Antigenic properties and population stability of a foot-and-mouth disease virus with an altered Arg-Gly-Asp receptor-recognition motif

Carmen M. Ruiz-Jarabo, Noemí Sevilla,† Mercedes Dávila, Gema Gómez-Mariano, Eric Baranowski and Esteban Domingo

Centro de Biología Molecular ‘Severo Ochoa’, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, 28049 Madrid, Spain

The antigenic properties and genetic stability of a multiply passaged foot-and-mouth disease virus (FMDV) clone C-S8c1 with an Arg-Gly-Gly triplet (RGG) instead of the Arg-Gly-Asp (RGD) integrin-recognition motif at positions 141 to 143 of capsid protein VP1 are described. Clear antigenic differences between FMDV RGG and clone C-S8c1 have been documented in ELISA, enzyme-linked immunoelectrotransfer (Western) blot and neutralization assays using site A-specific monoclonal antibodies and anti-FMDV polyclonal antibodies from swine and guinea pigs. The results validate with a live virus the role of the RGD (in particular Asp-143) in recognition of (and neutralization by) antibodies, a role previously suggested by immunochemical and structural studies with synthetic peptides. The FMDV RGG was genetically stable in a large proportion of serial infections of BHK-21 cells. However, a revertant virus with RGD was generated in one out of six passage series. Interestingly, this revertant FMDV did not reach dominance but established an equilibrium with its parental FMDV RGG, accompanied by an increase of quasispecies complexity at the sequences around the RGG triplet. FMDV RGG exhibited a selective disadvantage relative to other RGD-containing clones isolated from the same parental FMDV population. The results suggest that large antigenic variations can be prompted by replacements at critical capsid sites, including those involved in receptor recognition. These critical replacements may yield viruses whose stability allows them to replicate efficiently and to expand the sequence repertoire of an antigenic site.

Introduction

Foot-and-mouth disease virus (FMDV) is an aphthovirus of the family Picornaviridae, and is the aetiological agent of an important disease of cloven-hoofed animals (Bachrach, 1968; Pereira, 1981; Brown, 1994; Domingo et al., 1990). FMDV is genetically and antigenically variable as documented by the identification of seven distinct serotypes (A, O, C, Asia1, SAT1, SAT2, SAT3), 65 subtypes (Pereira, 1977) and multitudes of antigenic variants (Mateu et al., 1988). This antigenic variation is a reflection at the antigenic level of the quasispecies dynamics of FMDV in vivo (Domingo et al., 1980; Gebauer et al., 1988; Carrillo et al., 1990, 1998; Marilat et al., 1994; Vosloo et al., 1996; Taboga et al., 1997). The emergence of genetic and antigenic variants of FMDV during cytopathic and persistent infections, and their dominance through positive selection and random drift, has been extensively documented in cell culture (Sobrino et al., 1983; de la Torre et al., 1985, 1988; Mateu et al., 1989; Diez et al., 1989, 1990; Martínez et al., 1991; Hernández et al., 1992; Rieder Rojas et al., 1992; Borrego et al., 1993; Baranowski et al., 1998; Escarmís et al., 1998, 1999).

The G–H loop of capsid protein VP1 of FMDV is exposed and mobile on the surface of the virus particle (Acharya et al., 1989; Logan et al., 1993; Lea et al., 1994; Verdaguer et al., 1999). This loop participates in at least two processes in the natural life-cycle of the virus. It binds an integrin receptor (αvβ3) via a highly conserved Arg-Gly-Asp (RGD) triplet (Fox et al., 1989; Mason et al., 1994; Berinstein et al., 1995; Hernández et al., 1996; Jackson et al., 1997; Neff et al., 1998),
and it constitutes one of the major antigenic sites of FMDV involved in neutralization of viral infectivity (Strohmaier et al., 1982; Bittle et al., 1982; Pfaff et al., 1982). For FMDV of serotype C this antigenic site has been termed site A, and it is composed of a number of overlapping, continuous epitopes, as defined by reactivity of synthetic peptides with neutralizing monoclonal antibodies (MAbs), and by nucleotide sequencing of antibody-escape mutants (Mateu et al., 1989, 1990; reviewed in Mateu, 1995).

We have been interested in genetic and antigenic variation of FMDV using as reference virus a biological clone termed FMDV C-S8c1, derived from natural isolate FMDV C-Sta Pau Sp/70, a representative of the European subtype C1 viruses (Sobrino et al., 1983). In clone C-S8c1, the RGD triplet (positions 141 to 143 of VP1; Fig. 1) is an essential part of several site A epitopes, as evidenced by biochemical, genetic and structural studies (Novella et al., 1993; Verdaguer et al., 1995, 1996, 1998; Martínez et al., 1997). In particular, in a complex between the Fab fragment of neutralizing MAbs 4C4 or SD6 – which recognize two distinct epitopes within site A (Mateu et al., 1990) – and a synthetic peptide antigen representing the G–H loop of VP1, the complementary-determining regions (CDRs) H2 and H3 of the two antibodies established bond interactions with the same atoms of Asp-143 (Verdaguer et al., 1995, 1998). In these complexes, the synthetic peptide antigen showed a conformation very similar to the one previously determined for the authentic G–H loop of chemically reduced FMDV O1 BFS particles (Logan et al., 1993). In all cases, the RGD was in an open-turn conformation, preceding a short helical region which participates in several site A epitopes (Mateu et al., 1990; Logan et al., 1993; Verdaguer et al., 1995, 1998). Studies of the binding of substituted synthetic peptides to antibodies indicated that the RGD triplet plays an essential role in the interaction not only with 4C4 and SD6, but also with several additional site A-specific neutralizing MAbs raised against three different FMDV isolates of subtypes C4 and C6 (Verdaguer et al., 1998).

In spite of being a target of neutralizing antibodies, the RGD of FMDV is highly conserved (Domingo et al., 1990, 1992). Furthermore, none out of 82 independent MAb SD6-escape mutants of FMDV C-S8c1 included a replacement at the RGD triplet (Mateu et al., 1989; Martínez et al., 1997). These observations, together with the lethal character of most substitutions at the RGD motif when introduced into infectious clones, led to the conclusion that the triplet was essential for infectivity (Mason et al., 1994; Mateu et al., 1996; Leippert et al., 1997). An unexpected result was obtained with MAb SD6-escape mutants derived from a population of FMDV C-S8c1 passed 100 times in BHK-21 cells (termed C-S8c1p100). In this population, 5 out of 31 escape-mutants included a replacement at the RGD motif (Mateu et al., 1989; Martínez et al., 1997). These observations, together with the lethal character of most substitutions at the RGD motif when introduced into infectious clones, led to the conclusion that the triplet was essential for infectivity (Mason et al., 1994; Mateu et al., 1996; Leippert et al., 1997). An unexpected result was obtained with MAb SD6-escape mutants derived from a population of FMDV C-S8c1 passed 100 times in BHK-21 cells (termed C-S8c1p100). In this population, 5 out of 31 escape-mutants included a replacement at the RGD motif (Martínez et al., 1997). The molecular basis for this apparent relaxation of the requirement for the precise RGD sequence is not well understood but it may have been facilitated by the use of heparan sulfate (Jackson et al., 1996; Sa-Carvalho et al., 1997), or some receptor alternative to an RGD-binding integrin, by the multiply passed FMDV C-S8c1p100. The capsid of FMDV C-S8c1p100, or clone c10 derived from it, differed from the capsid of the parental FMDV C-S8c1 at six positions: residues 41, 46 and 197 of VP1, and residues 25, 173 and 218 of VP3 (Martínez et al., 1997). Position 197 of VP1 is located at the C-terminal region of the protein, within antigenic site C, defined by a weakly neutralizing MAb (Mateu et al., 1990), and it may also exert some effect on site D, a discontinuous antigenic site which is independent of site A in FMDV of serotype C (Lea et al., 1994). The other positions that distinguish FMDV C-S8c1p100 from FMDV C-S8c1 are outside the antigenic sites that have been defined for FMDV of serotype C (Mateu et al., 1989, 1990; reviewed in Mateu, 1995).
behaviour of a virus with an unusual sequence at the antigenic site A (Fig. 1). For immunological and growth-competition assays biological FMDV clones were amplified from about 10^5 p.f.u. to about 10^6 p.f.u.

For infection of confluent BHK-21 cell monolayers with FMDV in liquid medium, the cell culture medium was removed and the desired dilution of FMDV (0·2 ml per 60 mm diameter culture dish) was added onto the cell monolayer. Virus was adsorbed to cells for 1 h at 37 °C in 7% CO₂ with gentle rocking every 15 min; the inoculum was removed, the cells washed with DMEM, and then overlaid with DMEM containing 2% foetal calf serum (2 ml of medium per 60 mm diameter culture dish). Infections were allowed to proceed until cytopathology was nearly complete. FMDV was titrated by plating serial dilutions on BHK-21 cell monolayers. After the adsorption period of 1 h at 37 °C, the cells were overlaid with DMEM containing 2% foetal calf serum, 0·5% agar and DEAE-dextran (0·045 mg/ml) (de la Torre et al., 1985). Plaques were visualized 24 h post-infection by crystal violet staining of fixed cells.

MAbs, polyclonal sera, immunohistochemical assays and neutralization of infectivity. MAb SD6, 4C4, 5A2, 6D11, 5C4, 2A12 and 2E5, raised against FMDV of serotype C, have been described previously (Mateu et al., 1987, 1990; Lea et al., 1994). Their properties relevant to the present study are described in the text and in the appropriate table and figure legends.

Total Ig and fractionated polyclonal antibodies employed are those described by Mateu et al. (1995b). The antibodies were from vaccinated or convalescent swine that had been vaccinated or infected with FMDV C-Sta Pau Sp/70. Ig from the animal sera was purified by protein A–Sepharose affinity chromatography. Antibodies specific for antigenic site A of FMDV were purified by affinity chromatography on a column with peptide A21, representing amino acids 136 to 156 of VP1 (sequence YTASARGDLHLTIITTHARHLP; see Fig. 1) coupled to Sepharose. Sera are identified by the code employed by Mateu et al. (1995b), as indicated in the relevant figures.

Polyclonal antibodies raised against antigenic site A were obtained by inoculating guinea pigs with synthetic peptide A24 representing amino acids 133 to 156 of VP1 (sequence TTTYTASARGDLHLTIITTHARHLP[C]), which spans antigenic site A; see Fig. 1). The sera are those described by Borrego (1993) and Borrego et al. (1993) and they are identified as a, b, c, d, e and h as in the original reports.

Enzyme-linked immunoelectrotransfer (Western) blot assays were carried out as previously described (Mateu et al., 1987, 1988). The FMDV particles were partially purified by sedimentation through a sucrose cushion. Viral protein was quantified by densitometry of the Coomassie blue-stained protein bands separated by PAGE, including SDS (Laemmli, 1970) and 8 M urea in the gel (SDS–urea–PAGE) (Mateu et al., 1987, 1988).

Competition ELISA was used to quantify the reactivity of MAbs with FMDV C-S8c1 and RGG. ELISA plates were coated with FMDV C-S8c1 particles (amount equivalent to 1 pmol of capsid protein VP1) or peptide A21 conjugated to keyhole limpet haemocyanin (KLH; 5 pmol of peptide). The assay was carried out as described previously (Mateu et al., 1995a) and they using as competing antigen 0·5, 5, 20, 100 and 500 pmol (VP1 equivalents) of either FMDV C-S8c1 or FMDV RGG.

Neutralization of infectivity was carried out by a plaque-reduction neutralization assay as previously described (Mateu et al., 1987). Briefly, FMDV (100·0 to 200·0 p.f.u.) was incubated with serial dilutions of antibody for 1 h at 25 °C. Then the mixtures were plated on BHK-21 cell monolayers and plaques visualized by crystal violet staining. Neutralization curves are plotted as percentage of plaque-reduction versus the logarithm of the antibody dilution. To compare neutralization of FMDV C-S8c1 and FMDV RGG, the reciprocal of the dilution of MAb (or polyclonal fraction) that causes neutralization of 70% of p.f.u. (plaque-reduction neutralization titre 70 or PRNT<sub>70</sub>) is used.

Methods

Cells, viruses, and infections. BHK-21 (c-13) cells were cloned by end-point dilution from a cell population kindly provided by L. Carrasco, as previously described (Domingo et al., 1980; Sobrino et al., 1983). BHK-21 cell monolayers were grown in Dulbecco’s modification of Eagle’s medium (DMEM), supplemented with 5% foetal calf serum, at 37 °C in an atmosphere with 7% CO₂. Cells were subcultured every 2 to 3 days by seeding one-third to one-sixth of the number of cells reached at confluency.

FMDV C-S8c1 is a clone of isolate FMDV C-Sta Pau Sp/70 obtained from a single viral plaque on a BHK-21 cell monolayer, as previously described (Sobrino et al., 1983). FMDV C-S8c1p100 is a viral population resulting from 100 serial passages of clone C-S8c1 in BHK-21 cells at an m.o.i. of 2 to 4 p.f.u. per cell (Martinez et al., 1997). FMDV RGG is a mutant clone derived from population FMDV C-S8c1p100, selected for its resistance to neutralization by MAb SD6 (Martinez et al., 1997). Other MAb SD6-resistant clones derived from the same parental population are identified by the amino acid replacement at the SD6 epitope in antigenic site A (Fig. 1). For immunological and growth-competition assays
Results

Reactivity of FMDV RGG with site A-specific MAbs

FMDV RGG, which includes substitution Asp-143 → Gly within antigenic site A (Fig. 1), was isolated as a MAb SD6-neutralization-resistant mutant of FMDV C-S8c1p100 (Martínez et al., 1997). The epitope defined by MAb SD6 spans the RGD triplet (Mateu et al., 1989; Verdaguer et al., 1995, 1998). To determine whether substitution Asp-143 → Gly affected the interaction with MAbs which recognize other epitopes within site A, competitive ELISA, Western blot assays and neutralization of infectivity experiments were carried out with FMDV C-S8c1 and FMDV RGG, using MAbs SD6, 4C4, 5A2 and 6D11. By competitive ELISA, binding of each site-A MAb antibody tested was at least 20- to 3000-fold lower for FMDV RGG than for FMDV C-S8c1 (Table 1). The corresponding difference for site D-specific MAbs 5C4, 2A12 and 2E5 (Lea et al., 1994) was 16- to 11-fold (Table 1). The differences with some site D MAbs probably reflect an influence of substitution His-197 → Arg in FMDV RGG (Martínez et al., 1997). The ELISA results with site A MAbs agree with results of Western blot assays (data not shown). The behaviour of FMDV RGG was comparable to that of FMDV HR (Fig. 1), an escape mutant of FMDV C-S8c1 (Mateu et al., 1989).

Table 1. Quantification of binding of FMDV type C MAbs to FMDV C-S8c1 and FMDV RGG by competition ELISA

<table>
<thead>
<tr>
<th>MAb†</th>
<th>Antigenic site†</th>
<th>FMDV C-S8c1</th>
<th>FMDV RGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD6</td>
<td>A</td>
<td>46</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>4C4</td>
<td>A</td>
<td>6.8</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>5A2</td>
<td>A</td>
<td>6.4</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>6D11</td>
<td>A</td>
<td>0.3</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>5C4</td>
<td>D</td>
<td>64</td>
<td>103</td>
</tr>
<tr>
<td>2A12</td>
<td>D</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>2E5</td>
<td>D</td>
<td>7.5</td>
<td>80</td>
</tr>
</tbody>
</table>

* pmol of VP1 equivalents needed to reduce absorbance by 50% in the assay described in Methods.
† The origin of the MAbs used, and the characterization of the epitopes that each MAb defines within antigenic site A or D, have been described previously (Mateu et al., 1987, 1990; Lea et al., 1994).

As expected from the antibody-binding experiments, MAbs SD6, 4C4, 5A2 and 6D11 were unable to neutralize FMDV RGG even at the highest concentration tested (PRNT<sub>50</sub> < 0; Fig. 2). In contrast, PRNT<sub>50</sub> with FMDV C-S8c1 ranged from 1:5 to 3, in agreement with previous determinations (Mateu et al., 1987, 1990). Thus, substitution Asp-143 → Gly has a profound effect on the antigenicity of FMDV C-S8c1, as probed with site A-specific MAbs.

Antigenic distance between FMDV C-S8c1 and FMDV RGG estimated with swine polyclonal antibodies

To evaluate the overall antigenic distance between FMDV C-S8c1 and FMDV RGG, the neutralization of the two viruses by polyclonal sera from vaccinated or convalescent swine was quantified (Fig. 3). For each serum, neutralization was assayed with the total Ig, as well as with the fractions retained and not retained on a peptide A21-Sepharose column (Mateu et al., 1995b). Using total Ig, PRNT<sub>50</sub> values for FMDV RGG were 3- to 9-fold lower than for FMDV C-S8c1 (left panels in Fig. 3). This difference reached 6- to 12-fold when the Ig fraction retained on A21-Sepharose resin was used (right panels in Fig. 3). Thus, the antigenic diversification attained by FMDV RGG is detected by differences in neutralization titres using vaccinated and convalescent swine sera.

Antigenic distance between FMDV C-S8c1 and FMDV RGG estimated with site A-specific polyclonal sera

The anti-FMDV swine sera employed in the preceding experiments include antibodies directed to several sites of the
Fig. 3. Neutralization of FMDV C-S8c1 (●) and FMDV RGG (○) by sera from swine that had been either vaccinated (sera 13 and 474) or infected (VFAC and 175; convalescent swine sera) with FMDV C-Sta Pau Sp/70. The origins of the sera and their fractionation is described in Mateu et al. (1995b) and in Methods. The total Ig concentration (mg/ml) was 16, 16, 22 and 12 for sera 13, 474, VFAC and 175, respectively (Table 3 in Mateu et al., 1995b). Left panels: neutralization by Ig; middle panels: neutralization by the Ig fraction not retained (NR) on peptide A21-Sepharose resin; right panels: neutralization by the Ig fraction retained (R) on peptide A21-Sepharose resin. Percentage neutralization was determined as indicated in Methods.

Fig. 4. Neutralization of FMDV C-S8c1 (●) and FMDV RGG (○) by guinea pig polyclonal antibodies directed to antigenic site A of FMDV C-S8c1. The preparation and characterization of antipeptide A24 sera a, b, c, d and e have been described previously (Borrego, 1993; Borrego et al., 1993). Serum h was produced by inoculating a guinea pig with herpes simplex virus type 1, and was used as a negative control (Borrego et al., 1993). Each panel compares neutralization of C-S8c1 and RGG by the serum indicated at the top right. The percentage neutralization as a function of serum dilution was determined as described in Mateu et al. (1987) and in Methods.

FMDV capsid involved in neutralization of infectivity (Mateu et al., 1994, 1995b; Lea et al., 1994; Holguín et al., 1997). The fraction retained on A21-Sepharose resin was depleted of non-site A antibodies and also of site A-specific antibodies that displayed a low affinity for peptide A21, such as antibodies requiring an antigen conformation not represented in the
Fig. 5. Test of genetic stability of FMDV RGG. Top: FMDV RGG was serially passaged at an m.o.i. of 10 and 0-1 p.f.u. per cell, in triplicate, as indicated. ■, Biological clone; ○, uncloned populations. One of the triplicate passage series at an m.o.i. of 0-1 p.f.u. per cell included a small proportion (about 20%) of an RGD-containing revertant at passage 10 (p10). Infections in this particular series were continued up to passage 50. Bottom: Consensus nucleotide sequence around the RGD-coding region [nucleotides 3622 to 3642 of the FMDV genome (residue numbering as in Escarmís et al., 1999), encoding amino acids 139 to 145 of VP1]. Sequences correspond to the passage series in which reversion of replacement Asp-143 → Gly was observed; the passage number is indicated below each panel. To the right of the p50 panel, the nucleotide sequence around the RGD-coding region is given (non-coding strand as read from the gel on the left, and its complementary, coding-strand on the right), with the encoded amino acids. Nucleotides in parentheses indicate presence of double bands in sequencing gels.

E, Position of nucleotide 3635 in genomic RNA with coexistence of G and A at passages 15, 25, 50; ○, position of nucleotide 3641 with the dominance of C at passage 24 and similar amounts of C and A at passage 50. Viable genomes that demonstrate heterogeneity in the FMDV quasispecies at positions 3635 and 3641 were isolated (see text). Procedures for serial passages in BHK-21 cells and nucleotide sequence determinations are given in Methods.

peptide (see Discussion). To evaluate whether FMDV C-S8c1 and RGG differed with regard to polyclonal antibodies directed specifically to antigenic site A, we used a set of guinea pig polyclonal sera raised against synthetic peptide A24, representing residues 133 to 156 of the G–H loop of VP1 of FMDV C-S8c1 (Borrego et al., 1993). For sera a, c, d and e, PRNT values for FMDV RGG were 8- to 12-fold lower than for FMDV C-S8c1 (Fig. 4). In contrast, no significant difference of neutralization of the two viruses was observed using serum b (Fig. 4). This serum was also unusual with regard to selection of escape mutants of FMDV C-S8c1 (Borrego et al., 1993), and it may have a broader neutralization spectrum than the sera from the other animals (see Discussion). In spite of animal-to-animal variations in the antibody response to FMDV antigens, the results with polyclonal antibodies specifically directed against the G–H loop of VP1 of C-S8c1 show that FMDV RGG is several-fold more resistant to neutralization than FMDV C-S8c1. Thus, FMDV RGG differs considerably antigenically from FMDV C-S8c1 as quantified by a number of immunological assays using monoclonal antibodies and a variety of polyclonal antibodies (Table 1 and Figs 2, 3 and 4).

Genetic stability of FMDV RGG

Since FMDV RGG is unusual in lacking the RGD integrin-recognition sequence it was interesting to study its stability upon extended replication in BHK-21 cells. Two series of triplicate passages were carried out at high and low m.o.i. Sequence determination of the consensus viral genome at a number of passages revealed that partial reversion of the sequence RGG to RGD occurred in one out of the six passage series (Fig. 5). In all other series the RGG sequence remained
completely dominant for at least 20 passages. Interestingly, once the reversion occurred, the revertant virus, rather than becoming gradually dominant, coexisted with ancestor viruses maintaining the RGG sequence. The proportion of genomes encoding RGG and RGD remained in the range 40 to 60% from passage 15 to 50 (the relevant nucleotide position is indicated by a filled circle in the sequences depicted in Fig. 5). At passage 20 the population regained the ability to react with site A-specific MAbs in Western blot assays. The consensus nucleotide sequence in the capsid-coding region revealed no mutations except those at the region encoding site A (Fig. 5) (results not shown). Once this equilibrium had been established, a second mutation (C-3641 → A) resulting in amino acid replacement Ala-145 → Asp became gradually dominant (open circle in the sequences of Fig. 5). To ascertain that FMDVs harbouring an Asp residue at position 145 of VP1 were viable, 18 biological clones from population FMDV RGG p50 were obtained and the region encoding the G–H loop of VP1 was sequenced. The results demonstrated the coexistence in the FMDV RGG p50 quasispecies of viruses with the following sequences (amino acid positions 141 to 148 of VP1; see Fig. 1): RGDLAHLT (11 clones); RGGLDHLT (6 clones); GGLALHLTA (1 clone) (additional variant amino acids are underlined). Thus, FMDV RGG with Asp at position 145 of VP1 can be viable. Mutant FMDV RGG displays genetic stability which is statistically supported, but which is not absolute. At times, FMDV RGG may undergo a true reversion which, however, is followed by the establishment of a population equilibrium with the parental virus, and an increase in the quasispecies complexity around the RGD-coding region.

**Competition between FMDV RGG and other biological clones derived from population FMDV C-S8c1p100**

To study whether FMDV RGG showed a selective disadvantage relative to other biological clones which were also derived from population C-S8c1p100, competition experiments between FMDV RGG and each of six mutants derived from FMDV C-S8c1p100 (c10, A-138 → D, S-139 → I, L-144 → V and A-145 → V; sequences listed in Fig. 1) were carried out. The results of these competitions (Table 2) could be influenced not only by the relative fitness of the initial clones, but also by possible revertants arising from any of the competing mutants in the course of passaging. In all cases, the dominant genomes at passage 20 included RGD at positions 141 to 143. In competitions with L-144 → V and A-145 → V these mutants became unambiguously dominant over FMDV RGG, with no evidence of reversions. In the competition with c10, the dominant sequence could have arisen either from reversion of FMDV RGG or from a selective advantage of c10. In one of the series of the competition with A-138 → D, the dominant sequence could have been generated either from reversion of FMDV RGG or reversion of mutant A-138 → D. However, in the second series of this competition, the presence of a mixture of Asp and Ala at position 138 strongly suggests a selective advantage of A-138 → D over FMDV RGG (Table 2). Similar arguments apply to the competition with S-139 → I, in which this mutant displayed an advantage over FMDV RGG. The results suggest that FMDV RGG manifests a selective disadvantage relative to other mutants isolated from the same FMDV C-S8c1 population upon replication in BHK-21 cells.

**Table 2. Competition between FMDV RGG and other mutants derived from population FMDV C-S8c1p100**

<table>
<thead>
<tr>
<th>Competition virus*</th>
<th>Dominant genome† (passage 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c10</td>
<td>c10, c10</td>
</tr>
<tr>
<td>A-138 → D</td>
<td>A-138 → D, mixed</td>
</tr>
<tr>
<td>S-139 → I</td>
<td>S-139 → I, mixed</td>
</tr>
<tr>
<td>L-144 → V</td>
<td>L-144 → V, L-144 → V</td>
</tr>
</tbody>
</table>

* The origin of the mutants employed in the experiment is described in Methods, and the amino acid sequence of the G–H loop of VP1 for the different mutants is listed in Fig. 1. Each competition series was carried out in duplicate for a total of 20 passages at an m.o.i. of 2–4 p.f.u. per cell (for each infection 4–8 × 10³ BHK-21 cells was infected with 10⁶ p.f.u.). Competitions were started with about 50% of FMDV RGG and 50% of the competing FMDV.

† The dominant genome(s) in each of the two competition assays is indicated. Dominance is based on the consensus sequence observed at the region encoding amino acids 138–145 of VP1. Mixed means a mixture of about 50% of the two competing viruses, according to the band pattern in the sequencing gels. Procedures used for the infections and nucleotide sequence determinations are given in Methods. The interpretation of results in terms of dominance of initial virus versus reversion in the course of passage is given in the text.

**Discussion**

The antigenic properties of FMDV with a site A including amino acid replacements at the RGD triplet of VP1 could previously be examined only with synthetic peptides, taking advantage of the fact that peptides mimic faithfully the interaction of authentic site A with antibodies (Rowlands et al., 1983; Clarke et al., 1983; Mateu et al., 1989, 1990; Mateu, 1995). Using this approach, Novella et al. (1993) found that substitution of Asp-143 of VP1 by Ala resulted in the loss of multiple epitopes within antigenic site A of FMDV C-S8c1. In a screening of substituted synthetic peptides, Verdaguer et al. (1998) found that most replacements at position 143 adversely affected the interaction of the peptide antigens with site A-specific MAbs. In particular, substitution Asp-143 → Gly abolished recognition by MAbs SD6, 4C4, 5A2 and 6D11 (Verdaguer et al., 1998). The involvement of Asp-143 in paratope recognition has been supported also by structural studies (Verdaguer et al., 1995, 1996, 1998). The unexpected isolation of FMDV RGG (Martinez et al., 1997) opened the
opportunity of examining the antigenicity of a live virus harbouring replacement Asp-143 → Gly, particularly in neutralization assays. For FMDV of serotype C, the available evidence suggests that antigenic site A behaves quite independently of the rest of the viral capsid (Lea et al., 1994; Mateu, 1995). Therefore the behaviour of FMDV RGG with regard to reactivity with site A-specific monoclonal or polyclonal antibodies (Figs 2 and 4) must be strongly influenced by replacement Asp-143 → Gly, with a very unlikely participation of the substitutions found in other capsid residues of FMDV RGG. This is also supported by the reactivity with site A MAbs of population FMDV RGG p20, which contained about 50% true revertants. No other capsid replacement outside the G–H loop of VP1 was present in this population.

Serum b from a guinea pig immunized with peptide A24 neutralized FMDV RGG with a titre similar to FMDV CS8c1 (second panel in Fig. 4). Borrego et al. (1993) analysed neutralization-resistant mutants of FMDV C-S8c1 upon passage of the virus in the presence of serum a, b, c or d. Interestingly, in these experiments serum b selected resistant variants more rapidly, and with the largest repertoire of replacements within site A [Fig. 2 in Borrego et al. (1993)]. One possibility is that this particular animal generated antibodies directed to a broad spectrum of site A epitopes. As a result, the weight of replacement Asp-143 → Gly in reducing neutralization could have been smaller for serum b than for the other sera tested. Evidence for animal-to-animal variations regarding the antibody response to the same FMDV antigens has been reported (Mateu et al., 1995 b; Taboga et al., 1997).

The assignment of antibody populations from convalescent or immunized swine to the several antigenic sites of FMDV C-S8c1 is uncertain. Mateu et al. (1995 b) evaluated the immunodominance of site A in sera from swine that had been vaccinated or infected with FMDV C-Sta Pau Sp/70, including sera 13, 474, VFAC and 175 used in the present study. The immunodominance of site A was measured as the reactivity of the serum with peptide A19 (representing VP1 residues 138 to 156; Fig. 1), relative to the reactivity with the equivalent molar amount of peptide in the form of viral particles (Mateu et al., 1995 b). The ratio obtained varied considerably among sera from individual swine; for the sera used in the experiments reported here, values ranged from 0·41 for serum 13 to 0·15 for serum 474 [Table 2 in Mateu et al. (1995 b)]. Serum 474 is the one that showed the lowest PRNT10 difference between FMDV RGG and FMDV C-S8c1 (Fig. 3), which is consistent with a more limited representation of antibodies directed to site A, relative to antibodies directed to other antigenic sites. As expected, the difference between PRNT10 of RGG and C-S8c1 varied when the site A-specific Ig fraction was employed (Fig. 3). In addition to antigenic site A, FMDV C-S8c1 includes a complex discontinuous site D, site C (the carboxy-terminal region of VP1) (Lea et al., 1994; Mateu, 1995), and probably additional site(s) that have not yet been identified (Holguín et al., 1997). The relative abundance of antibodies directed to each of these sites (different from site A) in vaccinated or convalescent swine sera is not known. The differences in neutralization titres of FMDV RGG and FMDV C-S8c1 by non-site A antibodies could be influenced by any of the six amino acid replacements distinguishing the two viruses outside the G–H loop of VP1 (Martínez et al., 1997; see also Introduction).

Given the considerable antigenic distance between FMDV RGG and FMDV C-S8c1 documented here, and also the unusual nature of replacement Asp-143 → Gly, it was of obvious relevance to explore the genetic stability of FMDV RGG in the course of replication in BHK-21 cells. Regarding stability assays, the results (Fig. 5) illustrate the strong stochastic component that must influence many events in RNA virus evolution (Domingo & Holland, 1994). Had we not persevered with a 6-fold series of 20 passages each (Fig. 5), we would have concluded that FMDV RGG was genetically stable. Yet in one out of six passage series, evidence of reversion of substitution Asp-143 → Gly was obtained, perhaps facilitated by a low m.o.i. (and hence a higher number of replication cycles) in this group of passages. Surprisingly, however, the revertant virus, rather than becoming gradually dominant over FMDV RGG, established an equilibrium with FMDV RGG, the two viruses remaining at a surprising 40 to 60% proportion for at least 35 serial infections (from passage 15 until passage 50; Fig. 5). Several mechanisms may be operating to maintain the population equilibrium between FMDV RGG and its Gly-143 → Asp revertant. One is the possible formation of mosaic capsids composed of VP1 with Asp-143 and VP1 with Gly-143 in the same virions. Formation of mosaic capsids could be facilitated by the effective high m.o.i. in the second round of cell infection, occurring at each low m.o.i. passage: since each cell yields about 100 to 200 p.f.u. (Sobrino et al., 1983), ignoring effects of virus dilution into the culture medium, the m.o.i. during the second round of infection can be estimated at 10 to 20 p.f.u. per cell. Mosaic particles would carry randomly the two competing genomes blurring any possible capsid-dependent advantage of revertants over the parental FMDV RGG. Another possibility is that additional mutation(s) occurred in the FMDV RGG genome (in the capsid-coding region or elsewhere) that compensated for the fitness decrease due to the presence of Gly-143. Although replacement Ala-145 → Asp was associated with FMDV RGG genomes according to the clonal analysis at passage 50, it first became visible only when population equilibrium had been established (bottom of Fig. 5), rendering unlikely the possibility that this particular substitution was responsible for the establishment of the equilibrium between FMDV RGG- and RGD-containing revertants. The basis for this prolonged equilibrium and the possible effect of VP1 replacement Ala-145 → Asp on fitness of FMDV RGG are now under study.

In a second group of experiments, FMDV RGG was shown not to survive in competition with FMDV clones isolated also from FMDV C-S8c1p100 (Table 2). That the clones all
belonged to the same FMDV population (identical passage history) is crucial for the interpretation of the results since fitness of RNA viruses increases with the number of passages whenever large viral populations are involved in the infections (Martínez et al., 1991; Novella et al., 1995; Escamís et al., 1999). Therefore, in spite of its considerable genetic stability, the antigenically variant FMDV RGG is at a selective disadvantage over sibling clones that maintain the RGD triplet. The reason for this disadvantage, which may bear on receptor usage, is currently under investigation. An important question is whether FMDVs with substitutions at the signature RGD triplet do not imply loss of integrin recognition (Pfaff, 1997). Studies have established that substitutions at the signature RGD triplet can still enter cells via an integrin or whether they must use an alternative receptor. Work with other integrin ligands has established that substitutions at the signature RGD triplet do not imply loss of integrin recognition (Pfaff, 1997). Studies with FMDV are now facilitated by an increasing number of viable mutants with substitutions in the RGD. Particularly intriguing has been the isolation from the FMDV RGG p50 quasispecies of a biological clone bearing a GGG triplet instead of RGD. These profoundly altered, viable mutants, provide useful tools for studies of cell recognition by FMDV.

We are indebted to M. G. Mateu for excellent suggestions and for providing fractionated swine sera. We thank B. Borrego for the preparation of site-A specific guinea pig sera, and M. L. Valero and D. Andreu for providing synthetic peptides. Work was supported by grant DGES PM97-0000-C02-01, the EU Project FAIR 5 CT97-3665, and Fundación Ramón Areces. N.S. and C.M.R.-J. were supported by predoctoral fellowships from Comunidad Autónoma de Madrid.

References


Gebauer, F., de la Torre, J. C., Gomes, I., Mateu, M. G., Barahona, H.,...


Received 18 February 1999; Accepted 4 May 1999