

Differential restrictions on antigenic variation among antigenic sites of foot-and-mouth disease virus in the absence of antibody selection

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Clonal populations of foot-and-mouth disease virus have been serially passaged in cell culture to analyse variation in the absence of immune selection at different antigenic sites of the virus. Mutant frequencies at the RNA regions encoding two independent antigenic sites (sites C and D) were more than twentyfold lower than for antigenic site A (the G–H loop of VP1). Correspondingly, fixation of amino acid substitutions was very restricted in sites C

and D. In spite of such a restriction, neutralization assays using fractionated anti-virus polyclonal antibodies has provided direct evidence of significant antigenic variation in the absence of immune selection at sites unrelated to site A. It is proposed that the degree of tolerance to acceptance of amino acid replacements may modulate the variation at different antigenic epitopes of the same virus.

Introduction

Evidence for antigenic variation in the absence of immune selection has been obtained for several viruses (reviewed in Domingo *et al.*, 1993). Two mechanisms for such variation have been proposed: (i) selective forces unrelated to an immune response may nevertheless favour amino acid replacements that cause a change in antigenic specificity (Both *et al.*, 1983); (ii) fluctuations in quasispecies distributions – due to perturbation of population equilibrium (Domingo & Holland, 1988, 1994) – may result in the 'hitch-hiking' of mutations at sites where they are tolerated. These mutations can be regarded as neutral or nearly neutral in that they do not affect virus fitness substantially at the time of their incorporation in the viral genome. Antigenic sites, by virtue of being located at the surface of capsid proteins, will tend to tolerate amino acid replacements with higher frequency than other regions involved in structurally important interactions. The 'hitch-hiking' mechanism was termed the random change model of antigenic variation (Domingo *et al.*, 1993) to emphasize the stochastic and positive selection-independent nature of fixation of amino acid replacements. The model was proposed mainly

on the basis of variation of a major antigenic site, termed site A, upon passage of foot-and-mouth disease virus (FMDV) of serotype C (clone C-S8c1) in cell culture in the absence of anti-FMDV antibodies (Díez *et al.*, 1989, 1990; Borrego *et al.*, 1993).

FMDV is an important animal pathogen which belongs to the genus *Aphthovirus* of the family *Picornaviridae* (for a recent review see Rueckert, 1996). Seven distinct serotypes – termed A, O, C, Asia1, SAT1, SAT2 and SAT3 – and many antigenic variants of FMDV have been described (Pereira, 1977). Antigenic variation represents an important adaptive strategy for this virus and may contribute to decreased vaccine efficacy in the field (Feigelstock *et al.*, 1996, and references therein). Thus, it is important to gain insight into the mechanisms participating in dominance of antigenic variants in FMDV populations. Antigenic site A of FMDV is located within the G–H loop of capsid protein VP1 (Strohmaier *et al.*, 1982; Acharya *et al.*, 1989). In serotype C, site A is composed of distinct, overlapping, continuous epitopes (Mateu *et al.*, 1990). However, the G–H loop is not only a target for host antiviral antibodies; it is also involved in recognition of a cellular receptor via a highly conserved RGD triplet (Fox *et al.*, 1989; Mason *et al.*, 1994; Berinstein *et al.*, 1995; Hernández *et al.*, 1996) with participation of neighbouring residues (Rieder *et al.*, 1994; Mateu *et al.*, 1996). Antigenic variation in site A upon passage of FMDV C-S8c1 in cell culture in the absence of specific antibodies was reported previously (Díez *et al.*, 1989,

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1990; Borrego *et al.*, 1993). Because of the dual function of the G–H loop, such a variation could reflect either ‘hitch-hiking’ of amino acid replacements (in variant genomes displaying an increase in fitness mediated by mutations elsewhere in the genome) or fixation of amino acid replacements which optimize binding of virus to cells in culture. Even though no differences were detected between FMDV C-S8c1 and some antigenic variants regarding binding to BHK-21 cells (results quoted in Domingo *et al.*, 1993), a difference in cell binding has been recently documented for some antigenic variants of FMDV type A (Rieder *et al.*, 1994). In addition to site A, other antigenic sites have been characterized in FMDV C-S8c1 (Lea *et al.*, 1994; Mateu *et al.*, 1994). Thus, it was of interest to explore whether fixation of amino acid replacements in the absence of immune pressure occurred at those other antigenic sites also. The C-terminal segment of VP1 includes antigenic site C, which is composed of continuous epitopes (Mateu *et al.*, 1990). Antigenic sites A and C appear as topologically independent in serotype C (Lea *et al.*, 1994). In addition, a complex, major antigenic site, termed site D, has been defined in FMDV C-S8c1 (Lea *et al.*, 1994). This site involves at least part of the C-terminal region of VP1 (subsite D1, around residue 193), the B–B knob of VP3 (subsite D3, around residue 58) and the B–C loop of VP2 (subsite D2, around residues 72, 74 and 79) located near the capsid threefold axis. In addition, some other regions of the capsid of FMDV types A and O have also been found to be involved in epitopes (Thomas *et al.*, 1988a; Kitson *et al.*, 1990; Mateu, 1995). There is evidence that, in addition to site A, site D and perhaps other capsid regions may also be relevant to the antigenic diversification of FMDV in the field (Thomas *et al.*, 1988b; Feigelstock *et al.*, 1992, 1996; Mateu *et al.*, 1994, 1995). In the present report the reactivity, with antibodies directed to sites C, D and other epitopes unrelated to site A, of FMDV populations obtained upon passage of C-S8c1 in the absence of immune pressure is analysed. The rates of fixation of mutations are compared for the corresponding genomic regions. The results support the previous suggestion that there are severe restrictions to variation within antigenic site D (Lea *et al.*, 1994; Mateu, 1995). However, in spite of such restrictions, evidence of antigenic variation in the absence of immune pressure was found for epitopes unrelated to those in site A.

Methods

■ **Cells and viruses.** FMDV C-S8c1 is a plaque-purified derivative of the natural isolate FMDV C₁Santa Pau-Spain 70, a representative of the European serotype C viruses (Sobrinho *et al.*, 1983). FMDV C-S8c1, its monoclonal antibody (MAb)-resistant mutants HR or SN (Martínez *et al.*, 1991a), or mixtures of two populations were serially passaged in cloned BHK-21 cells at an m.o.i. of 0.2 to 2 p.f.u. per cell for a maximum of 100 passages as described previously (Sobrinho *et al.*, 1983; Díez *et al.*, 1989) (see Fig. 1).

■ **Monoclonal and polyclonal antibodies.** The MAbs used, and the identification and characterization of the corresponding epitopes,

have been described previously (Mateu *et al.*, 1990; Lea *et al.*, 1994). Serum immunoglobulins (Ig) from FMDV-vaccinated swine were fractionated by affinity chromatography using a synthetic peptide representing antigenic site A, coupled to a Sepharose matrix. Unbound Ig fraction included nonspecific antibodies and anti-FMDV antibodies directed to antigenic sites different from site A (Mateu *et al.*, 1995). The unbound fractions derived from animal 13 (NRf13) and from animal 53 (NRf53) (Mateu *et al.*, 1995) were used for virus neutralization assays.

■ **PCR amplification.** FMDV RNA was copied into cDNA, which was amplified by PCR. Primers used for PCR amplification and for nucleotide sequencing are as follows (numbering as in Díez *et al.*, 1990): 5′ GAAGGGCCCAGGGTTGGACT 3′ (complementary to positions 2246 to 2227 mapping in the 2A/2B-coding region of FMDV C-S8c1 RNA, except that residue 2236 is A in FMDV C-S8c1 RNA); 5′ CCTGTGGACGCTAGACA 3′ (complementary to positions 1546 to 1562 of FMDV C-S8c1 minus-strand cDNA); 5′ GTACTGTGTGTA-GTACTGCGC 3′ (complementary to positions 1209 to 1189 of C-S8c1 RNA, except that residue 1191 is G in FMDV C-S8c1 RNA) and 5′ CTAGAGACGCGCTTC 3′ (complementary to positions 406 to 421 of FMDV C-S8c1 minus-strand cDNA). The PCR reactions were carried out in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM each of the four deoxynucleotide triphosphates, and 0.01% gelatin. Reactions were initiated by incubation at 94 °C for 5 min, followed by 30 cycles consisting of incubations at 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min. Finally, the reaction mixtures were incubated at 72 °C for 10 min.

■ **Nucleotide sequencing.** Consensus nucleotide sequences were determined using the fmol method (Promega) on PCR amplified DNA, followed by treatment of the reaction mixture with terminal deoxynucleotidyl transferase to minimize ambiguities in sequencing gels derived from premature chain terminations (DeBorde *et al.*, 1986). The following genomic regions were sequenced: nucleotides 1591 to 1746 (amino acids 9 to 60 of VP1), nucleotides 1945 to 2193 (amino acids 127 to 209 of VP1), nucleotides 1036 to 1125 (amino acids 43 to 72 of VP3) and nucleotides 436 to 741 (amino acids 61 to 162 of VP2) of FMDV C-S8c1 (numbering as in Díez *et al.*, 1990). Primers used for nucleotide sequencing were the same used for PCR amplification. In addition, another primer was used to sequence the segment encoding site A: 5′ GCACGCTTCATGCGCAC 3′, complementary to positions 2102 to 2086 of FMDV C-S8c1 RNA.

■ **Immunological assays.** Plaque reduction assays (neutralization assays) and enzyme-linked immunoelectrotransfer blot (Western blot) assays were carried out as described previously (Mateu *et al.*, 1987).

Results

Mutant frequencies at genomic segments encoding different antigenic sites of passaged FMDV populations

Sixteen different FMDV populations were obtained by serial passages in BHK-21 cells of either FMDV C-S8c1 or its MAb-resistant mutants HR or SN (primary passages, Fig. 1A), or mixtures of two different populations (coinfections, Fig. 1B). RNA from 15 virus populations from independent passage series (series b of C-S8c1 was not analysed) and an additional population obtained from an intermediate passage (HRp57a, Fig. 1A) was reverse transcribed, amplified by PCR and sequenced, as detailed in Methods. The alignment of average

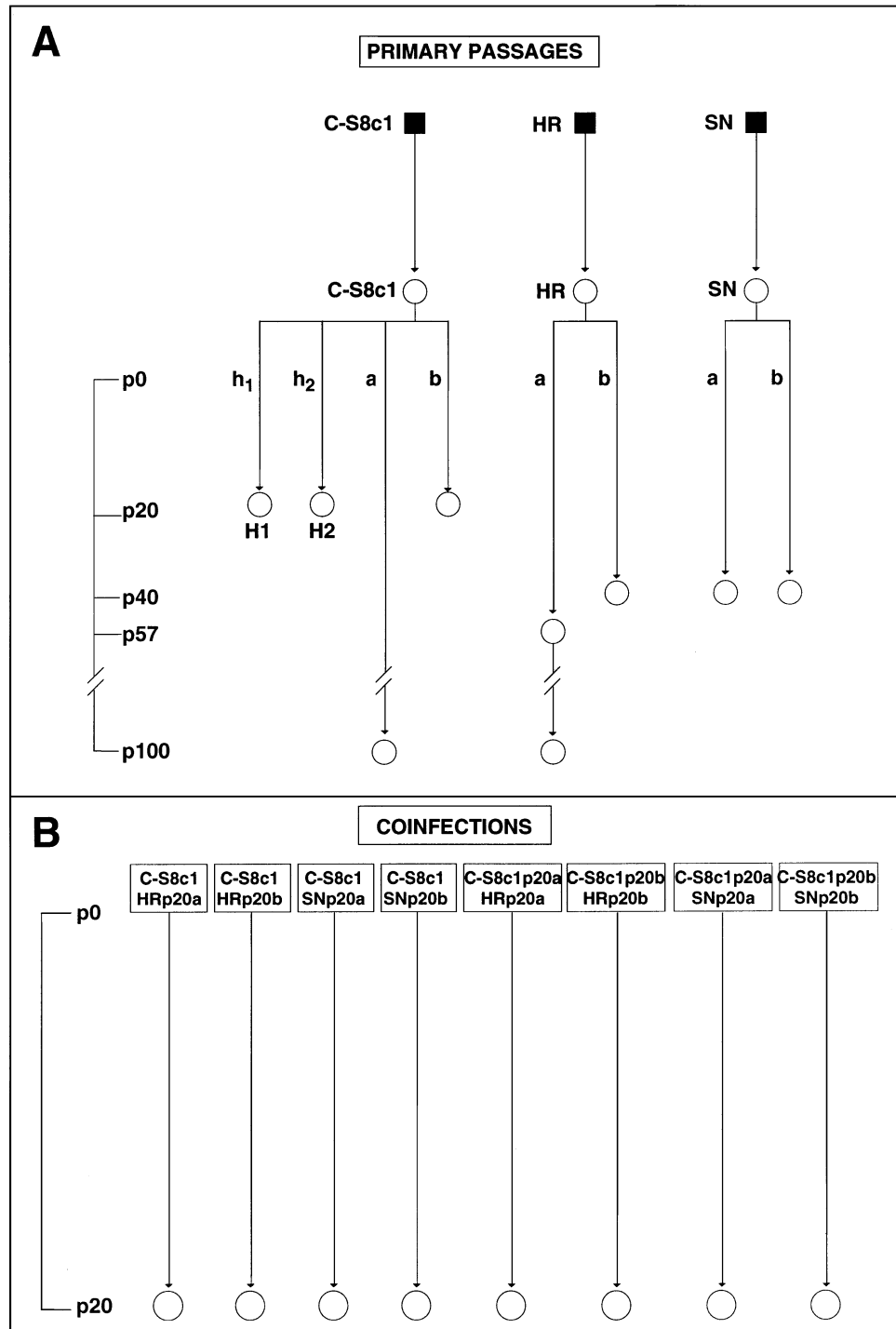
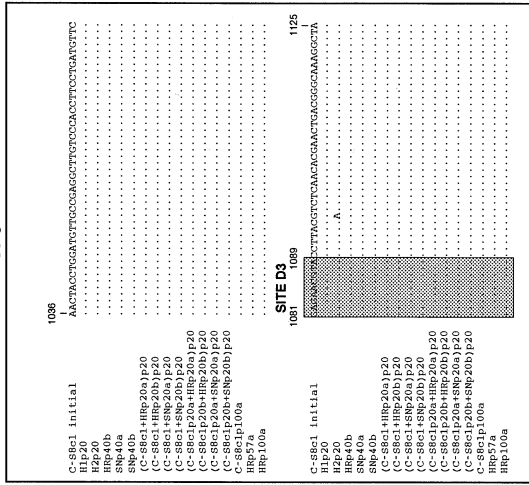
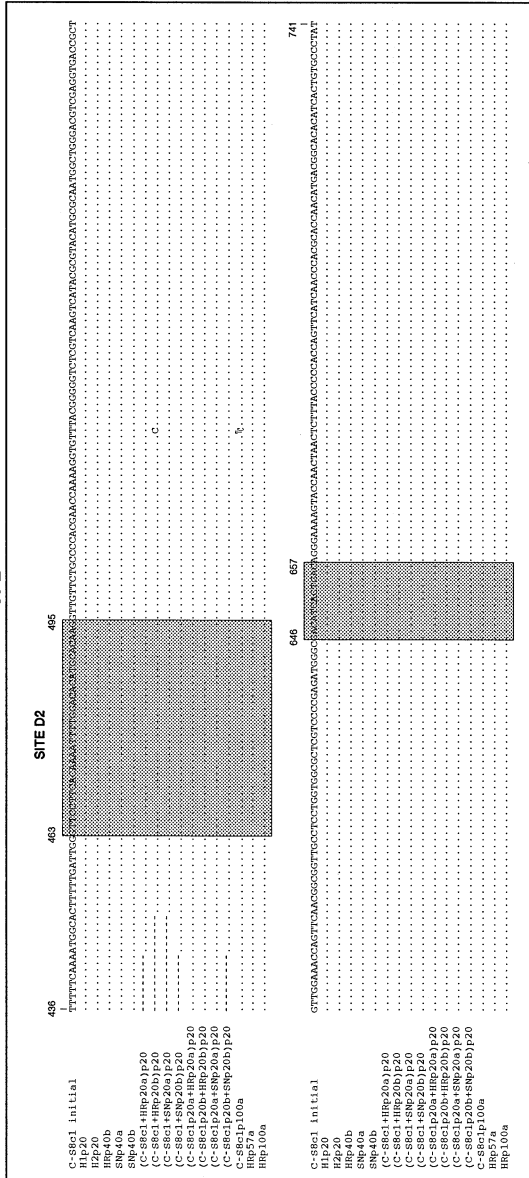


Fig. 1. Outline of passage histories of FMDV C-S8c1 and of MAb-resistant mutants HR or SN (which contain VP1 substitutions H146-R and S139-N, respectively) (primary passages, box A), and mixtures of populations (coinfections, box B). Symbols: a and b distinguish populations passaged in duplicate; p denotes the passage number; h_1 , h_2 correspond to passages in the presence of serum from a guinea-pig inoculated with herpes simplex virus type 1; they correspond to a negative control in the experiments described by Borrego *et al.* (1993). Viruses in all series were passaged in the absence of anti-FMDV antibodies. The passage number is indicated on the left. Plaque-purified viruses are represented by filled squares (■). All virus populations indicated by open circles (○), except C-S8c1 p20b, were analysed.

VP3



VP2



VP1

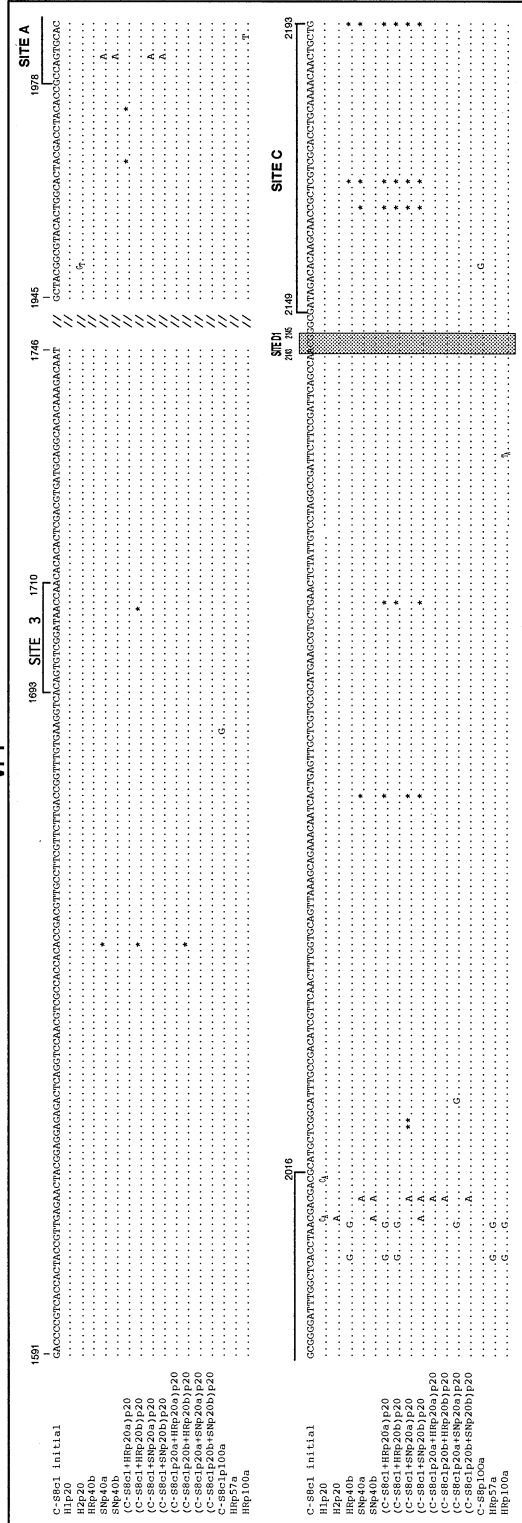


Fig. 2. Nucleotide sequence of genomic regions encoding part of capsid proteins VP1, VP2 and VP3 that include antigenic sites A, C and D of FMDV populations passed in the absence of immune selection (compare Fig. 1). The entire regions that have been sequenced are given to provide a visual assessment of occurrence of mutations in different capsid-coding regions, and the relative coding size of the three antigenic sites (A, C and D) considered in the present study. Only nucleotides which differ from those of the initial FMDV C-88c1 are indicated. Dots mark sequence identity with C-88c1. T_C, G_C, C_A and T_A indicate mixed populations, as revealed by double bands in the sequencing gels. Asterisks denote ambiguities in the gel, and dashes sequences that have not been determined. Numbering of P1 nucleotides is given in Methods. Shaded boxes and lines above the sequences delimit the segments found involved in sites A, C and D (plus site 3 of FMDV type O) as defined in Mateu *et al.* (1994).

Table 1. Mutant frequencies in 16 FMDV populations passaged in the absence of immune selection

Mutant frequency is calculated by dividing the number of mutations found relative to the FMDV C-S8c1 nucleotide sequence by the total number of nucleotides sequenced in the 16 populations analysed (Fig. 2). Double bands in sequencing gels, which are indicative of mixed populations, have not been considered mutations as regards the calculation of mutant frequency. Values were calculated considering nonsynonymous (Nonsyn.), synonymous (Syn.) or both types of mutation (Total) (first column).

Type of mutation	Antigenic site*			Flanking regions†	Average mutant frequency‡
	Site A	Site C	Site D		
Nonsyn.	2.7×10^{-2}	1.3×10^{-3}	$< 1.0 \times 10^{-3}$	2.8×10^{-4}	1.6×10^{-3}
Syn.	$< 1.6 \times 10^{-3}$	$< 1.3 \times 10^{-3}$	$< 1.0 \times 10^{-3}$	9.5×10^{-5}	7.8×10^{-5}
Total	2.7×10^{-2}	1.3×10^{-3}	$< 1.0 \times 10^{-3}$	3.8×10^{-4}	1.7×10^{-3}

* Antigenic sites of FMDV C-S8c1 are operationally defined as described in Mateu *et al.* (1994). Site A includes residues 138 to 150 of VP1; site C includes residues 195 to 209 of VP1; site D (and equivalent sites in other serotypes) include residues 70 to 80 and 131 to 134 of VP2, 58 to 60 of VP3 and 193 of VP1 (Lea *et al.*, 1994).

† The flanking regions considered are those outside the antigenic regions, and whose sequences are displayed in Fig. 2.

‡ Average frequency is that calculated from the total 12759 nucleotides that have been sequenced.

nucleotide sequences (Fig. 2) allowed calculation of mutant frequencies for the entire capsid regions analysed, and for the specific segments encoding antigenic sites A, C and D (Table 1). The results show a mutant frequency more than 20 times lower for site D- and C-coding regions than for the site A-coding region.

Lower frequency of amino acid replacements within antigenic sites D and C compared with site A

The differences in mutant frequency among the genomic segments analysed were also apparent at the amino acid level (Fig. 3). Amino acid replacements occurred preferentially on the capsid surface, within or near antigenic sites identified in FMDV. Substitutions within site A were fixed in most populations analysed. In contrast, only two substitutions were observed within or near the segments involved in sites C and D (Fig. 3).

Site A involves mainly residues 138 to 150 in the G-H loop of VP1 of FMDV type C. In the populations analysed, substitutions within this site included A-140 → V (in population HRp100a), T-148 → K or A, or T-149 → K (in many populations) or a mixture of variants with T-150 → T/K (in population H1p20). In addition, substitution H-154 → R within the G-H loop was found in population (C-S8c1p20a + SNp20a) p20. It must be noticed that most of the amino acid replacements detected within site A occurred at a single hypervariable triplet located at the C terminus of the G-H loop. Also, the redundancy of substitutions in independent evolutionary lineages suggests that the types of

replacements tolerated in this loop are limited (see Discussion). VP1 substitutions A-140 → V, T-148 → A or K, and T-149 → K were also seen as a result of the passage of the virus in the presence of anti-FMDV polyclonal antibodies directed to site A (Borrego *et al.*, 1993). Most of these substitutions affect site A recognition by at least one of the MAbs tested (Díez *et al.*, 1989, 1990; Martínez *et al.*, 1991a; data not shown). Though unlikely, anti-FMDV antibodies might be present in the foetal bovine serum used to culture cells, leading to immune selection of variants. To exclude this possibility, four independent virus populations were obtained by 20 to 30 serial passages of C-S8c1 or its mutant HR in the absence of bovine serum. Replacements at positions 148 and 150 of VP1 within site A were observed in one population and in biological clones derived from it. Accordingly, reactivity of these viruses with MAb 4G3 was abolished (data not shown).

Within the C terminus of VP1, which includes antigenic site C, only one replacement (H-197 → R), in population C-S8c1p100a, became dominant (Fig. 3). This replacement had no effect on the reactivity of the two MAbs (7JA1 and 6EE2 described by Mateu *et al.*, 1994) which have been used to define this site in type C viruses (data not shown). Within the segments which define antigenic site D of type C viruses (Lea *et al.*, 1994; Mateu *et al.*, 1994), and partially equivalent sites in other FMDV serotypes (Kitson *et al.*, 1990; Thomas *et al.*, 1988a; Saiz *et al.*, 1991), no substitutions were found in any population (Fig. 3). Replacement V-63 → I in VP3, located not far from the area assigned to site D, was found in population H2p20. However, this residue is not exposed on the capsid surface. In spite of no genetic variation being observed within

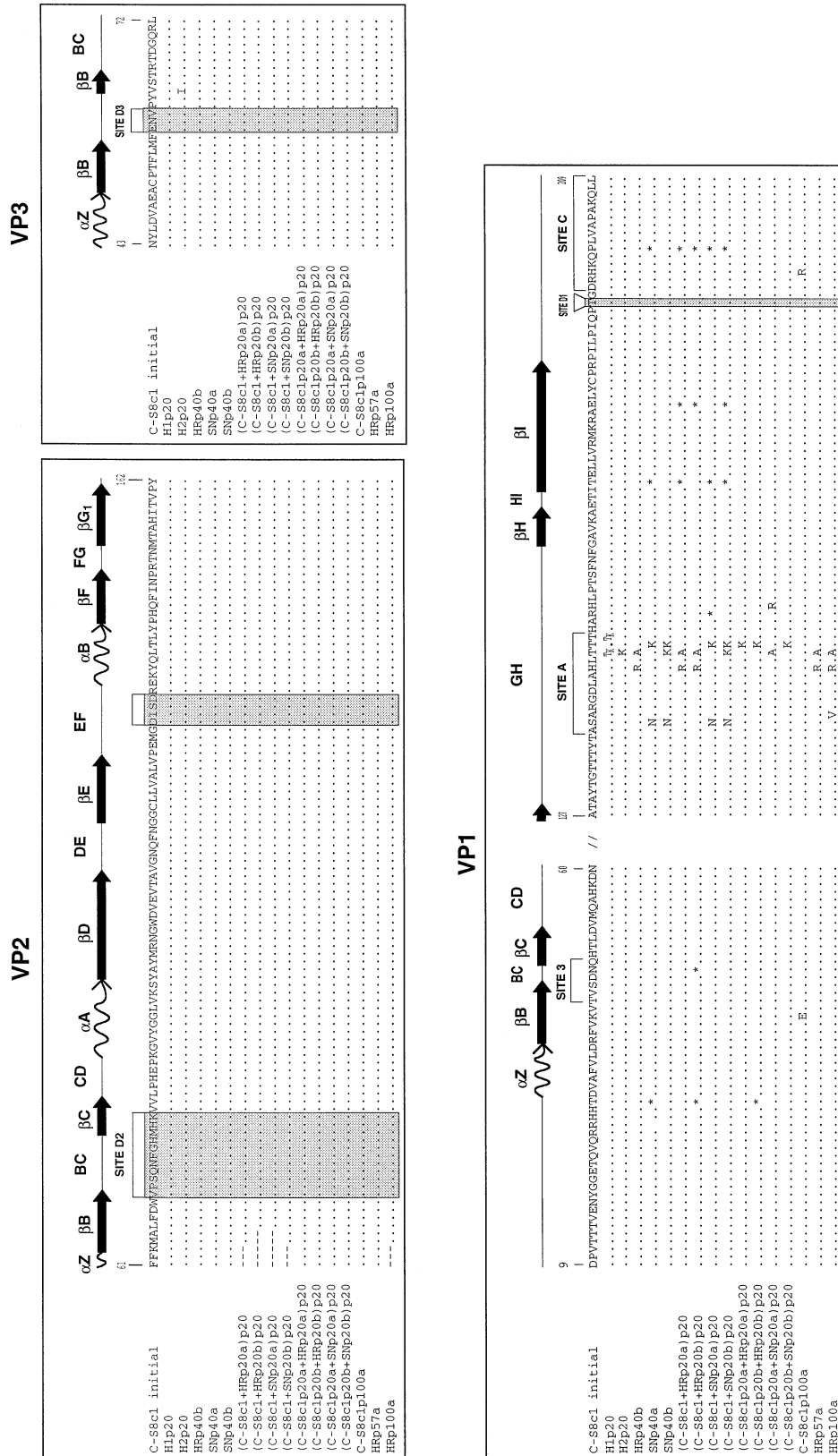


Fig. 3. Alignment of amino acid sequences of segments of VP1, VP2 and VP3 of FMDV populations passed in absence of immune selection. The amino acid sequences are those deduced from the nucleotide sequences shown in Fig. 2. The single-letter amino acid code is used. Only amino acid changes relative to FMDV C-S8c1 sequence are indicated. Dots mark sequence identity with C-S8c1. Two amino acids at a given position indicate a mixture of two nucleotides at the corresponding position (compare Fig. 2). Asterisks denote undefined amino acids due to sequence ambiguities. Dashes indicate sequences that have not been analysed. Each protein has been numbered independently, as indicated in Methods. On top of the C-S8c1 sequence the secondary structure motifs, determined from the X-ray crystallographic structure of C-S8c1 (Lea *et al.*, 1994), are shown. Symbols: thin line, loop and C termini; arrow, β -strand; wavy line, helix. Secondary structure assignment and nomenclature are according to Acharya *et al.* (1989). Boxes and lines above the sequences delimit antigenic sites A, C and D (plus site 3 of FMDV type O) as defined in Mateu *et al.* (1994).

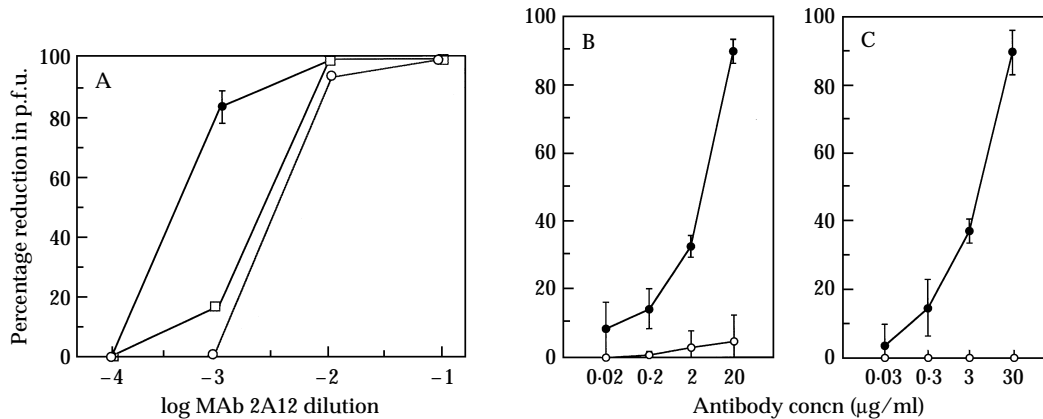


Fig. 4. Neutralization of virus populations FMDV C-S8c1 (●), C-S8c1p100a (○) and C-S8c1p61a (□). The origin of each virus population is described in Methods and depicted in Fig. 1. (A) Neutralization by site D MAb 2A12 (Lea *et al.*, 1994). (B) Neutralization by polyclonal antibodies from swine vaccinated with FMDV C-S8c1; the antibodies correspond to fraction NRF (depleted from site A-specific antibodies) of animal 13 described by Mateu *et al.* (1995). (C) Neutralization by NRF fraction of vaccinated animal 53 (Mateu *et al.*, 1995). Neutralization is expressed as percentage p.f.u. reduction (Mateu *et al.*, 1987). Values are the average of three independent experiments, each carried out in duplicate. Bars indicate standard deviations. Details of the neutralization assay are given in Mateu *et al.* (1987).

the segments so far found to be involved in site D, there was still the possibility that some of the capsid residues replaced in the passaged virus populations analysed could affect the reactivity of the virus with the MAbs used to define site D (Lea *et al.*, 1994). Thus, nine site D-specific MAbs were employed to test their ability to neutralize these populations as compared with the parental viruses. Population C-S8c1p100a was somewhat less efficiently neutralized than the parent virus by MAb 2A12 (Fig. 4A). However, no substantial differences were found for any of the other virus populations and MAbs tested (data not shown).

In addition to replacements found within or near recognized antigenic sites of type C viruses, replacement K-41 → E in VP1 was found in the population C-S8c1p100a. This replacement is located close to the B–C loop of VP1, within a variable region which so far has been found antigenic only for type O viruses (site 3 in Kitson *et al.*, 1990). Because no tested MAbs reactive with type C viruses recognize this region, a possible effect of that substitution on virus antigenicity could not be explored by using the available MAbs.

Antigenic variation in the absence of immune selection in epitopes unrelated to site A

Although no significant antigenic variation was observed in sites C and D as defined with MAbs, other epitopes not yet identified could be more tolerant to variation in the absence of immune selection. Thus, a fraction of anti-FMDV C-S8c1 swine polyclonal antibodies directed against epitopes which are unidentified but located outside antigenic site A (Mateu *et al.*, 1995; see Methods) was used to compare the neutralization of population C-S8c1p100a and the parental virus C-S8c1 (Fig. 1). The results (Fig. 4B, C) show that C-S8c1p100a is resistant to neutralization by non-site A antibodies derived from two

different animals. Comparison of amino acid sequences of the capsid protein of C-S8c1p100a with C-S8c1 revealed that within the entire capsid only four amino acid substitutions had occurred: A25 → V and Q218 → K in VP3, and K41 → E and H197 → R in VP1. Except for the residue at position 25 of VP3 which is in the interior of the capsid, one or a combination of the other three substituted residues could be antigenically critical for recognition by the polyclonal antibodies tested. The results suggest that very few amino acid changes fixed in the FMDV capsid in the absence of immune selection may be responsible for drastic antigenic variation in epitopes located outside the G–H loop of VP1.

Discussion

It is frequently assumed that only immune selection drives the fixation of amino acid replacements within antigenic sites. However, the occurrence of antigenic variation in the absence of immune pressure has been documented for FMDV and other viruses. The random change model predicts that antigenic variation can occur not only by antibody selection, but also because of the random replacement of any antigenically critical amino acid (Domingo *et al.*, 1993). The results reported here show that under the controlled environmental conditions provided by growth of the virus in cell culture, three antigenic sites of FMDV serotype C differ substantially in the degree of tolerance to amino acid substitutions in the absence of immune selection. This was previously suggested as a model to explain the different extents of antigenic diversification undergone by picornaviruses (Mateu, 1995). Different degrees of tolerance to accept amino acid replacements – because of varying degrees of stringency of structural and functional constraints – may modulate the variation at different antigenic sites of the same virus. At least for field isolates of FMDV type C, severe

restrictions to genetic variation of antigenically critical residues within site A are not limited to the conserved RGD motif but extend to a highly conserved neighbouring helical stretch, probably because of its involvement in receptor binding (Mateu, 1995; Verdaguer *et al.*, 1995; Mateu *et al.*, 1996). Accordingly, most substitutions fixed either in the absence or presence of MAbs or polyclonal antibodies occurred at two hypervariable stretches (VP1 positions 138 to 140 and 148 to 150), even though most of these residues were not as critically involved in site A antigenicity as other, more conserved site A residues (Díez *et al.*, 1989; Mateu *et al.*, 1990; Martínez *et al.*, 1991*a, b*; Borrego *et al.*, 1993; Domingo *et al.*, 1993; Verdaguer *et al.*, 1995; Mateu, 1995; this report). Other results suggested even more severe restrictions to variation within site D (Lea *et al.*, 1994): selection of escape mutants of C-S8c1 with MAbs directed to site D repeatedly led to some substitutions at only five residues within the epitopes. Examination of the C-S8c1 capsid structure revealed that most surface-exposed invariant residues within the area of site D were involved in interactions with other capsid residues, suggesting the existence of structural restrictions to genetic variation within this site (Lea *et al.*, 1994; Mateu *et al.*, 1994; Mateu, 1995). As described in Results, the only variation we detected in site D of FMDV passaged in the absence of immune pressure was a somewhat lower neutralizability by MAb 2A12 of population C-S8c1p100a relative to C-S8c1. Replacement of residue 197 of VP1, which is located not far from the site D area, as defined by our panel of MAbs, might explain this diminished neutralization. No other variation was observed within site D in any population tested in this study.

To show that optimization of some functional activity of antigenic site A – like its ability to bind to cells in culture – need not be necessarily invoked to explain antigenic variation in the absence of immune selection, variation of epitopes unrelated to site A should be detectable. This proved to be the case when polyclonal antibodies directed to epitopes not located within the G–H loop of VP1 were used as probes to study population C-S8c1p100a. Though the corresponding epitopes have not been located yet, all the available evidence indicates they are unrelated to sites A and C (Mateu *et al.*, 1995). The three substitutions of surface-exposed residues of C-S8c1p100a relative to its parent virus occurred outside the segments defined as antigenic in FMDV type C. However, the epitopes recognized by dominant antibodies within the two populations tested might be related to site 3 found in type O viruses (the B–C loop of VP1; Kitson *et al.*, 1990) or even to site D. This is under investigation, but it clearly points to the incompleteness of our current panel of MAbs for defining the antigenic structure of FMDV type C. It should be noticed also that antigenic sites are operationally defined; use of additional MAbs may result in further overlapping of epitopes which may blur the distinction between sites. Also, the immunodominance of the epitopes recognized by the polyclonal antibodies tested in this work in animal populations is unknown

(Mateu, 1995). In spite of these uncertainties, the results indicate that antigenic variation of FMDV without antibody pressure is not a feature exclusive to site A. The random occurrence of tolerated replacements may be a mechanism of antigenic diversification of highly variable RNA viruses operating in addition to direct positive selection by the immune system.

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