Fitness Alteration of Foot-and-Mouth Disease Virus Mutants: Measurement of Adaptability of Viral Quasispecies†

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We document the rapid alteration of fitness of two foot-and-mouth disease virus (FMDV) mutants resistant to a neutralizing monoclonal antibody. Both mutants showed a selective disadvantage in BHK-21 cells when passaged in competition with their parental FMDV. Upon repeated replication of the mutants alone, they acquired a selective advantage over the parental FMDV and fixed additional genomic substitutions without reversion of the monoclonal antibody-resistant phenotype. Thus, variants that were previously kept at low frequency in the mutant spectrum of a viral quasispecies rapidly became the master sequence of a new genomic distribution and dominated the viral population.

RNA viruses—including single isolates and clonal populations—consist of multiple variants collectively termed viral quasispecies (4–9, 11–13, 17, 18, 24, 29, 30). The proportion of each variant is determined by the rate at which it is generated by mutation as well as by its competitive fitness relative to all other variants present and arising in the population (2, 8, 10, 12–14, 17, 29). We are interested in the implications of quasispecies organization in determining antigenic diversification of foot-and-mouth disease virus (FMDV). This aphthovirus of the family *Picornaviridae* causes the economically most important disease of cattle (1, 9, 25). The antigenic heterogeneity of FMDV populations (9, 20–23, 26) is a major obstacle to the efficacy of synthetic vaccines. A major antigenic site of FMDV is located at residues 138 to 160 of capsid protein VP1 (21, 22), the binding site of several neutralizing monoclonal antibodies (MAbs). The epitope recognized by MAb SD6 involves amino acids 138, 139, and 146 of VP1 (22) of FMDV C-S8c1, a plaque-purified derivative of FMDV C-S8 (Santa Pau, Spain, 1970) (27). We have used two MAb SD6-resistant (MAR) mutants of FMDV C-S8c1—termed HR and SN, which contain substitutions H-146→R and S-139→N, respectively, in capsid protein VP1 (21)—to document that they show a clear selective disadvantage relative to their parental FMDV C-S8c1 population. However, this selective disadvantage was lost upon further passage of the mutants, even though the MAR phenotype and the relevant amino acid substitution were maintained.

The procedures for culturing BHK-21 cells and MAb SD6-secreting hybridomas and for FMDV infectivity assays have been described previously (6, 23, 27). The passage history of FMDV C-S8c1 in cell culture involved three successive plaque isolations and an amplification from about 10^5 to 10^8 PFU in BHK-21 cells (6, 27). Serial passages of FMDV were carried out by infecting a monolayer of about 2 × 10^6 BHK-21 cells with 4 × 10^6 to 8 × 10^6 PFU (multiplicity of infection, 2 to 4 PFU per cell). The progeny of each infection was used to infect a fresh cell monolayer at the

same multiplicity. The frequency of MAR genotypes was determined by plating about 10^5 PFU of FMDV C-S8c1 on monolayers of 10^7 BHK-21 cells with a 100-fold dilution of MAb SD6 ascitic fluid included in the agar overlay. This dilution of MAb completely suppressed plaque formation of 100 PFU of virus C-S8c1 and did not affect the PFU counts of FMDV HR and SN. Incubation of FMDV C-S8c1 with MAb prior to plating was avoided to prevent underestimation of MAR genotypes by phenotypic mixing (16, 31). For the plaque-reduction assays, about 100 PFU of FMDV was incubated for 2 h at 4°C either with the appropriate dilution of MAb SD6 in Dulbecco's modified Eagle's medium or with Dulbecco's modified Eagle's medium alone (23). A 1:1 dilution of MAb SD6 (supernatant of the hybridoma culture) was found adequate to decrease by more than 99% the number of PFU of FMDV C-S8c1, whereas the number of PFU of FMDV HR or SN was not affected. This dilution was used throughout the experiments.

Selective disadvantage of MAR mutants. Serial infections of BHK-21 cells were carried out in duplicate, using as starting populations FMDV C-S8c1, HR, SN, or mixtures of equal numbers of PFU of two viruses. Since HR and SN are resistant to neutralization by MAb SD6, the latter was used to determine the proportion of MAb-resistant mutants in the evolving populations. No change in the extent of neutralization of any of the viruses passaged individually was noted (Fig. 1A). In the coinfections with C-S8c1 and HR (Fig. 1B) or SN (Fig. 1C), FMDV neutralized by MAb SD6 became dominant, suggesting that C-S8c1 had a selective advantage over HR or SN. To ascertain that the dominant virus was C-S8c1, viral RNA from passages 1 and 15 was purified and the RNA encoding the relevant VP1 region was sequenced (Fig. 2). In agreement with the results of the neutralization assays, virus with a sequence diagnostic of C-S8c1 was present at passage 15, whereas at passage 1 the expected mixtures of C-S8c1 and mutant were found. FMDV C-S8c1, HR, and SN propagated separately each maintained unchanged sequences. The fitness of the mutants relative to C-S8c1 was 0.78 for HR/C-S8c1 and 0.68 for SN/C-S8c1 (Table 1).

The proportion of MAb-resistant mutants in viral populations has often been used to estimate the rate at which the mutants arose. However, for MAb-resistant mutants that
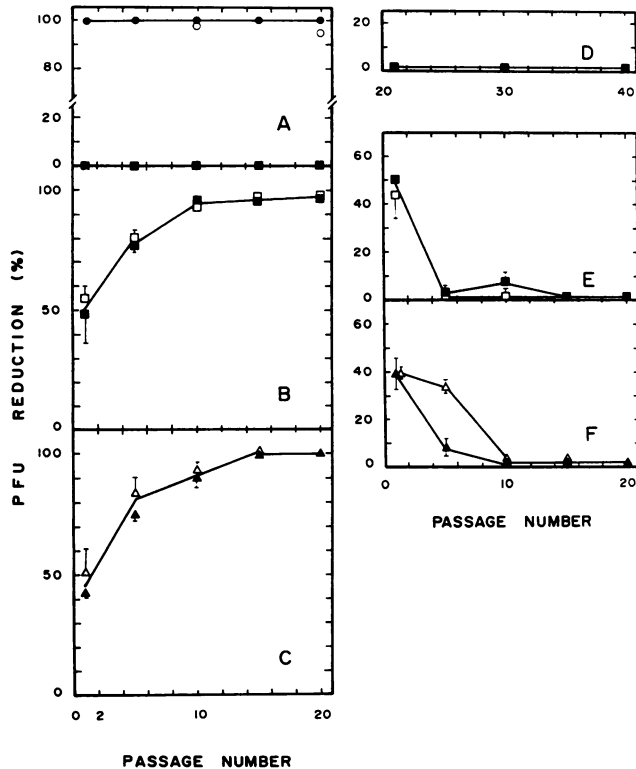

FIG. 1. Neutralization by MAb SD6 of FMDV populations serially passaged in BHK-21 cells. Passage conditions and neutralization assays are detailed in the text. Neutralization is expressed as percent PFU reduction (23) (average of three determinations, each with duplicate titrations; significant standard deviations are indicated). Each series of infections was carried out in duplicate, as shown by filled and empty symbols that correspond to a and b, respectively, in Fig. 2 and Table 1; when duplicate values were coincident, only filled symbols are shown. The viruses passaged serially were as follows. (A) FMDV C-S8c1 (●) and mutants HR and SN (■) (the two mutants yielded indistinguishable values). (B) Mixture of equal numbers of PFU of FMDV C-S8c1 and HR. (C) Mixture of equal numbers of PFU of FMDV C-S8c1 and SN. (D) Mutants HR and SN previously passaged 20 times. The four series gave indistinguishable values; passages (20 to 40 indicated in the abscissa) were the continuation of those shown in panel A for the two mutants. (E) Mixture of equal numbers of PFU of C-S8c1 and HR, the latter passaged 20 times. (F) Mixture of equal numbers of PFU of C-S8c1 and SN, the latter passaged 20 times.
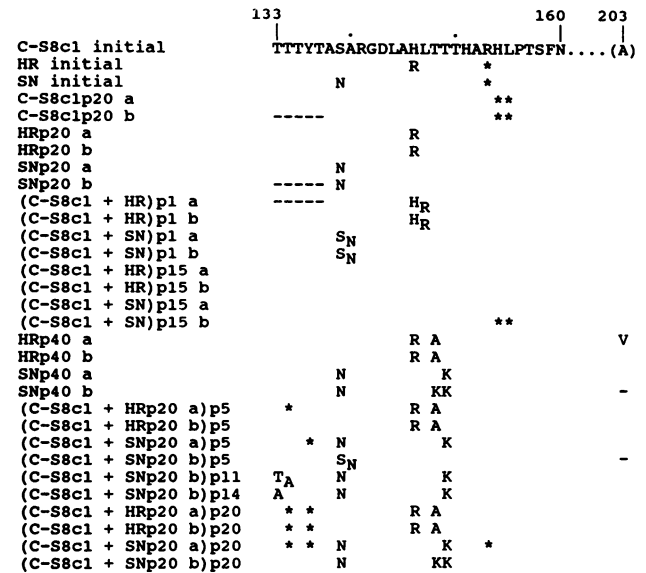

FIG. 2. Amino acid sequence (deduced from the corresponding nucleotide sequences) of a carboxy-terminal segment of VP1 of FMDV C-S8c1, HR, and SN and of populations serially propagated in cell culture. Symbols: a and b distinguish populations passaged in duplicate; p denotes the passage number (compare Fig. 1). The nucleotide sequence (28) encoding the VP1 segment shown for C-S8c1 is 5' ACTACGACCTACACCGCCAGTGCACGCGGGGATT TGGCTCACCTAACGACGACGCATGCTCGGCATTTGCCG ACATCGTTCAAC (nucleotides 397 to 480 in reference 28); alanine 203 is encoded by GCA (residues 607 to 609). The amino acid replacements were caused by the following mutations (given in parentheses): S-139→N (G-416→A); H-146→R (A-437→G); T-133→ A (A-397→G); T-148→A (A-442→G); T-148→K (C-443→A); T-149→K (C-446→A); A-203→V (C-608→T). T_A, S_N, and H_R indicate mixed populations as revealed by double bands in the sequencing gels. Asterisks denote undefined amino acids due to sequence ambiguities. Dashes indicate undetermined sequences. Sequencing was by primer extension and dideoxy-chain termination with FMDV RNA as the template (19, 28).

became resistant to neutralization by MAb SD6 (Fig. 1E and F). Nucleotide sequencing confirmed that C-S8c1 was overgrown by HR and SN, since the genomes at passage 40 included either H-146→R or S-139→N. The fitness of HRp20 and SNp20 relative to C-S8c1 was 1.21 and 1.24, respectively (Table 1). Remarkably, several additional mutations, each leading to an amino acid replacement, were observed in most populations (Fig. 2). In particular, T-148→A was fixed in HRp40 a and b (a and b indicate duplicate passages; see Fig. 2), and the former also included A-203→V, which distinguished population a from b. Likewise, SNp40 a and b both fixed replacement T-149→K, and b also included T-148→K. Most of these substitutions were present in the HR or SN that outcompeted C-S8c1. In the mixed population (C-S8c1 plus SNp20 b; see Fig. 2), a fleeting dominance of genomes with mutation A-397→G (leading to amino acid replacement T-133→A) at passage 14 was noted; such a sequence was no longer detected at passage 20, whereas a mixed population was present at passage 11 (Fig. 2). Thus, an important change in fitness occurred in fewer than 20 serial infections without loss of the MAb resistance phenotype.

In an independent study, Holland and colleagues have recently observed that greater-fitness variants of vesicular

show a selective disadvantage relative to their parental quasispecies, the mutation frequency [(2.5 ± 1.8) × 10^{-5} for mutants resistant to MAb SD6 (average of 16 determinations)] represents an underestimate of the mutation rate. Application of the equations derived by Batschelet et al. (2) indicates that the factor by which the mutant frequency is underestimated is approximately equal to the relative fitnesses of the mutant and wild type per passage (Table 1).

Rapid variation of fitness. To test whether the disadvantage of mutants HR and SN was a stable trait, 20 additional infections were carried out with the mutants passaged 20 times (mutants HRp20 and SNp20), used as the starting populations, individually and in competition with C-S8c1. When FMDV HRp20 and SNp20 were propagated alone, they maintained their MAb resistance phenotypes (Fig. 1D). In competition with C-S8c1—and contrary to the result with unpassaged HR and SN (Fig. 1B and C)—the population

TABLE 1. Analysis of competition between FMDV C-S8c1 and mutants HR and SN

Competing populations[a]	Slope[b]	SE[b]	Relative fitness[b]
(C-S8c1 + HR) a	−0.22	0.3	0.80
(C-S8c1 + HR) b	−0.28	−0.01	0.75
(C-S8c1 + SN) a	−0.38	−0.04	0.68
(C-S8c1 + SN) b	−0.39	0.05	0.67
C-S8c1 + HRp20 a	0.18	0.05	1.20
C-S8c1 + HRp20 b	0.21	0.08	1.23
C-S8c1 + SNp20 a	0.19	0.06	1.21
C-S8c1 + SNp20 b	0.24	0.05	1.27

[a] Letters (a, b, and p) used to describe populations are defined in the legend to Fig. 2.

[b] Slopes (given by the log-transformed proportion of mutants), standard errors, and relative fitnesses were calculated from the data of Fig. 1, as described previously (11). Following Batschelet et al. (2), after one passage (from n to $n = 1$, as defined in Fig. 1) the proportions of mutant (M_{n+1}) and wild type (W_{n+1}) are given by $M_{n+1} = K_M M_n - vM_n + uW_n$ and $W_{n+1} = K_W W_n - uW_n + vM_n$, respectively, in which u and v are the direct (W→M) and reverse (M→W) mutation rates, respectively, and K_M and K_W are the growth rate constants for mutant and wild type, respectively. The proportion of mutant genomes at passage $n+1$ is $p_{n+1} = M_{n+1}/(M_{n+1} + W_{n+1})$. Assuming that a large excess of wild type (W_n) containing a proportion of mutant $M_n = uW_n$ is used to infect the monolayer, then $p_{n+1} = (1 + K_M - v)u/(uK_M + K_W)$; solving for u leads to u = $K_W p_{n+1}/[1+K_M(1 - p_{n+1}) - v]$. We take $K_W = 1$ and $K_M = 0.78$ and 0.68 for mutants HR and SN, respectively. The relative error (e) in the experimental determination of mutant frequencies is e = $(p_{n+1}/u) - 1$, or e = $K_M(1 - p_{n+1}) - v$. Assuming that v and p_{n+1} are small compared with K_M (that is, $p_{n+1}, v < 10^{-2}$ [5, 17, 29]), the error is approximately equal to the relative fitness of mutant and wild type.

stomatitis virus were selected from viral clones within very few passages in new host cells or animals (15).

Because adaptive mutations or recombination events occur anywhere in the genome, we are currently measuring the relative fitness of FMDV C-S8c1, HR, SN, and other mutants at various passage levels to test whether adaptability increases gradually with passage number or at unpredictable intervals. If unpredictable variations of fitness do not occur (or if they occur only rarely), then the use of strains such as FMDV C-S8c1 with limited adaptation to a cell culture system constitutes an in vitro assay for the fitness variation of viruses. With the method employed here, fitness gain becomes an experimentally testable parameter in any host system. For example, attenuated vaccine strains should manifest little fitness gain upon multiplication in their hosts if they are to remain safe, stable vaccines. Also, variants resistant to antiviral agents (5) or able to replicate in vaccinated individuals (3) are initial components of the mutant spectra of viral quasispecies (5). However, they may become epidemiologically relevant only if they are capable of gaining fitness upon replication in their hosts.

We conclude that MAR mutants of FMDV that show a selective disadvantage relative to their parental populations are able to rapidly increase their competitive fitness. The selective disadvantage of MAR mutants underestimates the rate at which these antigenic variants actually arise in viral populations.

ADDENDUM IN PROOF

In agreement with our results, a selective disadvantage of MAR mutants of FMDV type A5 has recently been described (M. J. Gonzalez, J. C. Saiz, O. Laor, and D. M. Moore, J. Virol. 65:3949–3953, 1991).

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