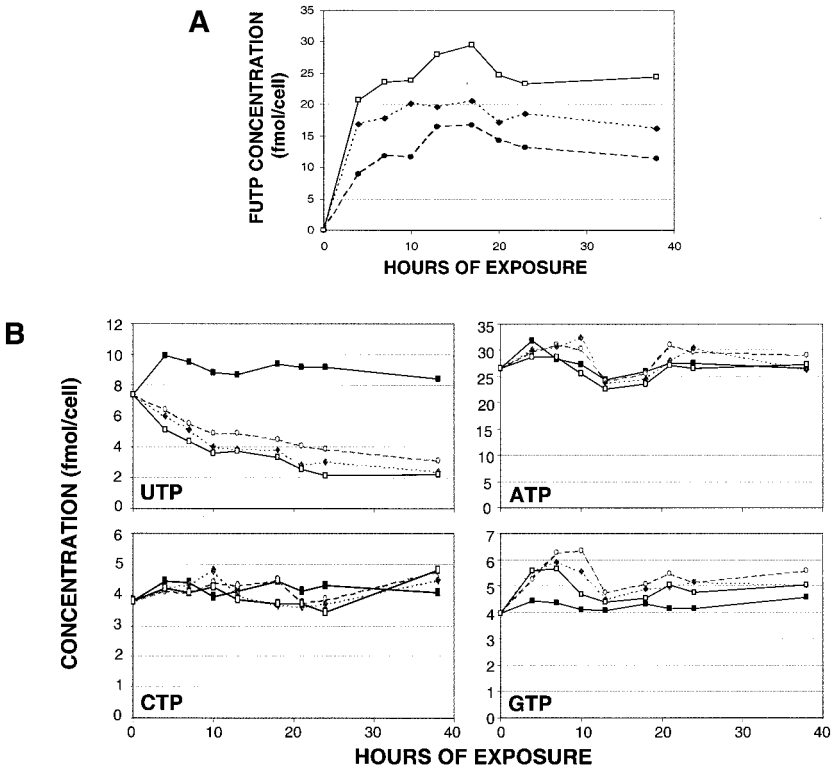

FIG. 1. FUTP accumulation and changes in intracellular ribonucleotide concentrations in FU-treated BHK-21 cells. (A) Intracellular FUTP concentrations were determined from BHK-21 cell cultures treated with FU (50 μg/ml) (dashed black lines with black circles), FU (100 μg/ml) (dashed black lines with black diamonds) and FU (200 μg/ml) (black lines with white squares). Assuming a cell volume of 3.6 ml/10^9 cells (55), the maximal intracellular FUTP concentration was approximately 7.5 mM. (B) Intracellular NTP concentrations were determined from BHK-21 cell cultures treated with Dulbecco's modified Eagle medium (DMEM) (solid lines with black squares), FU (50 μg/ml) (dashed gray lines with white circles), FU (100 μg/ml) (dashed gray lines with gray diamonds), and FU (200 μg/ml) (black lines with white squares). Note that the scale shown in ordinate is different in each graph. In all cases, samples were prepared for HPLC analysis at 4, 7, 10, and 13 h of exposure. After 13 h, the plates were mock infected (medium was removed and 200 μl of DMEM was added to mimic a real infection), and after 1 h, fresh medium with FU was added. NTPs were again extracted at 18, 21, 24, and 38 h (4, 7, 10, and 24 h after fresh medium was added).

different times of exposure to 50, 100, or 200 μg of FU/ml in the culture medium and subjected to high-performance liquid chromatography (HPLC) analysis as previously described (1, 41). The peaks of ATP, ADP, CTP, GTP, UTP, and FUTP were identified by comparing the observed retention times with those obtained with commercial nucleotide solutions. The peak of FUTP was the only additional peak in the nucleoside triphosphate (NTP) zone of the chromatograms (together with those of ATP, CTP, GTP, and UTP) that appeared in FU-treated samples, and its retention time was the same as that of commercial FUTP (Sierra Research). The ATP:ADP ratio was always above 20, indicating minimal loss of the terminal phosphate during sample preparation. Intracellular FUTP concentrations increased during the first 17 h of treatment with FU and decreased modestly after 18 h of exposure, reaching levels similar to those present after 10 h of treatment. These levels were maintained until at least 38 h of exposure to FU (Fig. 1A). Under our standard conditions of treatment with FU during FMDV infections (preincubation of cells with FU for 13 h followed by infection with FMDV for 24 h in the presence of FU [35, 52]), the intracellular FUTP content is between 23 and 30 fmol/cell (Fig. 1A). The relative concentrations of intracellular UTP, CTP, ATP, and GTP following exposure to 50, 100, or 200 μg of FU/ml were compared with those of parallel, untreated cultures (Fig. 1B). The most pronounced effect of FU treatment was a decrease of the intracellular concentration of UTP in a dose-dependent manner. After 10 h of exposure, 200 μg of FU/ml produced a 2.5-fold reduction in UTP levels, with respect to the control, and a 4.2-fold reduction after 24 h that was maintained until at least 38 h postexposure to FU. There was approximately ten times more FUTP than UTP in the cells during FMDV replication. There was a slight increase in GTP levels, although not in a FU dose-dependent manner, and there was no discernible effect on CTP and ATP levels (Fig. 1B). The same results were obtained when nucleotides were extracted from cultures treated for 4, 7, 10, and 24 h with FU (200 μg/ml) + G (4 mM) (after preincubation with FU for 13 h) (data not shown). We conclude that the maximum effects on nucleotide pools resulting from FU treatment occur during the period of FMDV infection under our experimental conditions (35, 52).

An essential contribution of mutagenesis versus inhibition in the extinction of FMDV. To investigate a possible advantage of using a mutagen, as opposed to using only antiviral inhibitors, to drive FMDV to extinction, we treated FMDVs differing up to 150-fold in relative fitness with different combinations of drugs. The mutagen and antiviral inhibitors used, as well as the procedures used to infect BHK-21 cells in liquid medium

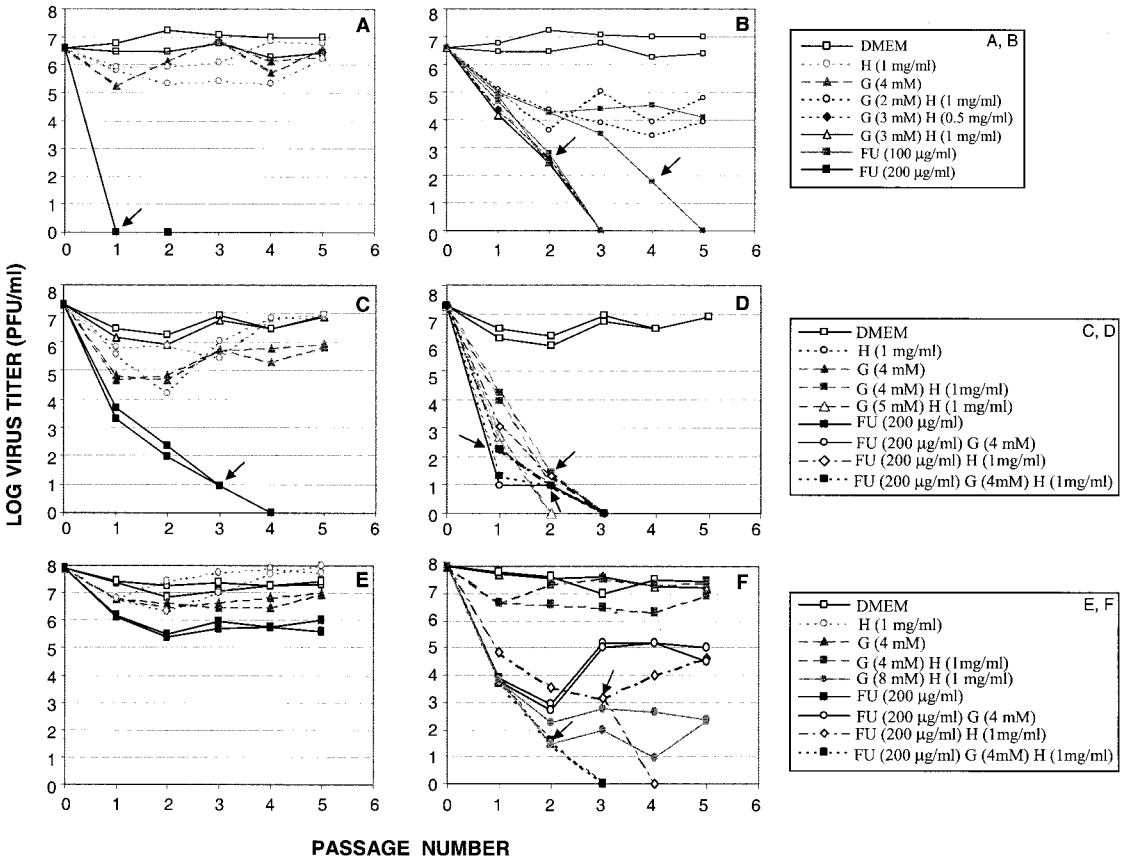

FIG. 2. Production of infectious virus upon passage of FMDVs H^5_{95} (A and B), REDpt60 (C and D), and MARLS (E and F) in the presence of different drug combinations in BHK-21 cells. Conditions for mutagenic and antiviral treatments have been previously described (35). In all cases, virus was tested by infecting 9×10^5 BHK-21 cells with 5×10^4 PFU of virus, and the next passages were carried out by infecting cells with 0.1 ml of supernatant from the previous passage. The multiplicity of infection can be calculated from the titers shown in ordinate. The legend panels identify drug treatments of the assays shown on their left. DMEM indicates culture medium in the absence of mutagen and inhibitor. Passages one to four depicted in panel E were published previously (35), and are included here for completeness. Preextinction populations are indicated by arrows. Viral extinction was ascertained by three additional blind passages in the absence of mutagen and inhibitors, with no evidence of infectivity or RT-PCR-amplifiable material in the supernatant of the third passage (35, 52). All titrations were done in triplicate. Standard deviations (not included in the plots) never exceeded 20%.

and for plaque assays, have been previously described (5, 12, 35). The concentrations of FU, G, and H (expressed in micrograms per milliliter for FU, milligrams per milliliter for H, and millimolar for G, to facilitate comparison of results of other laboratories) are indicated in parentheses following the corresponding abbreviation. Combination treatments are indicated with a "+" between the drugs employed in the treatment. The cytotoxicity of treatments with FU (200 μg/ml); G (4 mM); H (1 mg/ml); FU (200 μg/ml) + G (4 mM); FU (200 μg/ml) + G (4 mM) + H (1 mg/ml); and G (4 mM) + H (1 mg/ml) has been previously described (35). The rest of treatments used in this report were only cytostatic, except G (8 mM) + H (1 mg/ml), which was cytotoxic (85% viable cells after 38 h of treatment). For all three FMDVs analyzed, namely, H^5_{95}, REDpt60, and MARLS, when only one drug was used, either FU or an antiviral inhibitor, viral suppression was greater in the presence of FU and led to extinction of H^5_{95} and REDpt60 but not of MARLS (Fig. 2A, C, and E). Most of the combination treatments used had a similar antiviral effect in one passage (the reduction in viral titer was equivalent) whether they included FU or only combinations of the inhibitors G and H (Fig. 2B, D, and F).

FMDV H^5_{95} is a virus obtained after two passages of a mixture of five biological clones that were isolated after 95 plaque-to-plaque transfers of clone H5 (derived from C-S8c1p113 [16]) (18, 35); it has a relative fitness value of 0.05 with respect to our reference clone C-S8c1 (53). Combinations G (3 mM) + H (0.5 mg/ml) and G (3 mM) + H (1 mg/ml), which inhibited H^5_{95} production as much as FU (100 μg/ml), led to virus extinction in three passages, like two out of four series of passages in the presence of FU (100 μg/ml) (Fig. 2B). Combination G (2 mM) + H (1 mg/ml), which inhibited almost as much as the other combinations, failed to extinguish the virus, and mutants of H^5_{95} that are resistant to H emerged (Table 1). Since H^5_{95} in the presence of FU (200 μg/ml) became undetectable in one passage and the virus was extinguished in two passages, a lower concentration of FU (100 μg/ml) was used to compare with combination treatments without mutagen (in order to obtain comparable inhibitions with and without mutagen).

The origin of FMDV REDpt60 has been previously de-

TABLE 1. Substitutions found in FMDVs H^5_{95}, REDpt60, and MARLS subjected to treatments that did not lead to viral extinction

Viral population and treatment[a]	Substitutions in 2C[b]	Substitutions in the capsid[b]:	
		VP3	VP1
H^5_{95}			
G (4 mM)p5A	M159→L	ND	ND
G (4 mM)p5B	M159→L	ND	ND
H (1 mg/ml)p5A	ND	K173→T	—
H (1 mg/ml)p5B	ND	K173→T	—
G (2 mM) Hp5A	—	K173→E	—
G (2 mM) Hp5B	—	K173→K/E	—
REDpt60			
G (4 mM)p5A	M159→M/L	ND	ND
G (4 mM)p5B	M159→L	ND	ND
H (1 mg/ml)p5A	ND	—	R133→Q
H (1 mg/ml)p5B	ND	—	R133→Q
MARLS			
G (4 mM)p5A	M159→M/L	ND	ND
G (4 mM)p5B	C141→C/S	ND	ND
H (1 mg/ml)p5A	ND	T67→T/I, D68→D/H	K206→K/I, D52→D/A
H (1 mg/ml)p5B	ND	D68→D/H	—
G (4 mM) H (1 mg/ml)p5A	K169→R	T67→T/I, D68→D/H	N60→T
G (4 mM) H (1 mg/ml)p5B	C141→S	K173→E	S144→L
FU (200 μg/ml) G (4 mM)p5A	M159→L	ND	ND
FU (200 μg/ml) G (4 mM)p5B	M159→L	ND	ND
FU (200 μg/ml) H (1 mg/ml)p5B	ND	K218→K/E	K206→Q
G (8 mM) H (1 mg/ml)p5A	K169→R	—	—
G (8 mM) H (1 mg/ml)p5B	M159→L	M128→T	—

[a] The viral populations analyzed and the antiviral treatments used are described in the text. The viral populations analyzed are those described in Fig. 3; p indicates the passage number, and A and B distinguish duplicate series.

[b] The single-letter amino acid code is used. Amino acids are numbered independently for each protein (16, 17). When the antiviral treatment used contained G, the 2C protein was sequenced; when H was used, the capsid-coding region of the virus was sequenced; when both G and H were used, both regions of the genome were sequenced. Mixtures are indicated with the two amino acids separated with a slash. Substitutions are determined in each case by comparison with the consensus sequence of the corresponding parental virus. No amino acid substitutions were found in VP4 and VP2. —, absence of substitution; ND, not determined.

scribed (45). This virus has a relative fitness 1.5 times higher than that of C-S8c1. Treatment of REDpt60 with FU (200 μg/ml) + H (1 mg/ml) and with G (5 mM) + H (1 mg/ml) exerted the same antiviral effect in one passage, and treatments with FU (200 μg/ml) + G (4 mM) with or without H (1 mg/ml) exerted a higher antiviral effect, but all these treatments extinguished the virus (Fig. 2D). REDpt60 treated with G (5 mM) + H (1 mg/ml) was extinguished in two passages, and extinction with FU (200 μg/ml) + G (4 mM), FU (200 μg/ml) + H (1 mg/ml), and FU (200 μg/ml) + G (4 mM) + H (1 mg/ml) was attained in three passages. However, with FU (200 μg/ml) + G (4 mM) with and without H (1 mg/ml), no infectivity could be detected in a plaque assay in one passage (but virus was recovered in the second blind passage). It was unexpected that REDpt60 could be extinguished with FU (200 μg/ml) alone while C-S8c1, of comparable fitness, could not (35, 52). This difference may relate to mutations present in REDpt60 that, although not impairing fitness under standard culture conditions, may render this genome less tolerant to environmental perturbations, as previously described for VSV (43).

In the case of MARLS (5, 31, 33), a clone with a relative fitness 130-fold higher than that of C-S8c1, only treatments in which FU was present in combination with inhibitors were successful in driving the virus to extinction (Fig. 2F), even though a similar antiviral effect was exerted by the combination treatment G (8 mM) + H (1 mg/ml). One of the duplicates passaged in the presence of FU (200 μg/ml) + H (1 mg/ml), which exerted 1 logarithm less antiviral effect in one passage, was also extinguished. These results document an essential contribution of mutagenesis versus inhibition in driving FMDV populations to extinction.

Characterization of escape mutants to treatments with FU, G, and/or H. When extinction of H^5_{95}, REDpt60, or MARLS was not accomplished during the passages in the presence of different antiviral treatments (Fig. 2), viral RNA was extracted from the supernatant of the fifth passage, amplified by reverse transcription-PCR (RT-PCR), and sequenced to investigate the possible presence of drug resistance mutations. The nonstructural protein 2C was analyzed when the treatment contained G, because resistance to G has previously been found to map to this protein (3, 38–40, 48, 49). The entire capsid-coding region was analyzed when H was included in the treatment, because amino acids that affect binding to H are located in the capsid (4, 5, 24, 44, 47). Mutations conferring resistance to H have been studied (4, 44) and map to capsid amino acid positions usually representing a loss of a positive charge or, less frequently, the addition of a positive charge in the vicinity of an already existing one. Whenever FMDVs were not extinguished, viruses with mutations that confer resistance to G or H were selected (Table 1). Some other mutations appeared in VP3 and VP1, either in a dominant form or in a mixture with the wild-type amino acid, that do not confer a change in charge. Their possible contribution to H resistance has not been described (Table 1). All the changes, whether or not they

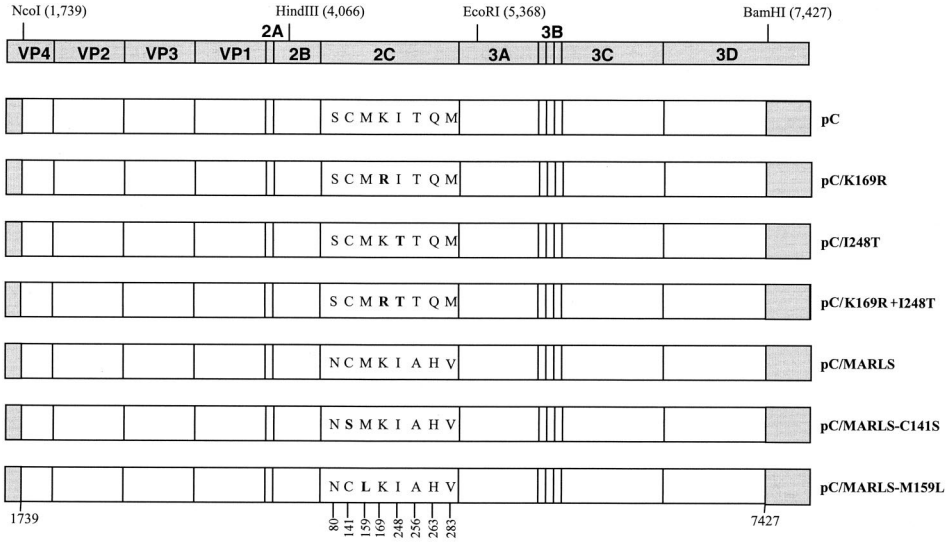

FIG. 3. FMDV chimeric plasmids. The scheme on the top of the figure represents FMDV C-S8c1 genomic regions encoding proteins VP4 to 3D. Numbering of FMDV genomic nucleotides is according to Toja et al. (54). Relevant restriction sites used for construction of chimeric cDNAs are indicated at the top, with the 5′ position of the restriction site given in parentheses. Below the FMDV C-S8c1 genome scheme are the different chimeric plasmids used in this study. In plasmid pC (34), the C-S8c1 FMDV region spans residues 1739 to 7427, which correspond to protein residues S33 of VP4 to W283 of 3D. The rest of the FMDV genome is that of FMDV O1K (serotype O) (shaded boxes) (60). Bold residues in the 2C box are those introduced to study their implication in resistance to G. The position numbers of amino acids in 2C that differ between C-S8c1 and MARLS and the positions on the 2C protein altered by site-directed mutagenesis are indicated at the bottom.

involved a change of charge, appeared in exposed positions of the capsid (32). All preextinction populations had the wild-type sequence, indicating lack of selection of resistance mutations, a fact that presumably was determinantal for the extinction observed.

Early work showed that G resistance mutations in FMDV map to protein 2C (48, 49), as in the case of poliovirus (2, 3, 39, 40), and a specific mutation in the carboxy-terminal part of 2C of FMDV O_6 was defined (49). We introduced the mutations that we found among FMDV clones and populations passaged in the presence of G in a chimeric infectious clone (references 34 and 60 and references therein) to determine whether the mutations conferred the G resistance phenotype (Fig. 3). Mutations leading to K169R and I248T were introduced, either individually or together, in the C-S8c1 background, and mutations C141S and M159L were introduced in the MARLS sequence context. Positions 169 and 248 of 2C were substituted frequently in C-S8c1 passaged in the presence of G and were found together in several G-resistant biological clones (results not shown). When introduced separately in an infectious clone, the second mutation arose promptly after transfection, indicating a selective advantage of a virus harboring the two replacements, even in the absence of G. Substitutions in positions 141 or 159 were found always in the context of multiply passaged FMDVs (never in the C-S8c1) and always as the only change present in 2C, never in combination, and thus we introduced these changes in the sequence context of MARLS 2C (which presents four amino acid changes with respect to C-S8c1 [Fig. 3]). The G resistance phenotype was characterized with a plaque assay in the presence or absence of G (the results for viruses with mutations K169R and I248T are shown in Fig. 4A). C-S8c1 and the wild-type chimera showed a reduction of 2.5 logarithms of viral production in the presence of 4 mM G, whereas the mutated chimeras showed no difference in viral production in the presence of 4 mM G. Substitution C141S conferred a resistant phenotype, with similar viral production in the presence or absence of G. The virus with M159L was partially resistant, and with 8 mM G, the viral production was somewhat less than 2 logarithms higher than for C/MARLS, although it was 1 logarithm lower than in the absence of G (Fig. 4B).

Viral RNA levels during C-S8c1 infection in the presence of FU (200 μg/ml) with and without G (4 mM). The amount of viral RNA during the replication of C-S8c1 in the presence of FU (200 μg/ml) with and without G (4 mM) was compared with that of a parallel infection in the absence of FU and G (Fig. 5). The presence of G led to a 1-logarithm decrease in viral production and in the intracellular and extracellular RNA levels. The reduction of intracellular and extracellular RNA levels observed in the presence of FU accounts for only 1/10 of the reduction of infectivity. From the data of Fig. 5, it can be estimated that in the presence of FU (with or without G), the ratio of infectivity to the amount of extracellular viral RNA at 24 h postinfection was 20-fold lower than in infections in the absence of FU. We conclude that during FMDV infections, the main force driving the virus to extinction is the mutagenic effect of FU rather than the decrease in RNA levels during the infection.

Mutagenic action of FU and the problem of virus extinction versus escape. FU inhibits the cellular enzyme TMP synthetase, which catalyzes the conversion of dUMP into TMP. Due to this activity, dTTP levels are significantly reduced, dGTP and dCTP levels are moderately reduced, and dATP levels are unaltered (20, 26, 36), but to our knowledge, no quantitations of the effect of FU on NTP pools have been reported. We have used this mutagen to drive FMDV into extinction (35, 52) and investigated whether the NTP levels in

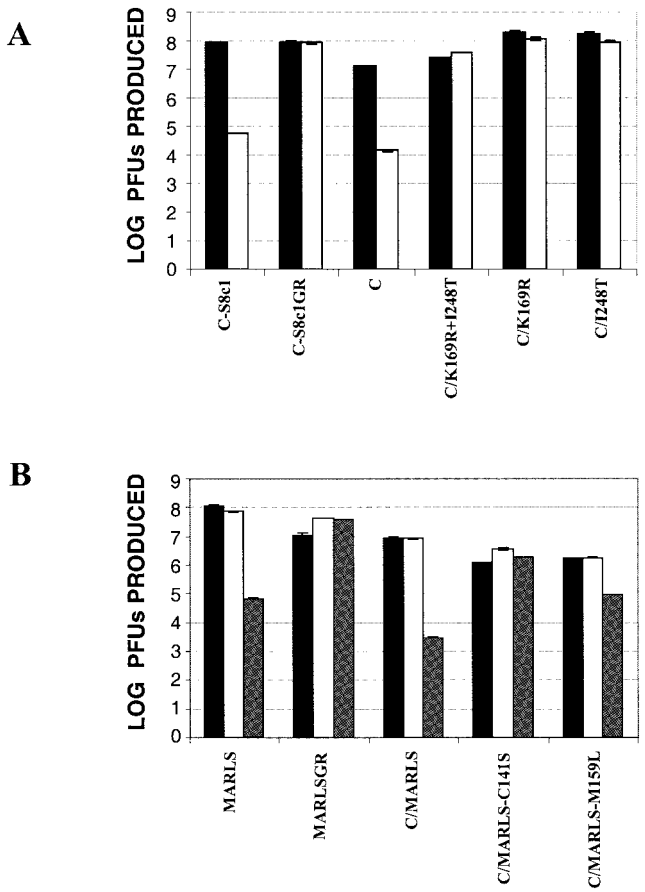

FIG. 4. Viral production in the presence or absence of G. Black columns represent viral production in the absence of G, empty columns indicate viral production in the presence of 4 mM G, and gray columns indicate viral production in the presence of 8 mM G. BHK-21 cells (2×10^6 to 4×10^6) were infected with serial dilutions of virus. After 45 min of adsorption, cells were overlaid with 0.5% agar medium with either 0, 4, or 8 mM G. After 24 h, cells were fixed with 2% formaldehyde and visualized by crystal violet staining. Viral production was estimated from the number of plaques and the viral dilution. The amino acid substitutions present in 2C of each virus tested are shown below the corresponding column. (A) Viruses derived from C-S8c1. C-S8c1GR is a G-resistant population obtained after four passages in the presence of 4 mM G. C is the virus obtained from chimera pC. (B) Viruses derived from MARLS. MARLSGR is a G-resistant population obtained after four passages in the presence of 4 mM G. Viruses were plated with 8 mM G because MARLS is partially resistant to G (there are four amino acid changes between the 2C of C-S8c1 and MARLS, and the 50% inhibitory concentration for MARLS is 2.7 times higher than that of C-S8c1) and because the difference in plaque formation was better assessed at higher concentrations of G. Sequence differences in protein 2C between the chimeras used in this experiment are depicted in Fig. 3.

BHK-21 cells were altered during this process. Mutagenesis might be mediated by the alteration in NTP pools, direct incorporation of FU into nascent viral RNA and the subsequent mispairing with G, or both. The results indicate that UTP is depleted in BHK-21 cells treated with FU but that the other NTPs remain at close-to-normal levels. In our system, FU accumulates in BHK-21 cells and is converted into FUTP, resulting in an FUTP concentration ten times higher than that of UTP in BHK-21 cells during replication of FMDV. This result suggests that the probable mechanism for FU-induced mutagenesis is direct incorporation into RNA, because it seems unlikely that the small alterations in NTP pools observed are responsible for the degree of mutagenesis induced by FU on FMDV (35, 52). Further studies with FMDV polymerase in vitro would be necessary to prove the incorporation of FUTP.

FU caused a 100-fold inhibition of C-S8c1 infectivity and a 10-fold reduction of the amount of viral RNA. This observation suggests that most of the RNA may be noninfectious due to accumulation of mutations, which we have reported previously using the same system (35, 52). These results are in agreement with the results showing that a 100-fold reduction of LCMV infectivity induced with FU treatment was associated with a 5-fold reduction in RNA synthesis (46). This provides further support to the evidence that, although FU can have other effects on cellular RNA species (20, 28, 36, 58) and on virus replication (19), mutagenesis is a major factor operating during FMDV and LCMV extinction (14, 21, 35, 46, 51, 52).

The efficient extinction of FMDV with combinations of FU and inhibitors has been previously described (35). Here we have documented that there is an advantage in the use of a mutagen over treatments without mutagen that achieve a similar degree of inhibition in relation to the selection of inhibitor-escape mutants. We have shown that for high-fitness FMDV, extinction is achieved only when a mutagen is present in the treatment. In the absence of FU, inhibitor-escape mutants were selected. However, intermediate- and low-fitness FMDVs could be extinguished with combination treatments without FU in the same or even a lower number of passages. REDpt60 is an intermediate-fitness FMDV population obtained after 60 plaque-to-plaque transfers (45). The accumulation of mutations associated with serial plaque-to-plaque transfers of FMDV (16, 18) may have rendered REDpt60 more susceptible to extinction than other viruses, such as C-S8c1, of equivalent relative fitness in BHK-21 cells (references 18 and 45 and data not shown). REDpt60 could be extinguished with FU in four passages under the same conditions in which C-S8c1 was not extinguished (52). The phenomenon underlying the sensitivity to extinction of REDpt60 could be contingent neutrality of accumulated mutations, first described with a VSV mutant that, despite showing fitness equal to that of the wild type under standard cell culture conditions, manifested a selective disadvantage when a number of environmental perturbations intervened during viral replication (43). Theoretical studies have also suggested the advantage of replicons with increased robustness against mutations (56). Other examples of extinction-prone combinations of mutations of viruses that show high fitness in a specific environment have been reviewed (11).

We have identified four replacements in the 2C protein that confer G resistance to FMDV serotype C, namely, C141S, M159L, K169R, and I248T. The resistance to G has been mapped in protein 2C for several picornaviruses, such as poliovirus (2, 3, 39, 40), echovirus-9 (25), and FMDV serotype O (48, 49). The determinants for G resistance in FMDV serotype C were not obvious, because one of the mutations that confer high G-resistance to poliovirus is N179→G (39), and in FMDV there is a G at this position. Introduction of the relevant mutations into cDNA clones to produce the corresponding infectious transcripts confirmed their association with the G resistance phenotype.

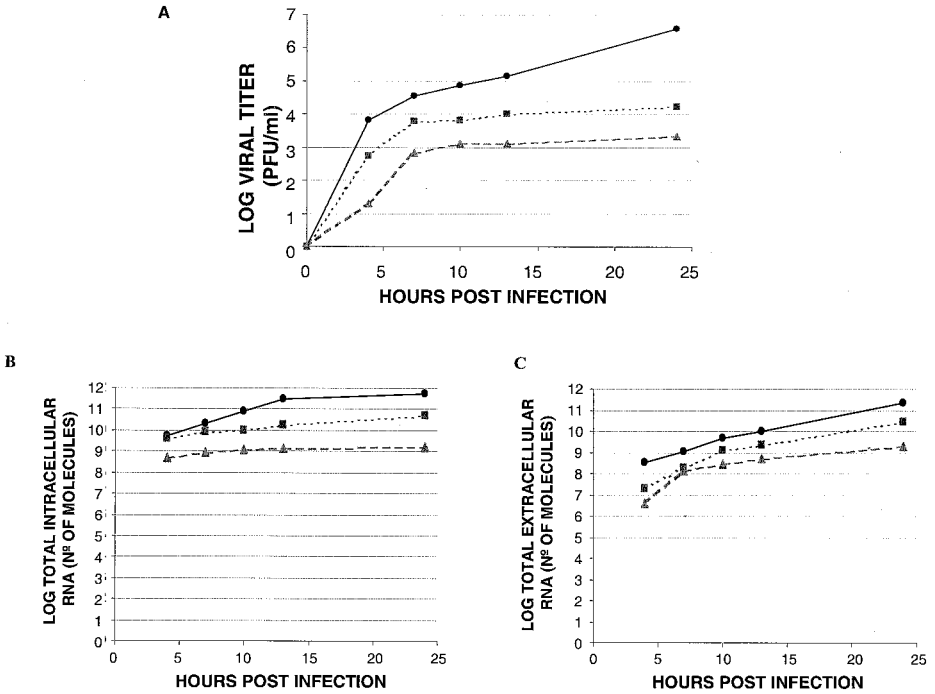

FIG. 5. Production of C-S8c1 infectious particles and RNA in one passage with different treatments. In all three panels, solid lines with circles represent infections in the presence of DMEM, dashed lines with squares denote presence of FU (200 μg/ml), and dashed lines with triangles represent treatment with FU (200 μg/ml) + G (4 mM). In all experiments, 9×10^5 BHK-21 cells were infected with 5×10^4 PFU of C-S8c1. (A) Viral production. Virus titers at different times after infection were determined by plaque assays on BHK-21 cell monolayers in triplicate; standard deviations (not included in the plots) never exceeded 25%. (B) Intracellular FMDV RNA level. Quantitation was done at the same time points at which virus in the media was titrated (panel A). RNA levels are the means of two independent quantitations. (C) FMDV RNA level in the culture medium. Quantitation was done at the same time points at which virus in the media was titrated (panel A). Extraction of C-S8c1 RNA has been previously described (35), and quantification was performed with the Light Cycler instrument (Roche) by using the Light Cycler RNA master SYBR Green I kit (Roche) and following the manufacturer's instructions. RNA levels are the mean of two independent quantitations. The amount of RNA at 0 h postinfection in panels B and C could not be determined because it was below the limit of reliable quantification of FMDV RNA (which is 3×10^4 RNA molecules under our experimental conditions).

Virus entry into error catastrophe is a promising new antiviral strategy (9, 10, 11, 14, 21, 23, 27, 29, 30, 35, 37, 46, 50, 51, 52) strongly encouraged by the recent observation of Ruiz-Jarabo et al. that FU treatment can prevent the establishment of a persistent LCMV infection in mice (46). However, a practical application of this antiviral strategy will require the finding of adequate, nontoxic, virus-specific mutagenic agents. Since low viral loads and low relative fitness favor virus extinction by enhanced mutagenesis (35, 51, 52), it may be necessary to design treatments to procure a low viral fitness at the onset of the mutagenic treatment and/or a low viral load by employing antiviral inhibitors and mutagens in succession or in combination. In these designs, it becomes critical to study the dynamics of viral extinction versus selection of inhibitor-resistant mutants that may mediate escape from extinction. The present report on FMDV has illustrated the contribution of mutagenesis to virus extinction, as well as escape of extinction through selection of inhibitor-resistant mutants whenever the intensities of the mutagenic and inhibitory activities were not commensurate with the fitness of the virus to be extinguished. The results presented here alert us to the need of a careful design of experiments to test the potential of enhanced mutagenesis in vivo and show that picornaviruses may provide adequate experimental systems to explore critical parameters before approaching more complex viral diseases.

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