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# Identification of Neutralizing Antigenic Sites on VP1 and VP2 of Type A5 Foot-and-Mouth Disease Virus, Defined by Neutralization-Resistant Variants†

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**Five neutralizing monoclonal antibodies (nMAbs) obtained against type A5 Spain-86 foot-and-mouth disease virus were used to generate a series of neutralization-resistant variants. In vitro and in vivo assays showed that the variants were fully refractory to neutralization by the selecting nMAb. On the basis of cross-neutralization and binding assays, two neutralizing antigenic sites have been located on the virus surface; one, located near the C-terminus of VP1, displayed a linear epitope, and the second, located on VP2, displayed two conformational epitopes. Nucleotide sequencing of RNA of the parental and variant capsid protein-coding region P1 has placed the amino acid changes at position 198 of VP1 for the first site and at positions 72 and 79 of VP2 for the related epitopes in the second site. The relative importance of these two sites in the biological properties of foot-and-mouth disease virus is discussed.**

Foot-and-mouth disease virus (FMDV), the only aphthovirus in the *Picornaviridae* family, is the cause of a severe, although rarely fatal, disease of cloven-hoofed animals. Because of drastic decreases in production, FMD results in serious economic losses when outbreaks of the disease occur. Like other picornaviruses, FMDV possesses a single-stranded positive RNA molecule more than 8,000 nucleotides long (2) encapsidated in an icosahedral capsid made of 60 copies each of four proteins: VP1, VP2, VP3, and VP4 (35). The virus presents a great antigenic variability extensively characterized in tissue culture and in the field (18, 27, 38, 39).

Of the four structural proteins, only isolated VP1, or fragments of this protein, has been shown to elicit neutralizing antibodies in animals (3, 12, 24, 25). A main antigenic site on FMDV has been identified around amino acid residues 140 to 160 of VP1, with the extreme carboxy terminus (residues 200 to 213) of that protein probably contributing to the antigenicity (9, 17, 34, 42).

In areas in which FMD is endemic, the disease can be controlled by use of inactivated virus vaccines, but it has been shown that substitutions in a single immunologically relevant site on VP1 occur within viruses from a single episode of the disease (39) and that some recent outbreaks in Europe are closely related to vaccine strains (8, 14). Molecular cloning and expression and synthetic peptide approaches based on VP1 sequences have produced successful experimental vaccines, but the antigens have shown less immunogenicity than expected (for a review, see reference 13). The antigenic variability of FMDV is an acknowledged impediment for the use of vaccines produced from virus grown in vitro. Studies of the epitopic diversity and determination of the amino acid changes involved in this diversity are important to the design of new, more broadly protective vaccines.

A recent approach to this problem has consisted of the isolation of neutralization-resistant variants and the characterization of the changes implicated in the escape from neutralization. As a result, two to four antigenic sites have been located on different virus serotypes (4, 6, 12, 28, 31, 32, 41, 44, 47). Major antigenic sites have been located on VP1 (residues 140 to 160 and the C terminus), but neutralizing epitopes have also been partially located outside VP1 for serotype O, suggesting that this serotype includes regions of VP2 (4). In addition, on the basis of crystallographic data (1) and the use of neutralization-resistant variants, substitutions in VP2 around amino acid 80 have been located very close to the so-called VP1 loop (11). In the case of FMDV serotype A, at least one epitope outside VP1 has been described (6, 12, 44).

In this report, we describe the selection of nine neutralization-resistant variants of FMDV type A5 strain Spain-86 (SP-86) using five different monoclonal antibodies (MAbs), which have defined three different epitopes (36). These variants allowed us to define two antigenic sites on FMDV A5: one is located at the C terminus of VP1 and displays a sequential epitope, and the other is located on VP2 and displays two conformational epitopes.

## MATERIALS AND METHODS

**Viruses and cells.** FMDV type A5 strain SP-86 and MAb-resistant variants were grown in monolayers of baby hamster kidney (BHK-21) cells and purified as previously described (5, 46). A continuous bovine kidney cell line (LFBK) was used for neutralization assays and the generation of variants (43).

**MAbs.** We have previously described the production and characterization of the neutralizing MAbs used in this work

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† This article is dedicated to the memory of the late Donald O. Morgan of Plum Island Animal Disease Center, who was a driving force in the initiation and advancement of monoclonal antibody studies of FMDV, which have helped us to gain a better understanding of FMDV antigenic structure and of immunity to FMD.

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TABLE 1. MAbs used to select variants

| MAb | Isotype[a] | Specificity[b] | Neutralization[c] | Classification[d] |
|---|---|---|---|---|
| 19DG5.1.1.[e] | IgG1 | 140S, 12S | + | cII |
| 23FB4.1.1.[f] | IgG3 | 140S, 12S | + | cII |
| 23MF4.1.2.[f] | IgG2b | 140S, 12S | + | cII |
| 23OG2.1.1.[f] | IgG3 | 140S, 12S | + | cII |
| 23HA6.1.1.[f] | IgM | 140S, 12S, VP1 | + | cIII |

[a] Ig, Immunoglobulin.
[b] Binding specificity in RIA for intact virus (140S) and 12S subunits and in Western blot for VP1.
[c] Neutralization ability in vivo (mouse protective dose) and in vitro (PRN).
[d] Classification on the basis of binding and neutralization capacity (41).
[e] Ethylenimine-inactivated A5 SP-86 virus was used as the eliciting antigen.
[f] Infectious A5 SP-86 virus was used as the eliciting antigen.

(36), and their behavior is summarized in Table 1. The endings of the designations (e.g., .1.1. in 19DG5.1.1.) for the clones used in the tests indicated in Table 1 are omitted elsewhere for simplicity.

**MAb-resistant variants.** Variants refractory to neutralization by the different MAbs tested were isolated by a plaque reduction technique (29). Monolayers of LFBK cells were grown in 9-$cm^2$ petri dishes, washed with phosphate-buffered saline, and infected with 10-fold dilutions of A5 SP-86 virus which had been preincubated with an equal volume of either an appropriate dilution of MAb or medium containing fetal calf serum. After incubation at 37°C for 1 h to permit virus attachment, the plates were overlaid with 0.7% agar incorporating a 1/50 dilution of either the same MAb or fetal calf serum. The plates were maintained at 37°C with 5% $CO_2$ for 48 h. After that, plaques developing in the presence of the selecting MAb were picked and cloned again under the same conditions. Half of the material recovered after the second plaque purification was incubated with 20 volumes of the selecting MAb for 1 h at 37°C and expanded on LFBK cells in 25-$cm^2$ tissue culture flasks. Only variants fully refractory to the selecting MAb were used for further studies. Variants were designated with a number preceding the name of the MAb used to obtain them (e.g., 1-DG5 was selected by using MAb 19DG5).

**Neutralization assays.** The isolated variants were tested for neutralization with the MAbs by plaque reduction assays and mouse protection assays performed as previously described (40). The titers were determined as a 50% mouse protective dose by the Spearman-Karber method (19) and as a 70% plaque reduction neutralization (PRN) value by the logit-log transformation method (45).

**RIA.** The purified viruses were radiolabeled with $^{125}I$ under mild oxidizing conditions (Iodogen; Pierce) (21), and then 140S virus particles were purified as previously described (36). Radioimmunoassays (RIAs) were performed by the procedure of Stave et al. (40). Titration endpoints were determined at the 30% antigen-binding capacity by logit-log transformed linear regression analysis (45).

**Sequencing viral RNA.** RNA from purified virions grown in BHK-21 cells was extracted with phenol-chloroform-isoamyl alcohol as described by Grubman and Baxt (22) and stored in 70% ethanol. The RNA integrity was monitored by agarose gel electrophoresis. Sequencing reactions were performed by the primer extension-dideoxynucleotide chain termination method directly from virus RNA by previously described procedures (6, 37). A total of 16 oligonucleotide primers (17-mers or 18-mers) complementary to selected areas of the coding region for the four structural proteins (VP1, VP2, VP3, and VP4) were used. Oligonucleotide primers were synthesized and purified as previously described (6, 26). Sequence information was stored and analyzed by using software provided under license from the Genetics Computer Group of the University of Wisconsin (16).

TABLE 2. In vitro neutralization titers of different MAbs to A5 SP-86 virus and neutralization-resistant variants

| Virus[a] | 70% PRN titer of MAb[b]: | | | | |
|---|---|---|---|---|---|
| | 19DG5 | 23FB4 | 23MF4 | 23OG2 | 23HA6 |
| A5 SP-86 | 2.36 | 3.74 | 2.59 | 4.30 | 1.72 |
| 1-DG5 | 0.00 | 0.00 | 0.00 | 0.00 | 1.38 |
| 2-DG5 | 0.00 | 0.00 | 0.00 | 0.00 | 1.41 |
| 1-FB4 | 0.00 | 0.00 | 0.00 | 0.00 | 1.68 |
| 2-FB4 | 0.00 | 0.00 | 0.00 | 0.00 | 1.86 |
| 1-MF4 | 0.00 | 0.00 | 0.00 | 0.00 | 1.74 |
| 2-MF4 | 0.00 | 0.00 | 0.00 | 0.00 | 1.56 |
| 1-OG2 | 0.00 | 0.00 | 0.00 | 0.00 | 1.77 |
| 2-OG2 | 0.00 | 0.00 | 0.00 | 0.00 | 1.39 |
| 1-HA6 | 1.56 | 1.93 | 2.13 | 1.77 | 0.00 |

[a] 100 PFU of virus was incubated with the supernatant of hybridoma culture dilutions in the assay.
[b] Each value is the logarithm of the dilution of antibody needed to reduce the number of plaques by 70%, as described in Materials and Methods.

## RESULTS

**Isolation of neutralization escape variants.** Five neutralizing MAbs produced against A5 SP-86 virus have been used to generate a battery of neutralization escape variants. The MAbs have been previously described (36), and a brief characterization of their properties is given in Table 1. These classifications were established on the basis of RIAs, Western immunoblots, and in vivo and in vitro neutralization assays. MAb 23HA6 defined a sequential epitope which bound to the entire virus (140S) and 12S subunits in a liquid-phase RIA and to VP1 in a Western blot assay. A conformationally dependent epitope which did not bind to any denatured structural proteins in Western blot assays was defined by MAb 19DG5. Finally, MAbs 23FB4, 23MF4, and 23OG2 were found to recognize the same conformational epitope; none of them bound to FMDV structural proteins in Western blots.

By using these five neutralizing MAbs, nine neutralization escape variants were isolated for this study. Two variants were selected with each MAb, except for MAb 23HA6, for which only one variant was isolated. All of the variants presented a growth pattern similar to that of the wild-type FMDV (A5 SP-86) in LFBK cell monolayers.

**Characterization of neutralizing MAb-resistant variants.** All variants were characterized by their capacity to be neutralized by each of the five neutralizing MAbs in a PRN assay (Table 2). None of the variants obtained under the pressure of MAbs 19DG5, 23FB4, 23MF4, and 23OG2 was still neutralized by any of these MAbs, but all of them continued to be neutralized by MAb 23HA6 with a pattern similar to that of the wild type. On the other hand, variant 1-HA6 (obtained by using MAb 23HA6) was neutralized by all of the MAbs except its selecting antibody, 23HA6. None of the variants presented a partial neutralization pattern with any of the MAbs, being either completely neutralized (PRN $> 1.70$) or fully refractory to neutralization (PRN $< 0.30$).

TABLE 3. In vivo neutralization titers of different MAbs to A5 SP-86 virus and neutralization-resistant variants

| Virus[a] | 50% MPD titer to MAb[b] | | | | |
|---|---|---|---|---|---|
| | 19DG5 | 23FB4 | 23MF4 | 23OG2 | 23HA6 |
| A5 SP-86 | 2.57 | 2.57 | 1.87 | 2.66 | 2.40 |
| 1-DG5 | <0.30 | — | — | — | — |
| 2-DG5 | <0.30 | — | — | — | — |
| 1-FB4 | — | <0.30 | — | — | — |
| 2-FB4 | — | <0.30 | — | — | — |
| 1-MF4 | — | — | <0.30 | — | — |
| 2-MF4 | — | — | <0.30 | — | — |
| 1-OG2 | — | — | — | <0.30 | — |
| 2-OG2 | — | — | — | <0.30 | — |
| 1-HA6 | — | — | — | — | <0.30 |

[a] 100 50% lethal doses of virus were incubated with the supernatant of hybridoma culture dilutions in the assay.

[b] MPD, Mouse protective dose; —, not determined. Each value is the logarithm of the dilution of antibody needed to protect 50% of the mice tested, as described in Materials and Methods.

The variants were also tested by examining their neutralization behavior in vivo with suckling mice (Table 3). In this assay, variants were tested only against the corresponding MAbs. The results were the same as those obtained in vitro: the variants were no longer neutralized by their selecting MAbs.

The binding pattern of the variants against the five MAbs was established by liquid-phase RIA using purified $^{125}$I-labeled virus (Table 4). As expected from the neutralization tests, all MAbs except 23HA6 bound to variant 1-HA6, and conversely, all of the variants except 1-HA6 reacted with MAb 23HA6. The variants generated with MAbs 23FB4, 23MF4, and 23OG2 formed a group unreactive with all of the MAbs except 23HA6. Interestingly, all of the MAbs except the selecting MAb, 19DG5, showed some reactivity with variants 1-DG5 and 2-DG5. These results confirm that 19DG5 recognizes a conformational epitope different from the one recognized by the 23FB4-23MF4-23OG2 MAb group (36).

TABLE 4. Antibody-binding titers of different MAbs to A5 SP-86 virus and neutralization-resistant variants

| Virus[a] | 30% ABC titer to MAb[b]: | | | | |
|---|---|---|---|---|---|
| | 19DG5 | 23FB4 | 23MF4 | 23OG2 | 23HA6[c] |
| A5 SP-86 | 3.04 | 4.45 | 5.33 | 4.10 | 3.15 |
| 1-DG5 | 0.00 | 0.99 | 2.09 | 1.31 | 2.19 |
| 2-DG5 | 0.00 | 1.27 | 2.35 | 1.30 | 3.58 |
| 1-FB4 | 0.00 | 0.00 | 0.00 | 0.00 | 2.43 |
| 2-FB4 | 0.00 | 0.00 | 0.00 | 0.00 | 3.39 |
| 1-MF4 | 0.00 | 0.00 | 0.00 | 0.00 | 2.81 |
| 2-MF4 | 0.00 | 0.00 | 0.00 | 0.00 | 2.85 |
| 1-OG2 | 0.00 | 0.00 | 0.00 | 0.00 | 2.55 |
| 2-OG2 | 0.00 | 0.00 | 0.00 | 0.00 | 2.81 |
| 1-HA6 | 3.05 | 5.94 | 6.00 | 5.96 | 0.00 |

[a] Purified $^{125}$I-labeled viruses were used in the assay.

[b] 30% ABC, 30% antigen-binding capacity (endpoint of 30% binding). Each value is the logarithm of the dilution of antibody needed to bind 30% of the maximum number of counts bound to the MAb.

[c] *Staphylococcus aureus* was armed with rabbit anti-mouse immunoglobulin M prior to immunoprecipitation of the antigen-antibody complex (40).

**Nucleotide sequence of the capsid region of the RNA of FMDV A5 SP-86 and sequences of the neutralization escape variants.** The entire nucleotide sequence of the P1 coding region for the capsid proteins of FMDV A5 SP-86 was determined, and the amino acid sequence for VP1, VP2, VP3, and VP4 was derived from the nucleotide sequence (Fig. 1). A comparison of the degree of homology and number of substitutions at the protein-coding level among several type A FMDVs, type O1, and type C1 for the four proteins is presented in Table 5.

The complete nucleotide sequences of all nine neutralization-resistant variants through the P1 region were also determined. Only a single nucleotide change was detected for each variant (Table 6). Changes in coding assignments were identified and located in the corresponding virus capsid protein. The single variant, 1-HA6, selected by MAb 23HA6 had a nucleotide change at position 2117 (A to G) that corresponds to an amino acid substitution at position 198 of VP1 (Asp to Gly). The group of six variants selected by MAbs 23FB4, 23MF4, and 23OG2 all had G-to-A change in VP2 at position 421 that corresponds to amino acid 72 of VP2 (Asp to Asn). A related but different epitope recognized by MAb 19DG5 selected two variants, 1-DG5 and 2-DG5, each with a single nucleotide substitution at position 443 (A to G or A to U, respectively). This corresponded to an amino acid change at position 79 of VP2 (Glu to Gly or Glu to Val, respectively).

## DISCUSSION

Recently, by using MAb-resistant variants, between two and four antigenic sites have been located on the surface of FMDV (4, 6, 12, 28, 31, 32, 41, 44, 47). By characterizing a battery of MAbs by using neutralizing, binding, and inhibition assays, we previously reported the presence of up to seven neutralization-related epitopes on the surface of FMDV type A5 (36). In the current study, we used five of these MAbs (Table 1) to select nine neutralizing MAb-resistant variants, which allowed us to define the presence of two independent antigenic sites on the virus surface, one located at the C terminus of VP1 and the other located on VP2 and consisting of two related but distinguishable sites.

The five MAbs used in this study were defined as representing three epitopes, one sequential (MAb 23HA6) and two conformational (MAb 19DG5 and MAbs 23FB4, 23MF4, and 23OG2) (36). Two neutralization-resistant variants were isolated with each MAb except 23HA6, for which only one variant was selected. All of the variants were fully refractory to neutralization by the selecting MAb either in vitro or in vivo. When the in vitro neutralization behavior of these variants was analyzed with the five MAbs (Table 2), the variants were classified into two main groups. The first one, represented by variant 1-HA6, was resistant to neutralization only by the selecting MAb (23HA6) but was still fully neutralized by all the other MAbs. The second group contains the remaining variants, which were neutralized only by MAb 23HA6. In vivo neutralization tests (Table 3) were performed with only the selecting MAb, and the results were in accordance with the in vitro tests.

Antibody 19DG5 was previously classified in a different group from 23FB4, 23MF4, and 23OG2 (36) but appeared by neutralization tests to be in the same group. However, when binding assays were performed (Table 4), the DG5 variants could be placed into a subgroup because MAbs 23FB4,

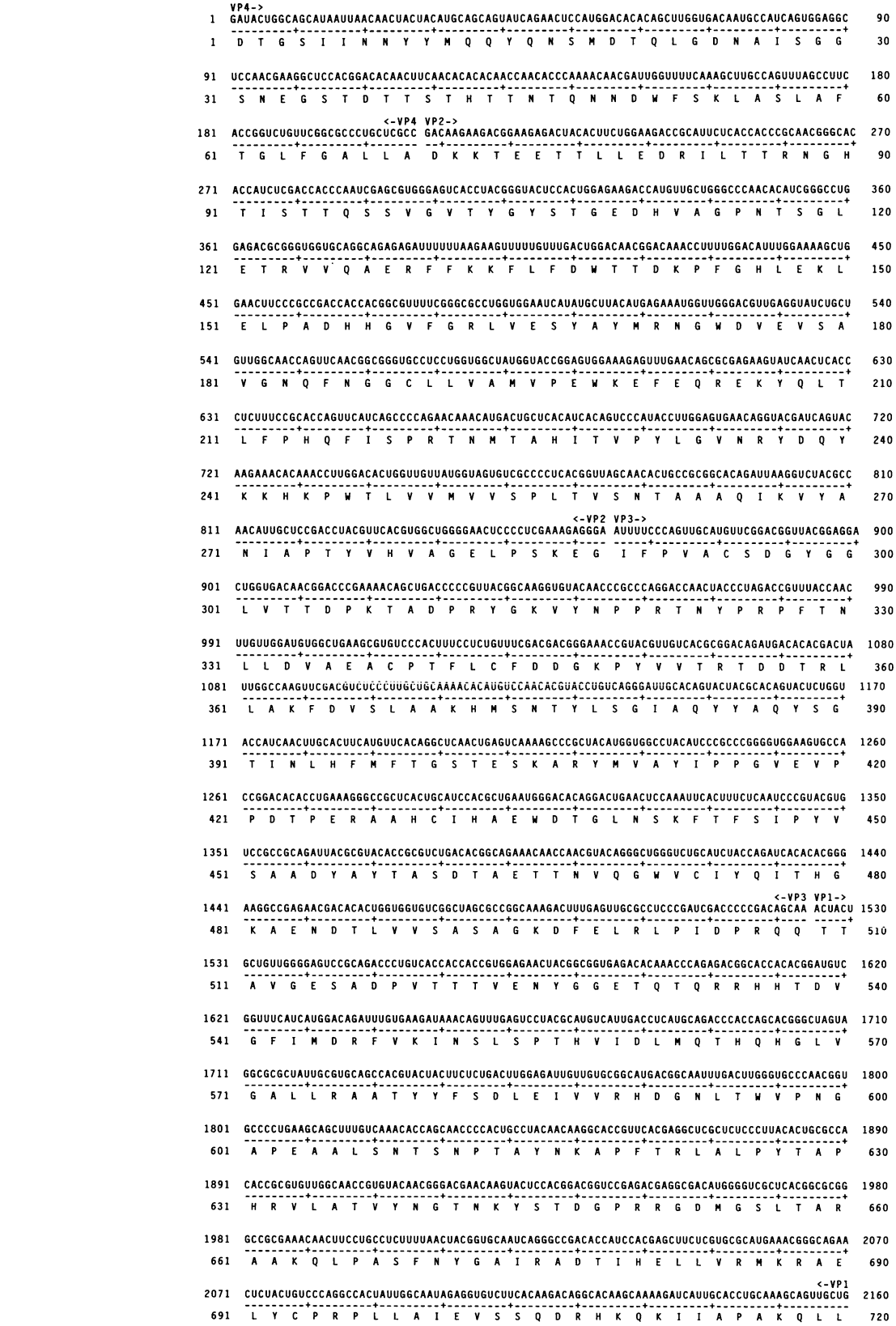


FIG. 1. Nucleotide and deduced amino acid sequence for the P1 region of FMDV type A5 SP-86. VP4 consists of nucleotides 1 to 207 (69 amino acid residues), VP2 consists of nucleotides 208 to 861 (218 amino acid residues), VP3 consists of nucleotides 862 to 1524 (221 amino acid residues), and VP1 consists of nucleotides 1525 to 2160 (212 amino acid residues).

TABLE 5. Amino acid homology and substitutions between FMDV type A5 SP-86 and other FMDV types in the P1 region

| Virus type[a] | % Identity (number of changes) compared with A5 SP-86[b] | | | |
|---|---|---|---|---|
| | VP1 | VP2 | VP3 | VP4 |
| A12 | 91.5 (18 + 1 i) | 95.4 (10) | 95.5 (10) | 97.1 (2) |
| A10 | 89.2 (23) | 94.0 (13) | 93.2 (15) | 94.2 (4) |
| A22 | 89.1 (23 + 1 i) | 89.9 (22) | 92.8 (16) | 95.7 (3) |
| O1 | 70.1 (63 + 1 i) | 81.2 (41) | 80.5 (43 + 1 d) | 95.7 (3) |
| C1 | 70.3 (63 + 3 d) | 77.1 (50) | 82.7 (38 + 2 d) | 95.7 (3) |

[a] References for viral RNA sequences used to determine amino acid sequences and relatedness: 6 and 33 (A12), 15 (A10), 11 (A22), 20 (01), and 7 (C1). Protein sequences were aligned by using the GAP program (16) to align each capsid protein to the equivalent A5 SP-86 protein.

[b] Numbers of substitutions which maximize homology are given in parentheses (i, insertion; d, deletion).

23MF4, and 23OG2 still bound the variants, even though they did not neutralize the viral infectivity. Similar results have been obtained with FMDV type O1 Brugge (41) and poliovirus type 1 (10). On the basis of these results, it has been confirmed, as suggested by others (23) and by us (36), that the presence of a neutralizing epitope on the virus surface does not imply that MAbs which bind to that epitope will always neutralize the viral infectivity. It has been suggested that this can be due either to shifts on the virus surface which may interfere with the epitope accessibility or perhaps to conformational changes in the epitope itself (6, 36).

The sequence of VP1-coding region of A5 SP-86 was previously reported by us (14), and now the first complete sequence of the P1 region of FMDV type A5 (VP4, VP2, VP3, and VP1) is presented (Fig. 1). When the coding assignments are compared with other FMDV type A sequences and with type O1 and C1 sequences available for the P1 region, the relative degree of variation for the four proteins can be seen (Table 5). As expected, the VP1 protein shows the greatest degree of variation within any serotype or strain. The variation from the type A5 SP-86 sequence is significantly higher for types O and C than for the other type A viruses (about 90% for the type A viruses but only about 70% for the others). The degree of homology is somewhat higher for VP2 and VP3 (90 to 95% for A types versus 77 to 83% outside type A). We suggest that there is less divergence outside of serotype A for VP2 and VP3. Capsid protein VP4 is highly conserved among the serotypes (94 to 97%), confirming that it contributes little to the antigenic diversity of the virus.

A comparison of the A5 SP-86 sequence with a consensus sequence derived from other FMDVs shows frequent coding changes in the VP1 sequence in the regions of residues 30 to 60, 130 to 170, and 190 to 213, consistent with the importance of this protein in the antigenic variability of FMDV (9, 17, 34, 42). Deletions and insertions of residues occur in the highly variable region between residues 130 and 150 and in the C terminus of VP1. These changes are likely to be tolerated by the virus because of the surface exposure and apparent disordered projection of the area between residues 130 and 160 out from the surface of the virion and the somewhat disordered nature of the C terminus (1). A consensus sequence can be made for VP2, VP3, and VP4 without the need for insertions and deletions of residues (except for one or two VP3 residues to accommodate the alignment of serotypes O and C with type A). A comparison of the VP2 sequence of A5 SP-86 with those of types A12 (33), A10 (44), and A22 (11) has revealed 22 amino acid changes between A5 and A22, 13 between A5 and A10, and 10 between A5 and A12 (Table 5). Most of these changes are located between amino acids 70 and 98, and there are also foci of changes between residues 131 and 134 and between residues 180 and 195. These areas may relate to sites on VP2 in close association with VP1 and surface features of the capsid (see below).

The sequencing of the P1 regions of the variant viruses supports the phenotypic characterization demonstrating the presence of two distinct antigenic sites on FMDV type A5 SP-86 (Table 6). The first site is located around residue 198 at the C terminus of VP1. This site was previously defined to be a linear epitope displayed on the virus, the 12S subunit, and denatured VP1 (36). Recent X-ray crystallographic data have shown the C terminus of VP1 crossing the twofold protomer interface and lying in the vicinity of the variable region (amino acids 140 to 160) of VP1 (1). In another study with type A12, two sets of escape variants containing a single VP1 change at residue 209 were found with two related MAbs (6). However, some variants isolated with one of the MAbs also had changes in the variable region of VP1 at residue 152. All variants were resistant to both MAbs whether there was a single substitution at the C terminus or there were dual substitutions including the variable region (amino acids 140 to 160) of VP1. This indicated that a single change at 209 could provide full resistance to neutralization but that the variable region probably contributed to the binding site as well. At this time, a single substitution at residue 198 near the C terminus of type A5 SP-86 VP1 and previous characterization of this site as a linear epitope found on denatured VP1 suggest a simple antigenic site.

The second site is located on VP2 and presents two related conformational epitopes, one recognized by MAb 19DG5 and the other by MAbs 23FB4, 23MF4, and 23OG2 (36). Sequencing the P1 region of these eight variants showed a single nucleotide and corresponding VP2 amino acid-coding change for each variant. The DG5 variants had an amino acid change at position 79 of VP2 (Glu to Gly or Glu to Val), and the six variants obtained with the other three MAbs all had the same base change corresponding to amino acid 72 of VP2 (Asp to Asn) (Table 6). Variants in which changes on VP2 (44) and VP3 (6, 12) in serotype A and VP2 in FMDV type O (4) contribute to the so-called immunodomminant site of VP1 have been described. However, a single variant for which a

TABLE 6. Nucleotide and amino acid changes in neutralization-resistant variants of FMDV A5 SP-86

| MAb | Variant | VP region | Nucleotide change (position) | Amino acid change |
|---|---|---|---|---|
| 19DG5 | 1-DG5 | VP2 | A (443) → G | Glu-79 → Gly |
| | 1-DG5 | VP2 | A (443) → U | Glu-79 → Val |
| 23FB4 | 1-FB4 | VP2 | G (421) → A | Asp-72 → Asn |
| | 2-FB4 | VP2 | G (421) → A | Asp-72 → Asn |
| 23HA6 | 1-HA6 | VP1 | A (2117) → G | Asp-198 → Gly |
| 23MF4 | 1-MF4 | VP2 | G (421) → A | Asp-72 → Asn |
| | 2-MF4 | VP2 | G (421) → A | Asp-72 → Asn |
| 23OG2 | 1-OG2 | VP2 | G (421) → A | Asp-72 → Asn |
| | 2-OG2 | VP2 | G (421) → A | Asp-72 → Asn |

solitary VP2 change occurred was found in only one study (residue 80 [44]). In the current work, only single VP2 substitutions were found for the second antigenic site. The area of VP2 consisting of residues 70 to 100 is highly variable, and amino acid 82 was predicted to lie adjacent to amino acid 135 of VP1, very near the major antigenic loop of VP1 (1, 12). However, for type A5 SP-86, the surface exposure and proximity of VP2 residues 72 and 79 to VP1 are not known. The contribution of VP1 sequences to the type A5 conformational epitopes found on VP2 cannot be predicted here. In continuing studies of the behavior of some of the variants after serial passage in vitro (21a), the VP1 linear epitope defined by variant 1-HA6 was more stable than the VP2 conformational variant epitope (represented by the OG2 variants). The linear epitope-containing virus persisted longer in serial passage, and in coinfection experiments the 1-OG2 conformational variant was rapidly outgrown by both variant 1-HA6 and wild-type FMDV. These data suggest that this position in the conformational epitope plays a critical role in the biological properties and survival of this type A5 variant. Also, a host range-selected variant of type A22 had changes nearby at residues 82 and 88 (and also a change at 207) of VP2, the only P1 residue changes from the parent strain (11).

Generally, the assignment of important antigenic sites on FMDV has centered on the sequences between amino acids 140 and 160 and the C terminus of VP1 (9, 32, 42). However, it has been established that VP2 and VP3 amino acid side chains are exposed on the virion surface (30), and areas of these two proteins have more recently been described as contributing to important antigenic sites (6, 12, 31, 44, 47). It was not possible to find evidence of immunodominance when variants representing different sites were assayed in neutralization tests with a bovine convalescent serum (47), and it has also been found that MAbs which bound to VP3 sequences competed with polyclonal sera from immune host animals as well as MAbs that bound to the VP1 region consisting of amino acids 140 to 160 (44). Two closely related viruses of type A22 showed different behavior in cell attachment, aggregation, and host range properties and had no differences in coding assignments of VP1 but did have three amino acid substitutions in VP2 (11, 12). This report adds support for the combined importance of VP1, VP2, and VP3 in the antigenic makeup of the virus.

In this report, we have described the presence of two antigenic sites on FMDV type A5 SP-86, one which appears to be located on VP2. The data presented support the ideas that epitopes located outside the hypervariable region of VP1 (amino acids 140 to 160) are very important in viral neutralization and that any approach to design new, more broadly reactive synthetic vaccines should include the study of epitopes located not only on VP1 but also on VP2 and VP3, since residues of these two proteins clearly contribute to conformational epitopes implicated in viral neutralization.

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## ADDENDUM

After the submission of this article, an article in which similar results were described for type O1 FMDV, showing residue changes in the VP2 protein at position 72 with a frequent change of Ser (analogous to Asp at position 72 in type A5) to Asn as seen in our study, was published (23a).

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