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Antigenic Heterogeneity of a Foot-and-Mouth Disease Virus Serotype in the Field Is Mediated by Very Limited Sequence Variation at Several Antigenic Sites

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Antigenic variation in a major discontinuous site (site D) of foot-and-mouth disease virus (FMDV) of serotype C has been evaluated with neutralizing monoclonal antibodies. Isolates representing the major evolutionary sublines previously defined for serotype C were compared. Extensive variation, comparable to that of continuous epitopes within the hypervariable immunodominant site A (the VP1 G-H loop), was found. The amino acid sequences of the complete capsids of three antigenically highly divergent FMDVs (C_1 Haute Loire-Fr/69, C_5 Argentina/69, and C_3 Argentina/85) have been determined and compared with the corresponding sequences previously determined for seven additional type C viruses. Differences in antigenicity are due to a very limited number of substitutions of surface amino acids accessible to antibodies and located within antigenic sites previously identified on FMDV. A significant number of residues at these positions were also replaced in monoclonal antibody escape mutants. Depending on the variants compared, replacements within site A or at site D, or at both sites, contributed significantly to their antigenic differences. Examples of divergence mediated by a few amino acid replacements were found among FMDVs of Europe and South America. The results suggest that within a serotype of FMDV, antigenically highly divergent viruses can arise in the field by very limited sequence variation at exposed key residues of each of several antigenic sites.

Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family, is the etiological agent of an economically important disease of cattle and other cloven-hoofed animals (for reviews, see references 24 and 47). Inactivated-virus vaccines are in use in many countries. However, a vaccine origin for some outbreaks in Europe has been proposed (8), and a nonvaccination policy has been compulsory in the European Community since 1992. These and various other reasons make a synthetic FMD vaccine an attractive possibility (13). Experimental peptide or recombinant vaccines (10, 15, 20) have been based mainly on a major continuous antigenic site of FMDV (site A) (10, 49, 56) located within a mobile surface loop (the G-H loop) of capsid protein VP1 (2, 32, 43). However, site A is genetically and antigenically hypervariable, even within each of the seven serotypes identified (36, 38; for a review, see reference 22). Single substitutions at critical residues can dramatically alter the antigenic specificity of this site (14, 28, 36, 39, 52). Thus, point mutations resulting in such critical substitutions in circulating FMDV may occasionally render a vaccine based on site A alone ineffective. Results on the extent of cross-protection of such vaccines are still very limited and inconclusive (21, 27).

Alternative approaches to synthetic anti-FMD vaccines include cocktails of synthetic peptides representing variant sequences and/or empty capsids (31, 51), which may present the immune system with several independent continuous epitopes as well as assembled epitopes. Discontinuous epitopes in antigenic sites independent of site A have been identified in FMDV serotypes A (7, 53, 57), O (29), and C (23), mainly by sequencing monoclonal antibody (MAb) escape mutants. These antigenic sites are as follows. (i) One site is the carboxy-terminal segment of VP1 (about 15 residues), termed site C. In FMDV of serotype O, site C forms with site A a discontinuous antigenic domain, termed site 1 (29, 44, 45, 58). However, sites A and C appear as independent continuous sites in serotype C (23, 39, 40). (ii) Another site is a discontinuous antigenic domain located close to the capsid threefold axis. In FMDV type C, this domain appears as a major antigenic site (site D) (23), which may be considered equivalent to sites 2 and 4 defined for type O (29). This antigenic region includes the B-C loop of VP2 (residues 70 to 80) and the B-B "knob" of VP3 (residues 58 to 61) in serotypes C, O, and A. A part of the VP1 carboxy terminus (around residue 193) is also involved in site D of FMDV type C. The VP2 E-F loop (residues 131 to 134) is involved in site 2 defined for FMDV type O. (iii) There is another independent antigenic site located close to the capsid fivefold axis. In FMDV type O, this site (designated site 3) involves the B-C loop of VP1 (residues 43 to 48) (29). Some substitutions found in escape mutants within or around site 3 appear to modify the antigenicity of site A (43). A site equivalent to site 3 occurs at least in FMDV type A; this site includes the H-I loop of VP1 (around residue 170) (7, 57). Finally, some epitopes found in

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FMDV type A, not assigned to any of the above-mentioned sites, involve other loops of VP3 (7, 57). The relative importance of the four antigenic sites identified in FMDV in determining recognition by the immune systems of target animals is not known. Also, the extent of conservation of FMDV epitopes in antigenic sites other than site A during the natural evolution of the virus has not been established. By comparing capsid sequences of representative isolates of each subtype and evolutionary subline of serotype C identified in Europe and South America and comparing reactivities of viruses with MAbs, we provide evidence that a very limited sequence variation in several or all four independent antigenic sites identified in FMDV constitutes the basis of its antigenic diversity in the field. This has several implications for the design of synthetic vaccines against FMDV.

MATERIALS AND METHODS

Viruses. Field isolates representative of serotype C in Europe and South America and belonging to the five defined subtypes (C_1 to C_5) were provided by the World Reference Laboratory (Pirbright, United Kingdom), Rhone-Merieux (Lyon, France), Laboratorios Sobrino (Olot, Girona, Spain), and DICOM-SENASA (Buenos Aires, Argentina) and are listed in Table 1. Virions were concentrated by sedimentation through a sucrose cushion and for RNA extraction were further purified by sucrose density gradient centrifugation as described previously (18).

MAbs. The neutralizing MAbs used have been previously characterized, and their epitopes have been located in different antigenic sites of FMDV (23, 38–41).

EID assays. The enzyme immunodot (EID) assays were carried out essentially as previously described (38). Briefly, the amount of virus was determined by polyacrylamide gel electrophoresis and by reactivity with nonneutralizing MAb 7BH11 (which recognizes a conserved epitope; see Fig. 1), and an equal amount of each FMDV variant was applied to nitrocellulose sheets which were then saturated with 5% bovine serum albumin in phosphate-buffered saline. The sheets were incubated with three 10-fold serial dilutions of supernatants of hybridoma cultures, and the amount of MAb bound was determined enzymatically with goat anti-mouse immunoglobulin G-peroxidase (diluted 1:2,000; Bio-Rad) and 4-chloro-1-naphthol as a substrate. Dots were quantified with a Joyce-Loebl densitometer.

Nucleotide sequencing. The nucleotide sequences of the genomic region P1 encoding the capsid proteins of C_1 Haute Loire-Fr/69 and C_3 Argentina/85c1 (in a virus designation, c1 denotes that the virus was plaque purified) were determined with viral RNA essentially as described previously (26). Purified RNAs of C_5 Argentina/69 and C_1 Serra Daró-Sp/81 (C-S15) were reverse transcribed to DNAs, and these were amplified by PCR and sequenced with the Femtomol sequencing kit (Promega). The region corresponding to the amino-terminal part of VP4 of C-S15 was sequenced to complete the P1 sequence previously determined for this virus (54).

Determination of potential accessibility to antibodies of capsid residues. The atomic coordinates of FMDV C_1 Santa Pau-Sp/70c1 (C-S8c1) have been determined at 3.5-Å (0.35-nm) resolution (1, 16, 23). The accessible surface area of C-S8c1 was calculated by the method of Lee and Richards (30). The accessible area is defined as the continuous path of the center of a spherical probe as it is rolled over the protein surface. For multimeric proteins, the calculation of accessible area may be made for the unique portion but must be performed in the context of the whole assembly. For FMDV, we achieve this by making the calculation for an icosahedral subunit in the context of a fringe of residues from symmetry-related units. To ensure that we did not consider the internal surface of the capsid, residues were considered to be potentially external only if they were at a capsid radius greater than 125 Å (12.5 nm). Residues are considered to be probe accessible if their accessibility in the C-S8c1 structure exceeds 10% of the amino acid accessibility in a Gly-X-Gly extended tripetide. Novotny et al. (42) have previously used probes with a radius of 10 Å (1 nm) to mimic the interactions between immunoglobulins and protein antigens. We considered a smaller probe size (2 Å [0.2 nm]) appropriate since we wished to detect not only residues that may themselves make contact with an antibody but also residues that, when mutated, may become contact residues.

TABLE 1. FMDV field isolates of serotype C used in the present study[a]

Evol. subline[b]	Virus designation[c]	Isolation date (month/year)	Place of isolation
I	C GC-Ger/26 (CGC)	Circa 1926	Germany
II	C_1 Loupoigne-Bel/53	5/1953	Loupoigne, Belgium
	C_1 Vosges-Fr/60	1960	Vosges, France
	C_1 Turup-Den/61	1961	Turup, Denmark
	C_1 Noville-Sw/65	1965	Noville, Switzerland
	C_1 Corneze-Fr/66	1966	Corneze, France
	C_1 Haute Loire-Fr/69	1969	Haute Loire, France
	C_1 Santa Pau-Sp/70 (C-S8)	1970	Santa Pau, Girona, Spain
	C_1 Bouches du Rhone-Fr/76	1976	Bouches du Rhone, France
	C_1 Serra Daró-Sp/81 (C-S15)	1/1981	Serra de Daró, Girona, Spain
	C_1 Monte Pulciano-It/88	6/1988	Monte Pulciano, Toscana, Italy
	C_1 Carpi-It/88	1988	Carpi, Italy
	C_1 Brescia-It/89	3/1989	Brescia, Lombardia, Italy
III	C_2 Pando-Ur/44	1944–1945	Pando, Uruguay
	C_2 997-UK/53	1953	United Kingdom
	C_4 Tierra del Fuego-Arg/66	12/1966	Rio Grande, Tierra del Fuego, Argentina
IV	C_3 Resende-Br/55	5/1955	Resende, Rio de Janeiro, Brazil
	C_5 Argentina/69	6/1969	Pehuajó, Buenos Aires, Argentina
	C_3 Argentina/83	3/1983	Daireaux, Buenos Aires, Argentina
	C_3 Argentina/84	9/1984	General Roca, Córdoba, Argentina
V	C_3 Santa Fe-Arg/75	1/1975	San Carlos, Santa Fe, Argentina
	C_3 Argentina/85	12/1984	Marcos Juárez, Córdoba, Argentina

[a] The FMDV type C isolates used (36, 38, 39, 41, 50) and the evolutionary (evol.) sublines (35) have been previously described.

[b] Sublines IV and V correspond, respectively, to groups V and VI of reference 35.

[c] In the text, C_1 after the virus designation denotes that the virus was plaque purified.

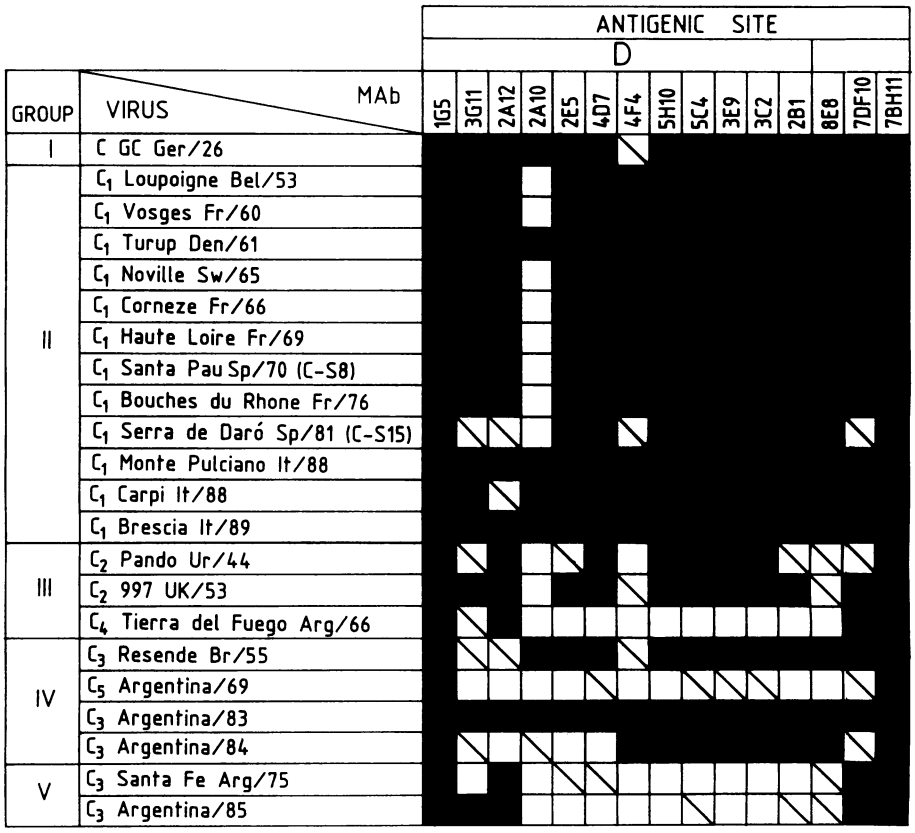

FIG. 1. Reactivities in EID assays of field isolates of FMDV type C with neutralizing MAbs directed to discontinuous epitopes in antigenic site D. The elements of the capsid involved in site D are described in the introduction. The discontinuous epitopes recognized by neutralizing MAbs 8E8 and 7DF10 have not been located. Nonneutralizing MAb 7BH11 recognizes a conserved epitope and is included as a positive control. MAbs 7DF10 and 7BH11 were elicited against FMDV C_3 Indaial-Br/71, MAb 8E8 was elicited against C_1 Vosges-Fr/60, and all other MAbs were elicited against C_1 Brescia-It/64. Virus groups refer to the different evolutionary sublines of FMDV type C as defined in Table 1. The places and dates of isolation of the viruses are given in Table 1. Reactivities are classified as positive (signal above 40% that of the control virus at a nonsaturating concentration of the MAb [■]), weak (signal between 10 and 40% [◹]), and negative (signal below 10% [□]).

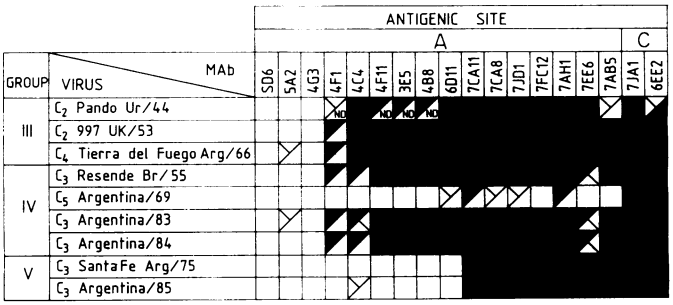

FIG. 2. Reactivities in EID and enzyme-linked immunoelectrotransfer blot (EITB) assays of field isolates of FMDV type C with neutralizing MAbs directed to continuous epitopes in antigenic sites A and C. For each virus-MAb combination, data from EID and EITB assays are indicated in the upper left and the lower right halves of the corresponding frames, respectively. Virus groups and reactivities are as indicated in the legend to Fig. 1. MAbs SD6 and 4G3 were elicited against FMDV C-S8c1; MAbs 5A2, 4F1, 4C4, 4F11, 3E5, 4B8, and 6D11 were elicited against C_1 Brescia-It/64; MAb 6EE2 was elicited against A_{12} 119; and all other MAbs were elicited against C_3 Indaial-Br/71. Data from EITB assays have been previously published (36, 38, 39) and have been adapted and included here for comparison with the EID data. EITB assays were not done (ND) for some combinations. The discrepancies (13% of the cases) did not affect the conclusions of our study. Some of the discrepancies (e.g., weak reactivity in the EID assay and no reactivity in the EITB assay) may be explained by the higher sensitivity of the former assay (38).

RESULTS

Similar antigenic variation in a complex discontinuous site D and the major continuous site A of FMDV serotype C. Our previous studies of the molecular basis of the antigenic variability of FMDV serotype C focused on continuous epitopes within the immunodominant antigenic site A (36–40). We have now extended the analysis to discontinuous epitopes located in a second major antigenic site (site D) which involves residues of VP1, VP2, and VP3 (see the introduction). EID assays were carried out with 22 field isolates representative of the five serological subtypes and the five evolutionary sublines identified for FMDV of serotype C in Europe and South America (4, 35, 36, 38, 46, 50) (Table 1) and with 12 neutralizing MAbs elicited against FMDV subtype C_1. These MAbs are directed to different discontinuous epitopes within site D (23). Two other neutralizing MAbs, which recognize discontinuous epitopes not yet located, were also tested. The results (Fig. 1) revealed 13 different patterns of MAb reactivity. All but one site D epitope were conserved in most European viruses (evolutionary subline II) (Table 1). In contrast, a significant degree of antigenic variation in site D was observed among isolates from Argentina and Uruguay (sublines III, IV, and V), even among viruses of the same subline. The epitope at site D recognized by MAb 1G5 was the only one strictly conserved among the viruses analyzed.

To compare the extent of variation of site D with that of site A, we tested the reactivities of the South American isolates listed in Fig. 1 with a panel of 18 neutralizing MAbs, directed to at least 14 different continuous epitopes in site A and 2 epitopes in site C, by using the EID assay (Fig. 2). Antigenic heterogeneity detected with site A MAbs was similar to that detected with site D MAbs. For example, the proportion of positive reactivities obtained with viruses from South America and with MAbs elicited with subtype C_1 viruses and directed to site A epitopes was 43%, whereas the same calculation with MAbs directed to site D epitopes was 49% (compare Fig. 1 and 2; for this comparison, we excluded the MAbs raised against C_3 FMDVs since these MAbs were all directed to site A). Thus, the degree of antigenic variability was not substantially higher in site A than in site D, in spite of the former being composed of continuous epitopes in a flexible loop and the latter being composed of discontinuous epitopes at the interface of three proteins.

Limited sequence variation in each of several antigenic sites of FMDV can give rise to antigenically highly divergent viruses. We have previously reported that the serologically different FMDVs C_3 Resende-Br/55 and C_3 Argentina/84 were indistinguishable in the amino acid sequence of antigenic site A but differed at other antigenic sites (25). Another significant example of the influence of capsid regions other than site A in antigenic diversification of FMDV is provided by a comparison of FMDVs C_1 Haute Loire-Fr/69 and C_5 Argentina/69. Both viruses include a critical replacement within antigenic site A (A-145 → V and H-146 → Q of VP1, respectively) which greatly alters the antigenic specificity of this site (36, 39; also, see the reactivities of C_5 Argentina/69 with site A MAbs in Fig. 2). C_5 Argentina/69 is so serologically highly divergent in relation to all other type C viruses (5) that it has been classified as a new subtype (46). However, C_1 Haute Loire-Fr/69 is not serologically different from other European C_1 isolates. This apparent discrepancy between serological results and MAb results was solved by comparing the reactivities of type C viruses with MAbs directed to epitopes within antigenic site D (Fig. 1). Site D of C_5 Argentina/69, but not that of C_1 Haute

FIG. 3. Amino acid sequences of the four capsid proteins of type C FMDVs. The amino acid sequences of FMDV C_1 Haute Loire-Fr/69, C_5 Argentina/69, and C_3 Argentina/85c1 were deduced from the nucleotide sequences and aligned with those previously published for seven other type C viruses (25, 35, 54). The single-letter amino acid code is used. Numbering is according to that for FMDV C-S8c1. Additions occurring in some viruses are not assigned numbers. For viruses isolated in Europe or South America, only the amino acid changes from the corresponding consensus sequence (EUR or SAM, respectively) are indicated. Amino acids which differ between the two consensus sequences are underlined. Symbols for primary structure: dot in the sequence, deletion; asterisk, ambiguity in the sequencing gels. Secondary structure assignments are also indicated. Since the hydrogen bonding pattern for C-S8c1 is the same as for O_1 BFS (23), the O_1 BFS assignments (2) have been used. Symbols for secondary structure: thin lines, N and C termini and loops; arrows, β-strands; wavy lines, α-helices. The structural elements are designated as in reference 2. The loops are named after the two β-strands they connect (e.g., the G-H loop connects β-strands G and H). On top of the first sequence (consensus EUR), bars indicate surface amino acids accessible to a surface probe with a 2-Å (0.2-nm) radius; they were deduced from the FMDV C-S8c1 atomic coordinates (see Materials and Methods). Amino acids 133 to 152 of VP1 of FMDV C-S8c1 were disordered (1, 23); all the available evidence (see also Results) suggests that they are surface-exposed residues. Boxes delimit surface amino acids separated in the sequence by a maximum of one internal amino acid. Triangles indicate the position—after sequence alignment—in which the relevant substitutions in MAb escape mutants of FMDV serotypes C (23, 28, 39, 40), O (6, 29, 44, 48, 55, 58), and A (7, 11, 53, 57) occurred. Boxes within boxes delimit amino acids replaced in escape mutants that are separated in the sequence by a maximum of two nonsubstituted amino acids. We consider these segments the antigenic regions identified in FMDV. Some of them constitute assembled antigenic sites (see the introduction). Note that this is a conservative approach, since only one or a few replacements have been selected with each MAb, while about 15 to 22 residues of a protein antigen may come into contact with antibodies in immune complexes (17; see also Discussion). Thus, the epitopes identified could also involve other amino acids spatially close to the antigenic segments defined here. Differences among FMDV serotypes regarding the structural elements involved in antigenic sites could be explained, at least in part, by their incomplete characterization due to the limited panel of MAbs available; compare, for example, the diverse antigenic domains identified by several groups in the same FMDV serotype (type A) (7, 11, 53, 57).

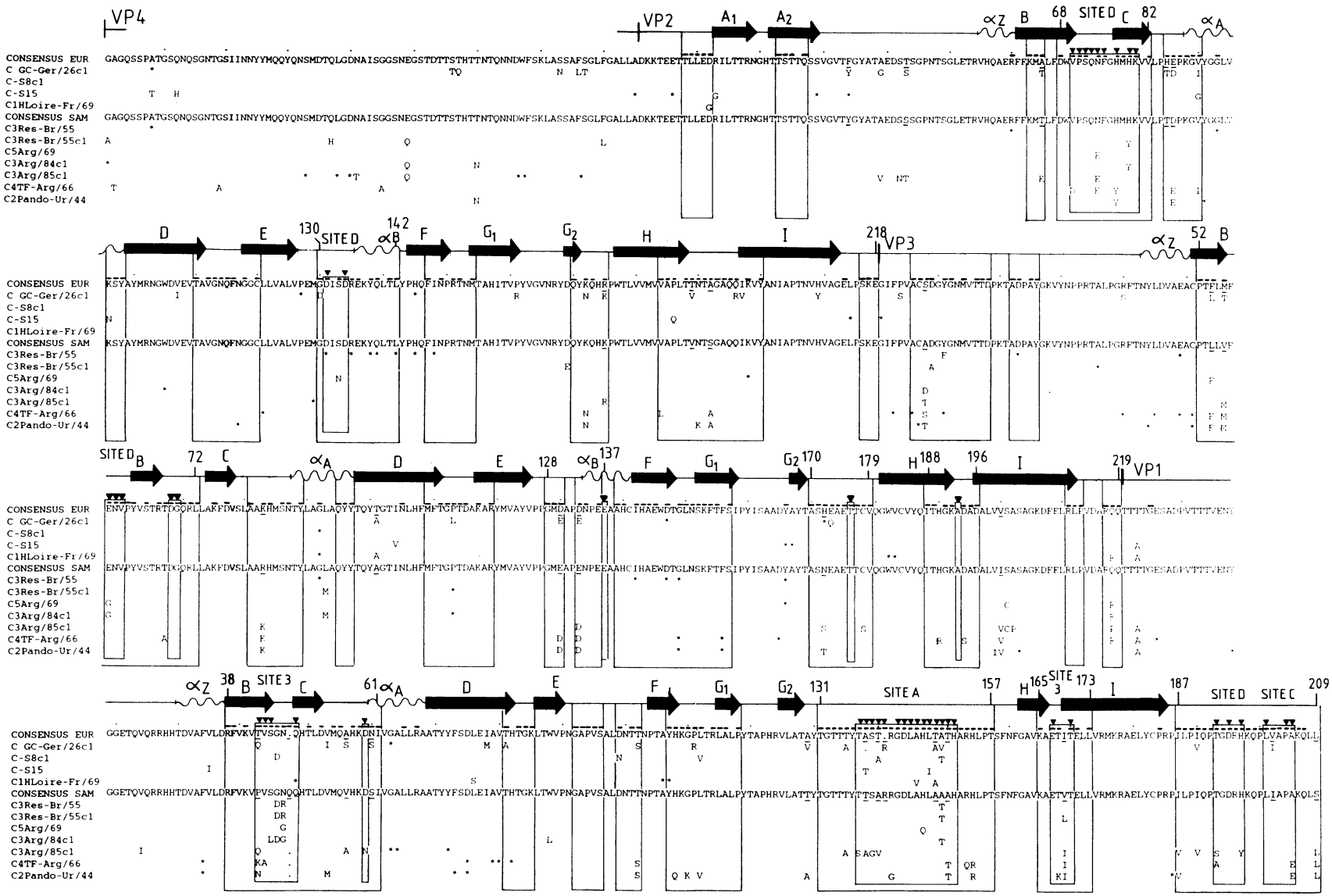
VP4
VP2
A1
A2
B
68
SITE D
C
82
CONSENSUS EUR
C GC-Ger/26c1
C-S8c1
C-S15
C1HLoire-Fr/69
CONSENSUS SAM
C3Res-Br/55
C3Res-Br/55c1
C5Arg/69
C3Arg/84c1
C3Arg/85c1
C4TF-Arg/66
C2Pando-Ur/44
D
E
130
SITE D
142
F
G1
G2
H
I
218
VP3
52
B
SITE D
72
C
D
E
128
137
F
G1
G2
170
179
188
196
I
219
VP1
38
SITE 3
61
D
E
F
G1
G2
131
SITE A
157
165
SITE 3
173
I
187
SITE D
SITE C
209

TABLE 2. Capsid surface amino acids which differ between FMDV C_1 Haute Loire-Fr/69, C-S15, and C-S8c1 of evolutionary subline II

Antigenic site[a]	Secondary structure[b]	Position	Residue[c]			Remark(s)[d]
			C_1 H. Loire	C-S15	C-S8c1	
A	VP1 G-H	138	A	**T**	A	MAR C
		140	T	T	**A**	MAR A
		145	**V**	A	A	MAR O, A
		147	L	**I**	L	MAR A
		148	**A**	T	T	MAR C, A
		149	A	A	**T**	MAR C, A
3	VP1 B-C	46	G	G	**D**	
None	VP2 N terminus	12	**G**	D	D	
	VP2 αA	90	V	**G**	V	
		96	K	**N**	K	
	VP2 βH	186	P	**Q**	P	Close to site D
	VP3 C terminus	218	**R**	Q	Q	
	VP1 E-F	99	D	D	**N**	Close to site 3
	VP1 F-G	112	L	L	**V**	Close to site 3

[a] "None" indicates that the differences were found outside the capsid segments identified as antigenic (see Fig. 3). Antigenic site 3 has been identified in FMDV serotypes O (29) and A (57) but not in serotype C (see the introduction).
[b] Secondary structure assignments are as in Fig. 3.
[c] Residues (single-letter amino acid code) which differ from a consensus European type C sequence are shown in boldface. C_1 H. Loire, C_1 Haute Loire-Fr/69.
[d] MAR C, O, and A indicate that the amino acid in the equivalent position, after sequence alignment, has been replaced in MAb escape mutants of serotypes C, O, and A, respectively. Other positions replaced in escape mutants are indicated in Fig. 3.

Loire-Fr/69, showed a substantially divergent antigenic specificity when compared with other genetically related viruses.

We have explored the molecular basis of the antigenic differences among these and other type C viruses analyzed with MAbs by comparing the complete capsid sequences of the viruses. The nucleotide sequences of the RNAs encoding the capsid proteins of C_1 Haute Loire-Fr/69, C_5 Argentina/69, and C_3 Argentina/85c1 were determined, and the deduced amino acid sequences (Fig. 3) were compared with those previously determined for seven additional field isolates of FMDV type C (25, 35). The alignment includes derived consensus sequences for European and American viruses (Fig. 3). By using the atomic coordinates obtained by X-ray crystallography of FMDV C-S8c1 (1, 16, 23), the amino acids of the capsid which

TABLE 3. Capsid surface amino acids which differ among FMDV C_5 Argentina/69, C_3 Resende-Br/55, and C_3 Argentina/84c1 of evolutionary subline IV

Antigenic site[a]	Secondary structure[b]	Position	Residue[c]				Remark(s)[d]
			C_5A	C_3R	C_3Rc	$C_3$84c	
A	VP1 G-H	146	**Q**	H	H	H	MAR C, A
		149	A	**T**	**T**	**T**	MAR C, A
D	VP2 B-C	74	**E**	N	N	N	MAR C
	VP2 βC	79	H	H	**Y**	**Y**	MAR C, A
	VP2 E-F	133	**N**	S	S	S	
	VP3 βB	54	**F**	L	L	L	
	VP3 B-B	58	**G**	E	E	**G**	MAR C, O, A
3	VP1 B-C	45	S	S	S	**L**	MAR O
		46	G	**D**	**D**	**D**	
		47	**G**	**R**	**R**	**G**	
None	VP3 N terminus	8	A	A	A	**D**	5 × axis
		9	D	D	**A**	D	5 × axis
		11	Y	**F**	Y	Y	5 × axis
	VP3 C terminus	218	**R**	Q	Q	**R**	

[a] "None" indicates that the differences were found outside the capsid segments defined as antigenic (see Fig. 3). In spite of not belonging to any of these segments, amino acid 54 is included within site D because it is located at the center of the structural domain corresponding to site D (23). Antigenic site 3 has been identified in FMDV serotypes O (29) and A (57) but not in serotype C (see the introduction).
[b] Secondary structure assignments are as in Fig. 3.
[c] Residues different from a consensus American type C sequence are shown in boldface. C_5A, C_5 Argentina/69; C_3R, C_3 Resende-Br/55; C_3Rc, C_3 Resende-Br/55c1; $C_3$84c, C_3 Argentina/84c1.
[d] MAR C, O, and A indicate that the amino acid in the equivalent position, after sequence alignment, has been replaced in MAb escape mutants of serotypes C, O, and A, respectively. Other positions replaced in escape mutants are indicated in Fig. 3. Residues 8, 9, and 11 of VP3 are located within a hole at the capsid fivefold axis (5 × axis) and are probably not accessible to antibodies.

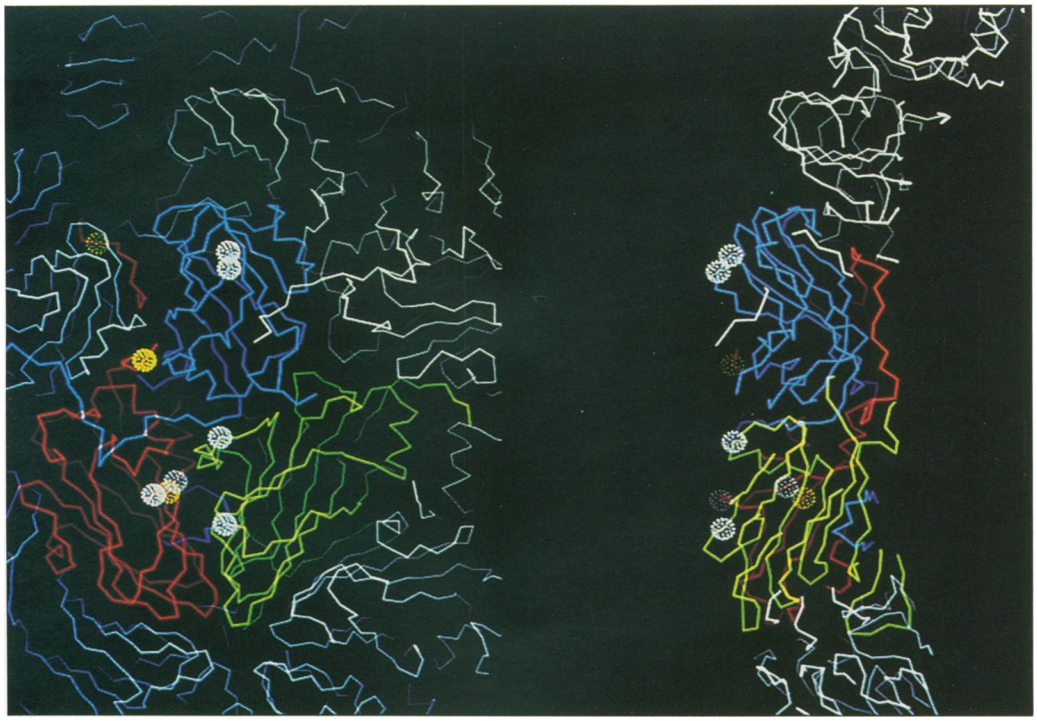

FIG. 4. Amino acid differences between the capsids of FMDV C_5 Argentina/69 and C_3 Resende-Br/55. Two orthogonal views of the biological promoter of FMDV C-S8c1 (1, 23) are shown as Cα traces. The image at the right is an edge view with the solvent-exposed surface on the left side and the virion interior on the right side. The proteins are colored conventionally (VP1, blue; VP2, green; VP3, red). VP4 is entirely internal and is omitted here for clarity. The surrounding portions of the capsid are shown as white Cα traces. Residues which differ between C_5 Argentina/69 and C_3 Resende-Br/55 are highlighted as dotted spheres. The two spheres colored white in the upper part of each view and the four spheres colored white in the lower part correspond to differences occurring in antigenic sites 3 and D, respectively; those colored yellow correspond to differences occurring outside identified antigenic segments. The surface-oriented G-H loop of VP1 (antigenic site A, containing two differences between these viruses) is disordered in C-S8c1 and is not shown (see text). A comparison of C_5 Argentina/69 and C_3 Resende-Br/55 based on the C-S8c1 structure is justified by the high degree of sequence identity among all three viruses and the overall structural similarity of FMDVs, even those of different serotypes (1, 2, 16, 23).

are accessible to a surface probe with a radius of 2 Å (0.2 nm) have been determined (Fig. 3). We have considered the possibility that such residues may also be accessible to antibodies and could potentially contribute to antigenicity (42). In addition to the accessible residues determined, all the available structural evidence (2, 32, 43) as well as the immunological and genetic evidence (Fig. 3) suggests that VP1 residues 133 to 152 within the G-H loop (which correspond to antigenic site A and were found disordered in FMDV C-S8c1) are also exposed on the virion surface. Considering that the antigenically most divergent type C viruses from Europe and South America currently available—some of which are thought to have escaped immunity conferred by the vaccines in use (4, 9, 34, 46)—are included in the alignment of Fig. 3, there is a remarkably limited number of amino acid differences among them. The amino acid sequence identity between any two viruses is higher than 92%, and the identity between the consensus sequences for European and American viruses is 96%. The capsids of the three subline II viruses compared (C_1 Haute Loire-Fr/69, C-S8c1, and C-S15) differ only in 9 to 10 surface amino acids (Table 2), many of them located within site A. Outside of site A, FMDV C_1 Haute Loire-Fr/69 differs from C-S8c1 and C-S15 in five surface residues. However, in most of these positions, C_1 Haute Loire-Fr/69 shows the same amino acids found in all other type C viruses sequenced. Such sequence conservation in antigenic segments other than site A agrees with MAb reactivity results and serological data for C_1 Haute Loire-Fr/69 (see above). In contrast, the viruses of subline IV from South America that have been compared (C_3 Resende-Br/55, C_5 Argentina/69, and C_3 Argentina/84c1), though genetically very similar to each other, are serologically remarkably divergent (Table 3) (25). Only 11 C_3 Resende-Br/55 capsid residues, 10 of them potentially accessible to antibodies, are replaced in C_5 Argentina/69 (Fig. 3). Figure 4 shows that most positions which differ between these two viruses are at the tips of surface loops and are highly accessible to antibodies. All but two of the exposed residues (F-11 → Y and Q-218 → R in VP3) are located within known antigenic sites of FMDV (Table 3). The critical residue replaced at VP1 position 146 within site A, which is responsible for the extreme antigenic divergence of C_5 Argentina/69 detected with site A MAbs (36), has been found repeatedly replaced in MAb escape mutants (28, 39, 40). Interestingly, some of the replacements within site D of C_5 Argentina/69 (amino acids 74 of VP2 and 58 of VP3; Table 3) also correspond to positions often found replaced in escape mutants isolated for FMDV of serotypes C (23), O (29), and A (53, 57). These results agree with observations made previously (23, 35) suggesting limited tolerance to amino acid replacements by the FMDV capsid and show that the extreme serological divergence of C_5 Argentina/69 within serotype C can be accounted for by very limited amino acid sequence variation within each of the antigenic sites of FMDV defined with MAbs.

Further evidence that each antigenic site identified in

TABLE 4. Site D amino acids[a] which differ between serologically divergent field viruses of serotype C

Secondary structure[b]	Position	Consensus residue[c]	Substituted residue[d]								Remark(s)[e]
			C_1	C_3R	C_3Rc	C_5A	C_384c	C_385c	C_3I	C_4TF	C_2P	
VP2 B-C	70	V								D		MAR O
	74	N				E		E	S	K		MAR C
	77	H								Y	Y	MAR O
VP2 βC	79	H			Y		Y					MAR C, A
VP2 E-F	133	S				N						
	134	D							E			MAR O
VP3 B-B	58	E				G	G					MAR C, O, A
VP1 C terminus	193	T						S		A		MAR C
	197	H						Y				MAR A

[a] A total of 23 amino acids lie within the peptide segments considered to be involved in site D (Fig. 3).
[b] Secondary structure assignments are as in Fig. 3.
[c] The consensus amino acids are derived from the 11 sequences shown in Fig. 3.
[d] Only variable positions within site D and the residues different from the type C consensus amino acids at these positions are shown. No ambiguities in the sequencing gels appeared within site D of any virus sequenced. C_1, C-S8c1, C-S15, and C_1 Haute Loire-Fr/69 showed the same sequence within site D, with no substitutions relative to the consensus C_3R, as did C_3 Resende-Br/55; C_3Rc, C_3 Resende-Br/55c1; C_5A, C_5 Argentina/69; C_384c, C_3 Argentina/84c1; C_385c, C_3 Argentina/85c1; C_3I, C_3 Indaial-Br/71-88, an isolate genetically and antigenically related to C_3 Argentina/85 (35, 38, 50); C_4TF, C_4 Tierra del Fuego-Arg/66; C_2P, C_2 Pando-Ur/44.
[e] MAR C, O, and A indicate that the amino acid in the equivalent position, after sequence alignment, has been replaced in MAb escape mutants of serotypes C, O, and A, respectively. Only the four positions indicated here as MAR C and residue 72 of VP2 were replaced in the 16 escape mutants of FMDV type C isolated with MAbs against six different epitopes within site D (23).

FMDV may contribute to different extents to the antigenic diversification of this virus is provided by comparison of C_3 Argentina/84c1 (25), C_3 Argentina/85c1, C_4 Tierra del Fuego-Arg/66, C_2 Pando-Ur/44, and C_1 viruses (which are representatives of different subtypes and evolutionary sublines [35]) with C_3 Resende-Br/55. The majority of differences in residues located at the capsid surface are within or very close to the four previously identified antigenic sites of FMDV (Fig. 3). Analysis with MAbs (Fig. 1 and 2) suggests that differences in sites A and D contributed to the antigenic divergence of C_3 Argentina/85c1 relative to C_3 Resende-Br/55. However, only variation at site D appears to have contributed significantly to the divergence of C_4 Tierra del Fuego-Arg/66. As observed with C_5 Argentina/69 (see above), amino acids 74 and 79 of VP2, 58 of VP3, and 193 of VP1—which probably contribute to the antigenic variation of site D observed among C_1 viruses, C_3 Resende-Br/55, C_3 Argentina/84c1, C_3 Argentina/85c1, C_4 Tierra del Fuego-Arg/66, and other type C viruses—are also replaced in escape mutants of serotype C; some of these residues are also replaced in escape mutants of other serotypes (Table 4). A substitution at position 74 of VP2 was found in most serological variants tested; replacements at this position may have a particularly drastic effect on the antigenic specificity of site D (compare Fig. 1 and Table 4). On the other hand, the serological divergence of C_1 viruses relative to C_3 Resende-Br/55 may be explained by differences in site A and perhaps in site 3 but not in site D (compare site D sequences and antigenicities of these viruses in Table 4 and Fig. 1). Finally, in spite of its being classified as a different subtype, C_2 Pando-Ur/44 reacted in a manner very similar to that of C_3 Resende-Br/55 with MAbs directed to sites A and D. However, several of the exposed amino acids in the B-C, F-G, and H-I loops of VP1 (within or around antigenic site 3) of C_2 Pando-Ur/44 were unique to this virus (Fig. 3) and may contribute to its serological divergence. This possibility cannot be tested at present since no MAbs directed to site 3 of FMDV type C are available.

DISCUSSION

Lack of correlation between genetic distance and antigenic divergence. The above-described results as well as the previous distinction between antigenically noncritical and critical residues within site A of FMDV—which can lead to antigenic diversification either through a gradual increase in antigenic distance mediated by accumulation of amino acid replacements or through drastic changes in antigenic specificity caused by single replacements, respectively (36)—point to a frequent lack of correlation within a FMDV serotype between genetic distance and antigenic divergence. FMDV C GC-Ger/26 (C GC), an early isolate which belongs to an evolutionary subline now probably extinct (35), is genetically the most divergent of the type C viruses which have been sequenced (Fig. 3). The capsid sequence of C GC differs from the consensus sequence for subtype C_1 in 46 amino acids, 40 of them outside antigenic segments as depicted in Fig. 3. In spite of such a considerable number of replacements, the overall reactivity of C GC with MAbs directed to three independent antigenic sites identified in type C is not substantially different from the reactivities of other C_1 viruses (see reference 38 for site A and Fig. 1 for site D), and C GC has served as a reference strain for the subtype C_1 viruses (46). The situation with C GC is in clear contrast to other examples described in Results concerning genetically closely related isolates of the same evolutionary subline which were nevertheless antigenically dissimilar. C_5 Argentina/69 is an extreme case of an antigenically highly divergent isolate (Fig. 1 and 2) classified as a new serological subtype in spite of belonging to the same evolutionary subline as C_3 Resende-Br/55 and other subtype C_3 viruses.

Each identified antigenic site may play a role in antigenic diversification of FMDV in the field. The striking genetic similarity of C_5 Argentina/69 to C_3 Resende-Br/55, the vaccine strain used in Argentina at the time of the emergence of C_5 Argentina/69, may suggest that the former virus arose from the vaccine strain itself (8). Remarkably, most of the few surface

amino acids replaced were located within the three major antigenic sites which have been identified in FMDV (Fig. 4). Thus, it seems that the emergence of new, highly divergent FMDV subtypes can occur through a mechanism of antigenic drift involving a very limited number of critical amino acid substitutions in the several identified antigenic sites. Significant variation in several antigenic sites relative to C_3 Resende-Br/55 was also detected in C_3 Argentina/85, a serological variant which replaced C_3 Resende-Br/55 as the type C vaccine strain in Argentina. Other serological variants (e.g., C_3 Argentina/84, C_4 Tierra del Fuego-Arg/66, and C_1 Haute Loire-Fr/69) differed in some but not all sites (see Results). In general, most of the capsid surface residues which differed among serological variants of FMDV type C were located within antigenic sites. These observations suggest that every antigenic site identified in FMDV is relevant to the recognition of the virus by the immune systems of target animals. Each antigenic site has participated, to a different extent depending on the isolates considered, in antigenic variation in nature.

Structural constraints, immune selection, and tolerance for change in the antigenic diversification of FMDV. The continuous antigenic site A located within the VP1 G-H loop has great flexibility, and so the structure of this region has proved elusive (1, 2, 23, 43). However, in FMDV O_1 BFS, reduction of a disulfide bridge between a residue at the start of the VP1 G-H loop and VP2 allows the loop to adopt a stable conformation (32), although even in this structure the loop remains among the most mobile surface-exposed regions. Thus, it may be less subject to structural constraints than other more rigid antigenic domains. The sequence hypervariability of site A may therefore reflect not only the selection exerted by the immune system but also tolerance of replacements fixed by chance events independently of immune selection (12, 19, 33). In contrast to site A, the discontinuous antigenic site D—at an area of contact between VP1, VP2, and VP3—shows more limited numbers and types of amino acid replacements. In fact, while the G-H and B-C loops of VP1, corresponding to sites A and 3, respectively, have been long recognized as hypervariable between serotypes and within a serotype (Fig. 3), the loops involved in site D epitopes are less variable in sequence (Fig. 3), even when different serotypes are compared (results not shown). The positions replaced within site D in field isolates of type C often coincide with positions repeatedly replaced in MAb escape mutants (Table 4). Air et al. (3) have noted that the limited number of substitutions selected by MAbs against influenza virus epitopes suggests that the number of amino acid changes which can lead to selection of new, epidemic-causing influenza viruses by antigenic drift may be very limited. Those authors suggest that the substituted amino acids may be those critically involved in interactions with the selecting antibody. However, our work (23) on mapping MAb escape mutations onto the structure of FMDV C-S8c1 suggests that structural constraints at antigenic site D may also significantly limit the mutations seen. The analysis of field variants of FMDV presented in this paper supports this view and suggests that, as for influenza virus, the variety of mutations leading to antigenic drift in FMDV may be highly restricted. However, the very few replacements found within site D of FMDV type C, and their combinations, must suffice to explain the high level of antigenic variation detected in this site. Thus, a limited number of tolerated replacements within epitopes does not necessarily preclude substantial antigenic variation.

In conclusion, antigenic variation of FMDV in the field is not determined solely by amino acid substitutions at the hypervariable site A within the G-H loop of VP1. Rather, each of the antigenic domains that have been identified and involve capsid proteins VP1, VP2, and VP3 may be subjected to considerable antigenic variation, as shown by the comparison of the most representative FMDV type C isolates from two continents. Such variation may involve the frequent selection of replacements at a few key positions on the viral capsid. Immune selection and tolerance to variation probably participate in defining the patterns of variation observed.

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