Influence of the Mutant Spectrum in Viral Evolution: Focused Selection of Antigenic Variants in a Reconstructed Viral Quasispecies

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RNA viruses replicate as complex mutant distributions termed viral quasispecies. Despite this, studies on virus populations subjected to positive selection have generally been performed and analyzed as if the viral population consisted of a defined genomic nucleotide sequence: such a simplification may not reflect accurately the molecular events underlying the selection process. In the present study, we have reconstructed a foot-and-mouth disease virus quasispecies with multiple, low-frequency, genetically distinguishable mutants that can escape neutralization by a monoclonal antibody. Some of the mutants included an amino acid substitution that affected an integrin recognition motif that overlaps with the antibody-binding site, whereas other mutants included an amino acid substitution that affected antibody binding but not integrin recognition. We have monitored consensus and clonal nucleotide sequences of populations passed either in the absence or the presence of the neutralizing antibody. In both cases, the populations focused toward a specific mutant that was surrounded by a cloud of mutants with different antigenic and cell recognition specificities. In the absence of antibody selection, an antigenic variant that maintained integrin recognition became dominant, but the mutant cloud included as one of its minority components a variant with altered integrin recognition. Conversely, in the presence of antibody selection, a variant with altered integrin recognition motif became dominant, but it was surrounded by a cloud of antigenic variants that maintained integrin recognition. The results have documented that a mutant spectrum can exert an influence on a viral population subjected to a sustained positive selection pressure and have unveiled a mechanism of antigenic flexibility in viral populations, consisting in the presence in the selected quasispecies of mutants with different antigenic and cell recognition specificities.

Introduction

The understanding of evolution of pathogenic agents poses unique challenges derived from their rapid replication, as compared with the hosts that they infect. RNA viruses display, in addition to rapid replication, mutation rates in the range of $10^{-3}$ to $10^{-5}$ substitutions per nucleotide copied (Drake 1993; Drake et al. 1998; Drake and Holland 1999; Domingo 2007) that are about 10^6-fold higher than those operating usually during DNA replication of their host cells (Holland et al. 1982; Friedberg et al. 2006). As a consequence of high mutation rates, RNA viruses replicate as complex distributions of variant genomes, termed viral quasispecies (Eigen and Schuster 1979; Eigen and Biebricher 1988; Domingo 2006; Domingo et al. 2008). Individual genomes that compose a viral quasispecies may vary phenotypically, as proven by the high frequency of antibody-escape, drug-escape, and cell tropism mutants present in viral populations, even those with a very recent clonal origin (reviewed in [Domingo 2006]). Viral quasispecies exhibit features of a complex system in that their properties are not necessarily predictable from the properties of their components. Rather, quasispecies behavior is often conditioned by complementation or interference exerted among components of the mutant spectrum (González-López et al. 2004; Crowder and Kirkegaard 2005; Grande-Pérez et al. 2005; Vignuzzi et al. 2006; Perales et al. 2007; Domingo et al. 2008). These findings with real viruses are coherent with the prediction of quasispecies theory that the target of selection is not a single genome, but rather the entire mutant distribution (Eigen and Biebricher 1988; Perales et al. 2005; Biebricher and Eigen 2006; Stich et al. 2007; Schuster and Stadler 2008).

Foot-and-mouth disease virus (FMDV) is an important animal pathogen (reviews in [Rowlands 2003; Sobrino and Domingo 2004; Mahy 2005]) that we have studied as a model for quasispecies dynamics during viral RNA genome replication in cell culture and in vivo (review in [Domingo et al. 2003]). Regarding the influence of the mutant spectrum on the behavior of a quasispecies, the studies with FMDV have documented the presence in mutant spectra of minority genomes that reflect those that were dominant at an earlier evolutionary phase of the same lineage and that we termed memory genomes (Ruiz-Jarabo et al. 2000, 2002, 2003; Arias et al. 2004). Memory genomes, which cannot be detected by a standard determination of the consensus sequence, can influence the evolution of a virus in vivo, as documented with HIV-1 (Briones et al. 2006). Also, the complementing–interfering interactions among components of a quasispecies (González-López et al. 2004, 2005; Perales et al. 2007) have as a corollary that fitness variations of FMDV can occur without a modification of the consensus sequence of the population (González-López et al. 2005), an observation that was also made with lymphocytic choriomeningitis virus (Grande-Pérez et al. 2005). Because, as currently defined for viruses, fitness quantitates the relative replication capacity of a virus in a given environment (Holland et al. 1991), the results with FMDV emphasize that very relevant biological traits (such as fitness) of a virus can be modified without a reflection in any alteration of the consensus genomic nucleotide sequence, the one traditionally measured in genetic studies (Domingo et al. 2008).

One of the major biological features of FMDV is its great antigenic variation and diversity in nature, mirrored in the identification of 7 serotypes, and multiple subtypes and variants (Rowlands 2003; Sobrino and Domingo 2004; Mahy 2005). Such antigenic diversity is a consequence of...
the general genetic variability of this virus and represents a difficulty for the design of effective anti-FMD vaccines (Domingo et al. 1980; Mateu, Andreu, and Domingo 1995; Rowlands 2003; Sobrino and Domingo 2004; Mahy 2005). A major, highly variable antigenic site of FMDV, present in all serotypes of the virus, is located at the exposed, mobile, G–H loop of capsid protein VP1 (Acharya et al. 1989; Logan et al. 1993; Lea et al. 1994). This loop has a dual function in the replication cycle of FMDV. It includes the amino acid sequence RGD, which is essential for the recognition of integrin receptors (Berinstein et al. 1995; Duque and Baxt 2003; Jackson et al. 2003; Berryman et al. 2005; Monaghan et al. 2005; Stewart and Nemerow 2007), and it is involved in the interactions with neutralizing antibodies (Mateu, Andreu, and Domingo 1995; Mateu, Camarero, et al. 1995; Verdaguer et al. 1995, 1996, 1998, 1999; Ochoa et al. 2000) (reviewed in [Mateu 1995]). In fact, the RGD establishes the key contacts for recognition of several monoclonal antibodies (mAbs), including mAb-SD6, as evidenced by both biochemical and structural studies (Mateu et al. 1990; Verdaguer et al. 1995; Ochoa et al. 2000).

Integrins do not provide the only pathway for FMDV entry into cells. FMDV passaged in cell culture can acquire the capacity to use heparan sulfate (HS) to bind to cells (Jackson et al. 1996). Multiple serial passages of FMDV clone C-S8c1 (our reference biological clone for evolutionary studies [Sobrino et al. 1983]) in BHK-21 cells resulted in selection of a virus population with the capacity to use alternative receptors (HS, and at least 1 additional, unidentified receptor) for cell entry, and the RGD triplet in VP1 became dispensable (Baranowski et al. 1998, 2000, 2001; Ruiz-Jarabo et al. 2003). The possibility of using several receptors resulted in an expansion of host cell tropism of FMDV (review in [Baranowski et al. 2003]). A consequence of the requirement or not of the RGD was a striking difference in the repertoire of mAb-escape mutants when selected using either the parental FMDV C-S8c1 clone or the multiply passaged population. In the latter case, many escape mutations affected the RGD triplet, which was strictly conserved among the mAb-escape mutants selected using the parental FMDV C-S8c1 (Martínez et al. 1997; Perales et al. 2005) (fig. 1). In a previous study, the ample collection of mutants resistant to mAb-SD6—whose continuous epitope is comprised between VP1 residues 138 and 147 (Mateu et al. 1990; Verdaguer et al. 1995)—was used to reconstruct a quasispecies with 19 mAb-SD6-escape mutants as minority components in a mutant spectrum immersed in the spectrum produced by C-S8c1. The reconstructed quasispecies was then subjected to replication in the presence or absence of mAb-SD6, and a clonal analysis showed that an ensemble of mutants was selected (Perales et al. 2005) and that the selected repertoire was partly influenced by the initial fitness of the individual mutants (Martin et al. 2006).

According to the description of viral populations as quasispecies, a mutant spectrum occupies a certain portion of sequence space (Eigen and Biebricher 1988). Sequence
space means the total number of possible sequences theoretically available to a viral genome (leading to viable or nonviable viruses), which is an astronomical number ($4^{5300}$ in the case of FMDV!). Despite real FMDV occupying a minute fraction of its theoretical sequence space, selective processes have been regarded as a “condensation” phenomenon or “localization” of sequences into a restricted area of sequence space (Eigen and Biebricher 1988; Nowak 2006). However, how this condensation process occurs in a real viral quasispecies subjected to a continuing and specific selective force has not been investigated.

In the present study, we have addressed the question of the evolution of the repertoire of antigenic variants of FMDV, present in a first step as minority components (each at a frequency of $2 \times 10^{-6}$ to $3 \times 10^{-6}$) in the mutant spectrum, when the selective pressure exerted by mAb-SD6 is maintained over several passages of the virus in BHK-21 cells. The objective has been to follow the evolution in mAb-SD6–escape mutant composition in serial infections, each with a cutoff frequency of variant genomes of about $10^{-6}$, following the founder infections (scheme in fig. 2). We have compared sequential samples of the population passed either in the presence or in the absence of mAb-SD6 and have analyzed statistically the types of dominant antigenic variants, their fluctuations, and the outcome of prolonged antibody pressure. In the presence of mAb-SD6, but not in its absence, the population focused toward one, single mutant that eventually became dominant (albeit accompanied by a cloud of other antigenic variants) and that affected the critical, tropism-determining RGD triplet. Interestingly, this same mutant was well represented in the population passaged in the absence of mAb-SD6, but it remained as a minority component of the mutant spectrum. Instead, another mutant that affected an antigenically relevant residue located outside the RGD became the dominant one in the absence of mAb-SD6 (albeit accompanied also by a cloud of other antigenic variants). We discuss the possible influence of relative fitness of different variants in the mutant compositions observed and implications for the influence of founder events in the outcome of evolution, for the sustained complexity of viral populations during selective processes, and for the coevolution of antigenicity and host cell tropism. We suggest some possible consequences for the modulation of infection processes in vivo.

Materials and Methods
Cells and Viruses
FMDV biological clone C-S8c1, derived from natural isolate C1 Sta Pau–Spain 70 (Sobrino et al. 1983), was serially passaged 100 times in BHK-21 cells at a multiplicity of infection (moi) of 2–4 plaque-forming units (pfus) per cell; this yielded population C-S8c1 p100. FMDV mutants resistant to mAb-SD6 were selected as previously described (Mateu et al. 1990). To ensure the absence of cross-contamination among cultures, mock-infected cultures were treated in parallel and maintained throughout the experiments. No evidence of infection of control cultures was obtained. Likewise, none of the total of 516 nt sequences determined throughout this study suggested any cross-contamination.

RNA Extraction, cDNA Synthesis, Polymerase Chain Reaction Amplification, and DNA Sequencing
Viral RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) as previously described (Escarmís et al. 1999). Procedures and primers used for reverse transcriptase–polymerase chain reaction (RT-PCR) amplification and nucleotide sequencing have been described (Ruiz-Jarabo et al. 2000, 2002; Perales et al. 2005). Reverse transcription was carried out using avian myeloblastosis virus RT (Promega, Madison, WI), and polymerase chain reaction (PCR) amplification was performed using Expand High Fidelity polymerase (Roche, Madrid, Spain) as specified by the manufacturers.

Quasispecies Origin
A FMDV quasispecies (termed C-S8c1+19m) was reconstructed by mixing about 10 pfu from each of 19 mAb-SD6–escape mutant viruses with $4 \times 10^6$ pfu from C-S8c1 as majority genomes. The 19 mutants used to reconstruct quasispecies C-S8c1+19m originated from the FMDV RED lineage (an antigenic variant with the sequence RED instead of RGD at VP1 positions 141–143, obtained as described in Martínez et al. (1997); Ruiz-Jarabo et al. 2000, 2003) at passages 15, 25, 45, 50, and 60 (Ruiz-Jarabo et al. 2002). The passage number at which each mutant was isolated was the following: G142 → E plus D143 → G (double mutant) and L147 → P; passage 15; H146 → P; passage 25; A138 → D, S139 → I, S139 → G, G142 → E, D143 → V, D143 → E, H146 → Q; passage 45; S139 → N, G142 → R, D143 → G, D143 → N, L144 → S, L144 → V, H146 → R; passage 50; and S139 → R, S139 → T; passage 60. The location of these substitutions (which confer the mAb-SD6–resistance phenotype and serve also to identify the origin of individual mutants in the clonal analyses) in the epitope recognized by mAb-SD6 is indicated in figure 1. Genomic residue 3797 is A in FMDV C-S8c1 and G in FMDV C-S8c1 p100. This position served as an additional indication among cultures, mock-infected cultures were treated in parallel and maintained throughout the experiments. No evidence of infection of control cultures was obtained. Likewise, none of the total of 516 nt sequences determined throughout this study suggested any cross-contamination.

Fig. 2.—Scheme of the passage history of FMDV quasispecies C-S8c1+19m and of clonal analyses at passages 2, 4, and 10. C-S8c1+19m consists of C-S8c1 (4 × 10^6 pfu) and a mixture 19 mAb-SD6–escape mutants (10 pfu each). The origin of C-S8c1 and of the 19 mutants is detailed in Materials and Methods and figure 1. Uncloned populations are represented as empty circles and biological clones as filled squares. Gray arrows indicate population passages in the absence of mAb-SD6; black arrows describe population passages in the presence of mAb-SD6; thin arrows describe the isolation of biological clones (virus from individual plaques), after dilution of virus and plating on BHK-21 cell monolayers. P followed by a number indicates passage number. Procedures for infection of BHK-21 cell monolayers, isolation of virus from biological clones, extraction of viral RNA, and nucleotide sequencing are described in Materials and Methods.
In parallel, C-S8c1 (4\textsuperscript{th}determines its relative fitness value (Martin et al. 2006). The increase in relative fitness of clonal populations (derived from the FMDV RED lineage) is in the range of 0.06–0.09 fitness units per passage (Arias et al. 2004). Therefore, the 19 mutants used to reconstruct the quasispecies may vary by 2.7–4.0 fitness units, and the passage number from which each of the 19 mutants was isolated determines its relative fitness value (Martin et al. 2006). In parallel, C-S8c1 (4 \times 10^6 pfu) alone and a mixture of the 19 mutants (about 200 pfu from each of the 19 mAb-SD6–resistant mutants) alone were used as control populations subjected to serial passages either in the absence or presence of mAb-SD6.

The p0 C-S8c1 + 19m populations, obtained by infecting BHK-21 cells at a moi of 1 pfu per cell in presence or absence of mAb-SD6, were passaged once in BHK-21 cells at a moi of 0.001 pfu per cell to yield population p1 (fig. 2). Populations p2–p10 were obtained after 9 serial passages at a moi of 1 pfu per cell, starting with population p1 in the absence or presence of mAb-SD6.

Viral Infections in the Presence or Absence of mAb-SD6

Procedures for cell culture, infections with FMDV of BHK-21 cell monolayers in liquid medium and plaque assays in semisolid agar medium, were carried out as described (Domingo et al. 1980; Sobrino et al. 1983; Perales et al. 2005). After virus adsorption for 1 h at 37°C, monolayers were washed once with 0.1 M phosphate buffer (pH 6.0) to inactivate unadsorbed virus due to its acid lability, and then, cells were washed extensively with Dulbecco modified Eagle’s medium (DMEM), and further incubated in DMEM containing 5% (v/v) fetal calf serum. MAb-SD6 (supernatant of hybridoma culture) was used at 1:5 dilution (a concentration calibrated previously that caused neutralization of about 99% of wild-type virus and allowed survival of a broad repertoire of escape mutants [Perales et al. 2005]). Samples were taken for titration of infectivity and clonal analysis, 24 h after infection. In the agar overlay, a 1:20 dilution of mAb-SD6 was added for clonal analysis of progeny produced in the presence of mAb-SD6 to maintain uninterrupted the selective pressure of the antibody. Mock-infected cultures were handled in parallel as control for the absence of contamination in each plaque assay.

Results

Variation of the Consensus Sequence of an Antigenic Site in a Complex Quasispecies Subjected to Sustained Antibody Selection

A viral quasispecies was reconstructed with FMDV clone C-S8c1 (Sobrino et al. 1983) (4 \times 10^6 pfu) and a mixture of 10 pfu of each of 19 mAb-SD6–escape mutants, each with an amino acid substitution within antigenic site A, as described in Materials and Methods and depicted in figure 1. The frequency of the individual escape mutants in the reconstructed quasispecies (C-S8c1 + 19m) was 2–3 \times 10^{-6}. This value is 10 times lower than the frequency of mAb-SD6–escape mutants found in the parental clonal population C-S8c1 [(2.5 \pm 1.8) \times 10^{-5}; see [Martínez et al. 1991]). As control populations, C-S8c1 alone (abbreviated C-S8c1) and the mixture of the 19 mutants alone (abbreviated 19m) were also subjected to serial passages under the same conditions. To investigate the evolution of the consensus sequences of the genomic region encoding the G–H loop of capsid protein VP1, the 3 populations (C-S8c1 + 19m, C-S8c1, and 19m) were subjected to 10 serial passages in BHK-21 cells, either in the absence or presence of mAb-SD6, under the conditions and neutralization intensity described in Materials and Methods and shown schematically in figure 2. Consensus nucleotide sequences of genomic residues 3573–3896—which encode the G–H loop of capsid protein VP1—were determined for each population at passages 1–10. Genomic residue 3797 differed between FMDV C-S8c1 and FMDV C-S8c1 p100 or the 19 mutants used to construct populations C-S8c1 + 19m and 19m. This mutation is stable (see Materials and Methods), and, therefore, G or A at position 3797 was an additional marker to identify the origin of the selected FMDV populations. The results (table 1) show a different pattern of evolution in the 3 lineages. In the control C-S8c1 quasispecies, passaged in the absence of mAb-SD6, no specific mutation was detected until passage 8. At passage 8, a single mutation (leading to amino acid substitution T148A, outside the SD6 epitope) appeared in mixture with the corresponding C-S8c1 wild-type nucleotide (A3649R, in which R means a mixture of A and G). Under the selective pressure of mAb-SD6, substitution S139R was mixed with the wild-type amino acid at passage 1 and remained present through passage 10, but never became dominant. (Dominant in this case refers to a residue that reached at least 75% in the population, as judged by the band pattern in the nucleotide sequence determination; the presence by a cloud of other mutants at the same position cannot be excluded because minority mutants are undetectable in the consensus sequence.)

In quasispecies C-S8c1 + 19m, passaged in the absence of mAb-SD6, substitution S139N appeared mixed with the C-S8c1 amino acid at passage 5 and N139 was dominant at passages 9 and 10. In the presence of mAb-SD6, S139R, and G142R were detected in a mixture with the wild-type amino acid at passage 1 through passage 5. Then, the populations at passages 6 and 7 showed only the G142R substitution mixed with the G142 wild-type amino acid. Finally, R142 became dominant at passage 8 and remained dominant at passage 10.

In the population composed only of 19 mAb-SD6–escape mutants passaged in the absence of mAb-SD6, substitution S139G was present, mixed with the C-S8c1 amino acid at passage 1, and remained as a mixture with similar composition through passage 10. In the presence of mAb-SD6, the outcome was different and overtly dynamic in that in the first 3 passages A138D was the only substitution...
Table 1
Mutations and Corresponding Amino Acid Substitutions at the G–H Loop of Capsid Protein VP1 in the Consensus Genomic Sequence of FMDV Populations, Passaged Either in the Absence or Presence of mAb-SD6

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<th>FMDV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>mAb-SD6&lt;sup&gt;b&lt;/sup&gt;</th>
<th>C-S8c1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>C-S8c1 + 19m&lt;sup&gt;d&lt;/sup&gt;</th>
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<sup>a</sup> The origin of the FMDV populations C-S8c1, C-S8c1 + 19m, and 19m is detailed in Materials and Methods.

<sup>b</sup> Absence (−) or presence (+) of mAb-SD6 during the serial infections.

<sup>c</sup> The genomic region analyzed corresponds to residues 3573–3896 of the FMDV genome, which encodes the G–H loop of capsid protein VP1 (residue numbering is that reported by Escarmis et al. [1996]). Nucleotide mixtures: M = A + C; R = A + G.

<sup>d</sup> These populations contain Guanine (G) at residue 3797 as in FMDV C-S8c1 p100 (as a marker of virus origin and the minority genomes used to reconstruct quasispecies C-S8c1 + 19m and 19m).

<sup>e</sup> These populations contain Adenine (A) at residue 3797, as in FMDV C-S8c1 (as a marker of virus origin and absence of contamination).

<sup>f</sup> The populations (and mutations found in them) that were subjected to clonal analysis (table 2) are highlighted with boldface letters.

detected, mixed with the wild-type amino acid, and 2 additional mutations, S139G and G142R, were present at passage 4. From passage 5 through 7, a mixture of A138D and G142R was present. Finally, G142R was the only substitution detected, mixed with the C-S8c1 amino acid, from passages 8 to 10. This is the same outcome observed with quasispecies C-S8c1 + 19m. The only difference was that in C-S8c1 + 19m quasispecies the G142R was the only variant detected in the consensus sequence, and in the 19m quasispecies the consensus sequence displayed a mixture of G142R and the wild-type amino acid. In the presence of selection by mAb-SD6, both the reconstructed quasispecies C-S8c1 + 19m and the quasispecies made exclusively of the 19m mAb-SD6–resistant mutants converged toward the same dominant genomes encoding the antigenically critical substitution G142R at the G–H loop of VP1.

These results show that both, in the absence or presence of antibody selection, minority components of the mutant spectrum, which were introduced in the reconstructed quasispecies affected the evolution of the viral population with consequences for its composition in antigenic variants, a salient phenotypic trait for a virus.

Continuous Antibody Selection Leads to Dominance of 1 Variant in a Cloud of a Different Class of Antigenic Variants

One of the objectives of the present study was to investigate whether a sustained antibody pressure would focus the selection on some specific variant or would promote a wide set of antigenic variants. To probe into the evolution of the repertoire of mAb-SD6–escape variants when selection by antibody was maintained, a clonal analysis of the mAb-SD6–escape mutants of the C-S8c1 + 19m quasispecies, at passages 0, 2, 4, and 10 in the absence or presence of mAb-SD6, was performed (fig. 2). MAb-SD6 was included in the agar overlay for the analysis of the population passages in the presence of mAb-SD6 (see Materials and Methods). The number of biological clones analyzed per sample ranged from 46 to 72. The results (table 2) show that, under the selective pressure of mAb-SD6, multiple mAb-SD6–escape mutants coexisted at each passage examined and that at passage 10 the repertoire became focused toward G142R (49 out of 58 clones analyzed; \( P < 0.001; \chi^2 \) test), in agreement with the analysis of the consensus...
The accumulation of S139N was already statistically significant at passage 2, and at passages 4 and 10, the repertoire of sampled variants became focused toward S139N (45 out of 57 clones analyzed; P < 0.01, \( \chi^2 \) test), dominant substitutions at passage 10 are highlighted with boldface letters. In contrast, in the passages carried out in the presence of mAb-SD6, the only position at which the wild-type amino acid was not prevalent was position 139, in the absence of mAb-SD6, the only position at which the wild-type amino acid was not prevalent was position 139, which was dominated by N instead of S (table 2 and fig. 3).

The variability at positions 142, 143, and 144 reflects the influence of the minority components introduced in quasispecies S8c1 + 19m because residues G142 and D143 rank as high conserved when FMDV isolates of different serotypes are compared (Domingo et al. 2003). The focusing toward a specific variant was reflected in a concomitant decrease of the variability index. In the passages carried out in the absence of mAb-SD6, the only position at which the wild-type amino acid was not prevalent was position 139, which was dominated by N instead of S (table 2 and fig. 3). In contrast, in the passages carried out in the presence of mAb-SD6, the positions at which the wild-type amino acid was not prevalent were positions 139, 142, and 143; at these 3 positions, R dominated instead of G, and V instead of D, respectively (table 2 and fig. 3).

Examination of the repertoire of antigenic variants, sampled as biological clones in the sequential viral populations, has led to the following observations: 1) Both, in the absence and the presence of mAb-SD6, in all the clones analyzed that included a replacement in the G–H loop of sequence of this population (compare with table 1). A statistically significant accumulation of mutants with substitution A138D was also observed at passages 2 and 4 (P < 0.001, \( \chi^2 \) test, in both passages), as well as G142R at passages 0 and 4 (P < 0.001, \( \chi^2 \) test, in both passages) and at passage 2 (0.005 < P < 0.01, \( \chi^2 \) test), S139R at passages 0 and 2 (P < 0.001, \( \chi^2 \) test, in both passages), and D143V at passage 0 (0.01 < P < 0.025, \( \chi^2 \) test). Thus, the continuous selection by mAb-SD6 led to a population dominated by a virus lacking the critical RGD triplet at position 141–143 of VP1.

In the absence of mAb-SD6, a very limited number of antigenic variants was found among the clones sampled at passage 0, and multiple mAb-SD6–escape mutants coexisted during subsequent passages, presumably favored by the higher fitness of the 19 mutants used to reconstruct the quasispecies, relative to C-S8c1 (see Discussion). At passage 10, the repertoire of sampled variants became focused toward S139N (45 out of 57 clones analyzed; P < 0.001, \( \chi^2 \) test), in agreement with the analysis of the consensus sequence of this population (compare with table 1). The accumulation of S139N was already statistically significant at passage 2 (0.001 < P < 0.005; \( \chi^2 \) test) and at passage 4 (P < 0.001; \( \chi^2 \) test). No other substitution acquired a significantly higher level relative to the repertoire of substitutions at each passage, except virus with substitution H146R at passage 2 (0.01 < P < 0.005, \( \chi^2 \) test). Thus, in the absence of selection by mAb-SD6, the population evolved to become dominated by a virus harboring replacement S139N, whereas replacements at the critical RGD triplet were observed only in the minority components of the mutant spectra (2 clones out of 57) (table 2).

The amino acid variability index (Wu and Kabat 1970), applied to the different mAb-SD6–escape mutants obtained at each position of the SD6 epitope of the C-S8c1 + 19m quasispecies, shows the minimal variability index at positions 140, 141, 145, and 147 and values above 4 in some passages at positions 139, 142, and 143 (fig. 3). The variability at positions 142, 143, and 144 reflects the influence of the minority components introduced in quasispecies S8c1 + 19m because residues G142 and D143 rank as high conserved when FMDV isolates of different serotypes are compared (Domingo et al. 2003). The focusing toward a specific variant was reflected in a concomitant decrease of the variability index. In the passages carried out in the absence of mAb-SD6, the only position at which the wild-type amino acid was not prevalent was position 139, which was dominated by N instead of S (table 2 and fig. 3). In contrast, in the passages carried out in the presence of mAb-SD6, the positions at which the wild-type amino acid was not prevalent were positions 139, 142, and 143; at these 3 positions, R dominated instead of G, R instead of G, and V instead of D, respectively (table 2 and fig. 3).
VP1, the replacement corresponded to one of the variants used to reconstruct the initial quasispecies C-S8c1 + 19m (compare figs. 1 and 2 and table 2). 2) In the absence of mAb-SD6, 3 out of the 6 variant amino acids scored at passage 10 were characteristic of antigenic variation in the absence of immune selection (Borrego et al. 1993; Domingo et al. 2003), and the population focused toward one of such variants (S139N) (fig. 1 and table 2). 3) Under selection by mAb-SD6, all 5 variant amino acids scored at passage 10 were characteristic of antigenic variation in the presence of immune selection (Borrego et al. 1993; Domingo et al. 1993, 2003), and the population focused toward a variant observed under antibody selection (G142R) (fig. 1 and table 2). 4) The FMDV populations evolved through complex mixtures of antigenic variants both in the absence and presence of mAb-SD6 leaning toward dominance of 1 specific mutant. However, only in FMDV populations subjected to selection by mAb-SD6, the evolution was focused toward a drastic antigenic variation associated with G142R, which abolished the key RGD triplet in VP1. The dominance of this mutant was only possible as a consequence of its being seeded, albeit at a very low frequency, in the parental quasispecies. The results emphasize the importance of low-frequency variant genomes in guiding viral evolution under continuing positive selection.

**Discussion**

Because of the high mutation rates operating in the course of viral RNA–replication and retrotranscription (Drake 1993; Drake et al. 1998; Drake and Holland 1999), RNA viruses evolve as complex distributions of related genomes termed viral quasispecies (Eigen and Schuster 1979; Eigen and Biebricher 1988; Biebricher and Eigen 2006; Domingo et al. 2006, 2008). Replicating viral RNA genomes are not molecularly defined entities because at each time point they are composed of a weighted average of multitudes of related sequences, referred to as mutant spectra or mutant clouds. Because these multiple genomes, either in isolation or in interaction with other related genomes of the same cloud, can express different phenotypic traits, high mutation rates, and quasispecies dynamics are determinants of adaptability of viral populations (Figlerowicz et al. 2003; Pfeiffer and Kirkegaard 2005; Vignuzzi et al. 2006; Domingo et al. 2008). As argued elsewhere (Domingo et al. 2008) both enzymological and virological considerations render unlikely that high mutation rates (the main reason to generate mutant clouds) are just an unavoidable consequence of rapid replication rates of RNA viruses. Rather, high mutability can serve as an adaptive factor of RNA viruses, as also documented for mutator bacteria (Giraud et al. 2001; de Visser 2002; Henrichreise et al. 2007; Sundin and Weigand 2007). Despite the obvious interest of defining not only consensus sequences but also the mutant spectrum composition in replicating viral quasispecies, most studies on viral evolution are still based on comparisons of consensus sequences and ignore the intricacies of the composition of mutant spectra. The composition of the mutant spectrum is particularly relevant in the course of antibody selection because a possible cloud of escape mutants will offer multiple but not identical stimuli to the immune system, itself composed of a distribution of nonidentical, related cells (Braciale et al. 2007; Nathanson and Ahmed 2007).

The question we have addressed in the present study is how the repertoire of the selected mAb-escape variants in a complex quasispecies would evolve when and highly specific antibody selective pressure was maintained with the same intensity for several rounds of viral replication. It must be clarified that in the experiments described here, there is a statistical indetermination in the composition of the initial viral populations regarding the presence of mAb-SD6–escape mutants, as previously documented (Perales et al. 2005). This is because each individual mAb-SD6–escape mutant introduced to reconstruct quasispecies C-S8c1 + 19m was present at a frequency lower than required to ensure its inclusion in the initial infection. Beginning at passage 1 (fig. 2), this indetermination was excluded due to the moi used for the successive infections. Because during passages 1–10, the moi at each passage was 1 pfu per cell and about $2 \times 10^6$ cells were infected per passage (see Materials and Methods), any mutant at frequency of $10^{-4}$ or

**Fig. 3.—**Amino acid variability index at each residue of the SD6 epitope, upon passage of C-S8c1 + 19m quasispecies. Amino acids residues correspond to the epitope defined by mAb-SD6 in FMDV C-S8c1 (underlined in fig. 1). The variability index is the ratio between the number of different amino acids found at each position in the analysis of biological clones (given in table 2) and the frequency of the most common amino acid at the same position. Bars correspond to the populations analyzed, as indicated in the box. Asterisks indicate positions for which the dominant amino acid was not the wild-type amino acid. Note the high variability index at residues 142–144, as compared with natural FMDV isolates (see text).
higher had to be represented in the inocula. Therefore, several alternative outcomes of the evolutionary process were possible. One possible outcome was a steady-state representation of variants, either in the absence or presence of mAb-SD6. Alternatively, because the initial relative fitness of the different variants used to reconstruct the C-S8c1 + 19m quasispecies was not identical (Martin et al. 2006), relative fitness could dictate the dominance of some escape mutants over others, upon replication in the absence or presence of the antibody. The results show that neither of these 2 possible outcomes was observed in the evolved populations. In the passages of C-S8c1 + 19m quasispecies in the absence of mAb-SD6, mutant S139R displayed the highest fitness value of the 19 mutants entered in the quasispecies (Materials and Methods and Martin et al. [2006]), and yet it was scored as a single clone in passage 2 and was not represented at passages 4 and 10 (table 2). Likewise, mutants G142R, D143G, L144S, and H146R had an initial fitness comparable with that of S139N, and yet they remained as a minority in the course of the serial passages in the absence of mAb-SD6. The majority component in the reconstructed quasispecies, which is the parental clone C-S8c1, was not selected as dominant variant. Therefore, probably due to continuing genetic variation in the course of the serial passages, initial fitness values were not maintained in the evolving quasispecies, but they had sufficient weight to prevent dominance of the genomes with the lowest fitness that constituted a majority in the initial population. Although affinities of the different mAb-SD6–escape mutants for mAb-SD6 have not been measured, they could influence the dominance of some mutants over others. It is unlikely, however, that such differences in affinity were sufficient to play a prominent role because all mAb-SD6–escape mutants were initially isolated under identical environmental conditions (including concentration of mAb-SD6 and culture medium), leading to mutants that produced similar plaque size in the presence of mAb-SD6 (Perales et al. 2005; Martin et al. 2006). As with fitness, affinities did not dictate a consistent progression of dominances with passage number and multiple factors must have affected the evolutionary outcome.

The mutant compositions derived from the clonal analyses agree with the consensus sequences determined for the uncloned populations passed in the absence or presence of mAb-SD6 (compare tables 1 and 2). For example, genomes encoding VP1 replacement S139N were introduced of mAb-SD6 (compare tables 1 and 2). For example, genomes encoding VP1 replacement S139N were introduced in the initial C-S8c1 + 19m quasispecies at a frequency of $2 \times 10^{-6}$ to $3 \times 10^{-6}$. Yet, genomes with this substitution became detectable at passage 5 in the absence of mAb-SD6 and remained present throughout passage 10. Thus, the comparison of the evolution of consensus genomic sequences of quasispecies C-S8c1 and C-S8c1 + 19m in the absence of mAb-SD6 illustrates the influence of a minority component in the mutant spectrum, in determining the evolution of an entire viral population (table 1).

The continuous selection by mAb-SD6 in C-S8c1 + 19m quasispecies had as a consequence the focusing of the selected population toward genomes encoding replacement G142R (tables 1 and 2). This is a substitution that was associated only with antibody selection in previous comparisons of antigenic variants of FMDV obtained either in the presence or absence of antibody selection (Borrego et al. 1993). This is in contrast with the same population (C-S8c1 + 19m) passaged in the absence of mAb-SD6, which evolved toward genomes encoding replacement S139N, a substitution previously observed in antigenic variation of FMDV in the absence of antibody selection (Borrego et al. 1993). This differential enrichment of genomes with G142R versus S139N in the presence and absence, respectively, of mAb-SD6 is a highly relevant biologically because the former substitution abolishes a critical RGD motif, whereas the second does not. It could be argued that this decisive difference in dominance of antigenic variants was unrelated to the antibody pressure and that it was determined by a founder effect due to the initial low moi inoculation to produce population p1 (fig. 2 and Materials and Methods).

This is very unlikely because in the presence of mAb-SD6 substitution G142R was represented among the genomes that evolved in the absence of the antibody (4, 1, and 2 clones at passages 2, 4, and 10, respectively; see table 2). Thus, if this substitution conferred some advantage independently of selection by mAb-SD6, its frequency should have increased in the course of passages carried out at a moi of about 1 pfu/cell, and this was not observed. Conversely, S139N, which became dominant in the absence of mAb-SD6, was also represented by one clone at passages 2 and 4 of the population that replicated in the presence of mAb-SD6 (table 2). Therefore, founder events did not bias the representation of the substitutions that became subsequently dominant either in the presence or absence of mAb-SD6.

In conclusion, with continuous antibody selection, a viral population can evolve toward dominance of some mutation types, yet the population is composed of a mutant cloud that includes representatives of alternative phenotypes: when a substitution that abolished the critical integrin recognition RGD motif became dominant, mutants conserving the RGD were still present in the mutant cloud. Likewise, when mutants with the conserved RGD triplet dominated, mutants that lack the RGD were still present in the mutant cloud. This documents a molecular mechanism for antigenic and receptor recognition flexibility in an evolving viral quasispecies. Selection of antigenic variants from complex viral quasispecies has been documented in vivo (Ciurea et al. 2000; López-Bueno et al. 2003). Furthermore, coevolution of antigenicity and cell tropism has been described for several virus-host systems (Baranowski et al. 2003; Stewart and Nemerow 2007). Therefore, the observation reported here that the viral response to a specific antibody selection proceeds with the presence of clouds of antigenic variants with different host cell tropism is likely to be relevant to the antibody response in vivo and virus persistence in the infected host. Indeed, clouds of antigenic variants may underlie the continuous escape to neutralization of some persistent, highly variable RNA viruses such as Hepatitis C virus or HIV-1 (Ciurea et al. 2001; Richman et al. 2004; Bowen and Walker 2005; Frost et al. 2005).

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