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Antigenic Sites on Foot-and-Mouth Disease Virus Type A10

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A set of monoclonal antibodies was used to isolate nonneutralizable foot-and-mouth disease virus variants, and the RNAs of the variants were sequenced. Cross-neutralization studies and mapping of the amino acid changes indicated two major antigenic sites. The first site was trypsin sensitive and included the VP1 140 to 160 sequence. The second site was trypsin insensitive and included mainly VP3 residues. Two minor sites were located near VP1 169 and on the C terminus of VP1. Comparison with poliovirus type 1 and human rhinovirus 14 showed a similarity in the immunogenicity of comparable sites on the viruses.

The VP1-coding regions of foot-and-mouth disease virus (FMDV) strains isolated during 18 recent European outbreaks and of vaccine strains were determined by cDNA sequencing (3). Comparison of the sequences revealed that most of the outbreak viruses were closely related to the vaccine strains (3). Clearly, the use of synthetic peptides as immunogen would reduce the hazards associated with large-scale production of infectious virus. Much work has been directed toward elucidating amino acid sequences that can be used to prepare peptide vaccines that will, in turn, induce neutralizing and protecting antibodies.

Two findings in particular have led to the focus of attention on VP1 and fragments derived from it. First, VP1 was found to be the only capsid protein capable of eliciting neutralizing antibodies against FMDV (1, 11). Second, trypsin treatment of FMDV resulted in cleavage of only VP1 and a drastic reduction of infectivity and immunizing activity (27). Strohmaier et al. have shown that peptides representing the two trypsin-sensitive areas of the virus (VP1 138 to 154 and 200 to 212) were able to induce neutralizing antibodies (25). Inoculation of a synthetic peptide encompassing VP1 141–160 of FMDV O1 protected guinea pigs against challenge with virus (4); the 200–212 peptide was less immunogenic (4, 25).

After a single vaccination, a chemically synthesized peptide containing VP1 residues 141 to 158 and 200 to 213 protected two of nine cattle against challenge with virulent O1 virus (7).

A systematic search for antigenic peptides on VP1 was performed by synthesizing all hexapeptides of VP1 and measuring the binding activity to rabbit antisera (8) and to a panel of monoclonal antibodies (MAbs) raised against FMDV type A10 (14, 17). Two reactive sets of peptides were identified in the trypsin-sensitive sites of VP1, and antisera raised against these peptides neutralized the virus (17).

Another approach to identifying antigenic sites is to isolate variants of FMDV that escape neutralization by a MAb, to sequence their genome, and to locate the mutations responsible for the escape from neutralization. This procedure has been followed for several picornaviruses. Using a panel of MAbs against poliovirus types 1, 2, and 3, researchers established an immunodominant locus on VP1 (around amino acid 100) for types 2 and 3 and several distinct antigenic sites for poliovirus type 1 (18). Similar conclusions were reached for poliovirus type 1 by Wimmer and coworkers (6, 28). Sherry et al. identified four antigenic sites on human rhinovirus 14 (HRV14) (22, 23). When sequences of poliovirus and HRV14 were aligned (A. Palmenberg, personal communication), the antigenic sites on both viruses were in similar positions. Examination of the three-dimensional structures of both viruses (10, 20) revealed that each antigenic site forms a protrusion from the viral surface. Since the three-dimensional structures of these two viruses, as well as that of mengovirus (13), are very similar, the antigenic sites of FMDV are probably located on similar protrusions. This supposition disagrees, however, with serological results for FMDV, for which only VP1 sequences were thought to elicit neutralizing antibodies (see above). Nonetheless, nonneutralizable variants of FMDV type A10-Holland were found to be charge altered in capsid proteins VP1, VP2, and VP3 (2; S. Barteling and A. Thomas, unpublished results). In this study the RNAs of these variants were sequenced to localize the epitopes of the MAbs and the resulting antigenic map of FMDV A10 is compared with those of HRV14 and poliovirus types 1, 2, and 3.

MATERIALS AND METHODS

MAbs. MAbs against FMDV A10, inactivated with acetyl ethyleneimine, were isolated as described for type O1 (16) and serologically characterized (2, 14, 17). In this report the MAb number is preceded by a code number indicating the group of MAbs that bind to the same antigenic site. For example, MAb1.11 means that MAb11 belongs to group 1. Definitions of an epitope and an antigenic site are from Wimmer et al. (28).

Virus. FMDV A10 (A-Holland) was isolated during an outbreak in cattle in the Netherlands in 1942 and was passaged several times in cattle. The virus was cloned three times in BHK cells and grown in BHK monolayer and suspension cells. A10-61 was isolated in 1961 from an A10-Holland derivative and was used by Boothroyd et al. to determine the nucleotide sequence (5).

Nonneutralizable variants were isolated as described previously (2). Briefly, 100 μl (0.2 μg) of BHK-cloned virus was incubated with threefold dilutions of ascites fluid. Goat anti-mouse immunoglobulin and protein A were added. After incubation and centrifugation, supernatant virus was diluted and plated in microtiter plates. Variants were cloned three times.

MAb1.11, MAb1.14, MAb3.27, and MAb3.29 mutants were isolated as described for HRV14 (22, 23) and cloned

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TABLE 1. Reaction of nonneutralizable variants with MAbs

MAb[a]	Neutralization titer of FMDV variants, escaping neutralization by:									
	MAb1.6	MAb1.11	MAb1.14	MAb2.18	MAb3.9	MAb3.10	MAb3.27	MAb3.29	MAb3.7[b]	MAb15
Group 1										
MAb1.4	r[c]	R[c]	r							
MAb1.5	R	R	R	r	r			R		
MAb1.6	R	R				r				
MAb1.11	R	R	r							
MAb1.14	r	R	R					R		R
Group 2										
MAb2.13	r		r	R	r					
MAb2.18				R	r					
Group 3										
MAb3.7[c]									R	
MAb3.9					R	R	R	R		
MAb3.10					R	R	R	R		
MAb3.17					R	r	R	R		
MAb3.21					r		ND[d]	ND		
MAb15										R

[a] MAbs described in this table were serologically characterized earlier (2, 14, 17). Members of groups 1 and 2 and MAb15 do not bind to trypsin-treated virus in ELISA, in contrast to group 3 MAbs (2).
[b] See text for classification of MAb3.7.
[c] r, Log_{10} neutralization titer decrease of 0.5 to 1.5; R, log_{10} decrease of 1.5 or more.
[d] ND, Not done.

three times successively. Finally, the virus was propagated in suspension cells and purified via CsCl centrifugation.

RNA isolation. Purified virus was extracted twice with phenol-chloroform-isoamyl alcohol (50:49:1, vol/vol/vol) three times with chloroform, and twice with ether. After precipitation in 70% alcohol, the RNA yield and integrity were monitored by agarose gel electrophoresis.

RNA sequencing. Primers (Biosearch model 8600) were purified by high-pressure liquid chromatography and extracted twice with ether. Annealing and sequencing with avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.) and [α-^{32}P]dCTP were performed essentially as described previously (21, 24). Some of the sequencing reactions were done with [α-^{35}S]dATP because they gave cleaner autoradiograms. In general, we sequenced only the genome region of the virus mutants that coded for the capsid protein with an altered pI. In the early stages of this work, however, several mutants were sequenced more completely. Furthermore, whenever escape from neutralization by a specific MAb involved different sites on the genome, these sites were sequenced for all variants raised with the MAb.

Neutralization assay and ELISA. A microneutralization test involving BHK cells and 100 50% tissue culture infective doses of virus per well was carried out as described previously (15). In the enzyme-linked immunosorbent assays (ELISAs) polyclonal rabbit anti-FMDV A10 serum purified by protein A was coated onto the plates at 1 μg/ml. A virus variant or parent virus was added at 0.5 μg/ml. Further steps were performed as described previously (15).

RESULTS

Characterization of MAbs. A panel of MAbs was raised in our laboratory against inactivated FMDV A10 (2, 16). Nonneutralizable variants of FMDV were isolated and used to characterize the MAbs (2). The neutralization titers of the MAbs toward the parent virus and the mutant viruses were determined (Table 1). The MAbs, except for MAb3.7 and MAb15, fell into three main groups. Members of the first two groups and MAb15 did not bind to trypsin-treated virus in ELISA (Table 1), in contrast to members of the third group and MAb3.7, which did. The classification of MAb3.7 is discussed below.

It has been shown that MAb1.11 bound to the peptide GDLGSLA (17), part of the trypsin-sensitive site of amino acids 140 to 160 of VP1. Results in Table 1 suggested that MAbs 1.4, 1.5, 1.6, and 1.14 bound to or near VP1 140 to 160 as well. MAb2.18 bound to the C-terminal part of VP1 (17), the second trypsin-sensitive site of VP1.

Variants selected with MAb3.7, MAb3.9, and MAb3.10 were isolated, and their capsid proteins were analyzed on isoelectric focusing gels. All mutants were charge altered in VP3, except for one MAb3.9 mutant, for which the pI of the capsid proteins was identical to that of the parental virus (results not shown). These results indicate that the antigenic site of the MAb group 3 is located on VP3.

We raised a second panel of MAbs with freshly isolated native FMDV A10 to gain more information on the relationships between the MAbs (Table 2). The MAbs were reacted with the variants from Table 1 in a neutralization test. MAb28 and MAb30 fell clearly into group 1, and MAb24 fell into group 2, whereas five new MAbs fell into group 3, since they did not react with MAb3.9-resistant mutants. Although all MAbs, except for MAb3.7, neutralized MAb3.7 variants, a small titer reduction was obtained with MAb3.23, indicating a link between the epitopes of MAb3.7 and group 3 MAbs.

MAb3.29 neutralized the MAb1.11, MAb2.18, MAb3.7, and some MAb3.9 variants as parental virus, whereas MAb3.27 gave only a reduction in titer with certain MAb3.9 variants (Table 2). Initially, we assumed that these MAbs bound to an independent antigenic site. Therefore, MAb3.27 and MAb3.29 variants were isolated (22, 23). The capsid proteins were charge altered in VP2 for the three MAb3.29

TABLE 2. Reaction of nonneutralizable variants with a new set of MAbs

MAb	Isotype[a]	ELISA[b]			MNT						
		A10	A10-trypsin[c]	Competition with bovine serum[d]	A10	Variants against:					
						MAb1.11	MAb2.18	MAb3.9	MAb3.7	MAb3.27	MAb3.29
MAb1.28	G2A	6.0	–	2.4	3.5	R					
MAb1.30	G2A	6.7	–	2.7	3.5	R					
MAb2.24	G3	5.8	–	<1.3	2.5		R				
MAb3.23	G2A	6.3	5.6	3.1	4.0			R	r	R	r
MAb3.25	G2A	5.4	5.4	2.9	4.0		r	r		R	r
MAb3.26	G2A	6.0	5.8	2.7	4.3		r	R		R	
MAb3.31	G2B	5.8	5.5	3.0	3.3		r	R		R	R
MAb3.32	G2B	6.3	6.1	3.3	3.5			R		R	
MAb3.27	G2A	6.3	6.2	2.3	4.0			r		R	
MAb3.29	G2A	6.1	6.0	2.9	4.0						R

[a] Isotype determination was performed in ELISA. MAbs were bound to coated A10 virus. Isotype-specific rabbit antibodies were added, and binding was visualized by using anti-rabbit immunoglobulin conjugated to peroxidase.

[b] ELISA was performed as described in Materials and Methods. The numbers indicate the antibody dilutions, resulting in an absorbance of twice the background value.

[c] Virus was incubated at 0.5 mg per ml of phosphate-buffered saline with 0.5 mg of trypsin for 15 min at 37°C. Then 0.7 mg of soybean trypsin inhibitor was added per ml, and virus was diluted to 0.5 μg/ml and added to the ELISA plates (see Materials and Methods). –, Log_{10} titer smaller than 2.0.

[d] Convalescent-phase bovine serum was obtained as follows. At day 1 a low dose of FMDV vaccine was given, followed at day 28 by a challenge with live FMDV A-Holland. Two weeks after recovery from an FMD infection, serum was prepared. The serum was serially diluted threefold and mixed with a just-saturating amount of the MAb indicated. Binding of the MAb was measured by anti-mouse immunoglobulin-conjugated peroxidase. The log_{10} dilution of bovine serum reducing the absorbance of the serum-negative control by 50% is given. Preimmune serum: <1.3. r and R are defined in Table 1, footnote *b*.

variants; of these, two were changed in VP3 as well. The three MAb3.27 variants were changed in VP3 (data not shown). Table 1 shows that MAb3.27 and MAb3.29 belonged to group 3 MAbs, since MAb3.27 and MAb3.29 variants were resistant to neutralization by group 3 MAbs. Other MAbs neutralized these variants as the parent virus, except for MAb3.29 variants, which were resistant to MAb1.5 and MAb1.14 (see below).

The two fusions yielded 10 MAbs that bound to trypsin-sensitive sites; 7 of these belonged to group 1, and 3 belonged to group 2. A total of 11 MAbs (or 12 when MAb3.7 is included) bound to trypsin-resistant epitopes (group 3) (Tables 1 and 2). It is unknown whether the different antigens used in the fusions of native and inactivated virus are responsible for the different number of MAbs obtained from groups 1 and 3.

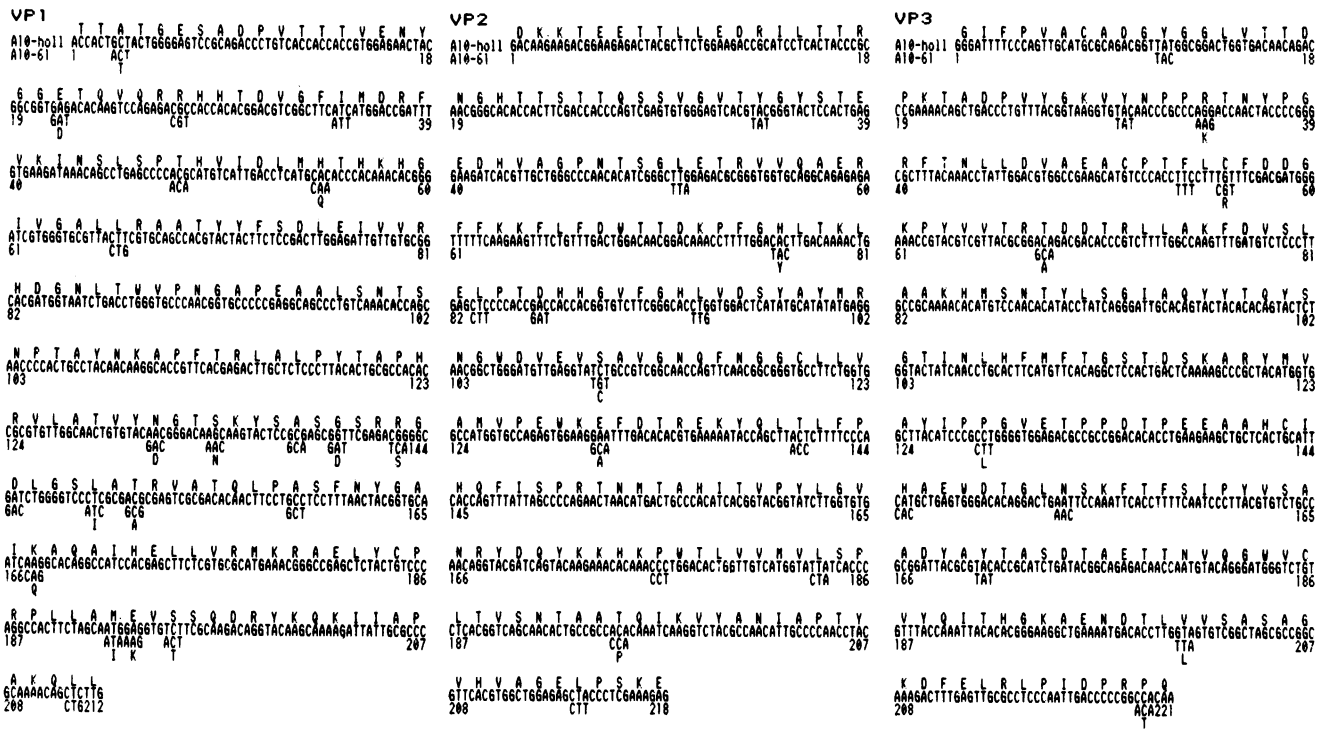

FIG. 1. Nucleotide and deduced amino acid sequence of VP1, VP2, and VP3 of FMDV A10. Triplets and amino acids of A10-Holland and differences from the A10-61 sequence (5) are shown. The amino acid sequence is given in the one-letter code.

TABLE 3. Amino acid changes in nonneutralizable variants of FMDV A10

MAb	VP region	Site	Amino acid change
MAb1.6	VP1	142	R → G (3×[a])
		144	G → D
		147	G → R
MAb1.11	VP1	146	L → R
			L → P (2×)
			L → P and VP2 132 F → V
		149	L → P
MAb1.14	VP1	149	L → F and VP1 157 L → P
		150	A → E
		151	T → K (2×)
		152	R → Q
		153	V → D (2×)
			V → F
MAb2.18	VP1	204	I → L
MAb3.7	VP3	58	D → G (3×)
		59	D → G
		61	K → E (2×)
MAb3.9	VP3	70	D → A
			D → G
		139	E → K (2×)
		195	A → V
MAb3.10	VP3	70	D → A
			D → G (4×)
			D → G and VP2 133 D → N
			D → N (2×)
			D → V (2×)
MAb3.27	VP3	69	deleted
	VP3	139	E → K
			E → K and VP3 136 T → I
MAb3.29	VP2	80	K → E
			K → E and VP3 70 D → G
			K → E and VP2 196 Q → H and VP3 70 D → N
MAb15	VP1	169	Q → R (2×)

[a] Indicates the number of independently isolated variants with this mutation. Double mutations are given on one line.

All MAbs mentioned in Table 1, except MAb3.10 and the members of group 2, competed with a set of neutralizing sera from vaccinated or convalescent cattle or pigs for binding to FMDV in ELISA; this indicates that the antigenic sites of groups 1 and 3 induced neutralizing antibodies (25a). To evaluate the MAbs mentioned in Table 2 (MAbs 23 to 32), we also studied these antibodies in a competition (Table 2). Again, all MAbs competed with the convalescent bovine serum, except for MAb2.24; thus, antigenic site 2 proved to be relatively unimportant. Other polyclonal sera (from pigs protected by vaccination, convalescent pigs, or guinea pigs) reacted similarly to the bovine serum in the competition assay. Preimmune sera or sera from pigs that were vaccinated with doses too low for protection did not compete with any of the MAbs tested.

Escape mutants. RNA from cloned parent A10-Holland virus was isolated, and the nucleotide sequence of the genome was determined by the dideoxynucleotide method (21, 24) as described in Materials and Methods. This sequence was compared with the published sequence of FMDV A10-61 (5) (Fig. 1). Six amino acids differed in the VP1 131 to 151 region, confirming the variability among different subtypes in this region (3). A total of 23 amino acid residues were changed in the structural proteins VP1, VP2, and VP3; of these, 13 were found in VP1.

(i) Group 1. The sequence of the variants was compared with the parental A10-Holland sequence, and the mutations are shown in Table 3 and Fig. 2. All group 1 selected variants, except for one (see below), were mutated in the VP1 140 to 160 region. This was expected from earlier data, which revealed binding of MAb1.11 to the 144 to 150 peptide GDLGSLA (17) and from the trypsin sensitivity of all group 1 MAbs in ELISA (Table 1). Classification of group 1 MAbs, based on cross-neutralization (Table 1), was consistent with the mutations found.

An interesting double mutation was observed for one MAb1.11 variant. VP1 146, as well as VP2 132, was changed, which suggests a link between the VP1 140 to 160 area and VP2 residues (20). A relationship between VP2 or VP3 residues and the VP1 140 to 160 sequence has already been suggested for type O1K (29). The structure of picornaviruses, deduced by X-ray crystallography examination (10, 13, 20), suggests that the residues around VP2 135 adjoin the residues of VP1 140 to 160. Variants with only a VP2 mutation have not yet been isolated, and so we are not able to determine whether VP2 residues form part of the antigenic site.

MAb1.11 bound to the synthetic peptide GDLGSLA (VP1 144 to 150) in ELISA (17). The mapped mutations (Table 3) agree with the loss of binding in ELISA when VP1 146 L or VP1 149 L is replaced by any amino acid except isoleucine (17). Correlation between the mapping of mutations and the peptide ELISA, as done for MAb1.11, was impossible for MAb1.6, since this MAb did not bind to synthetic peptides in the 140 to 160 region. Apparently, amino acids from this region constitute only part of the MAb1.6 epitope, or the epitope cannot be mimicked by a linear synthetic peptide.

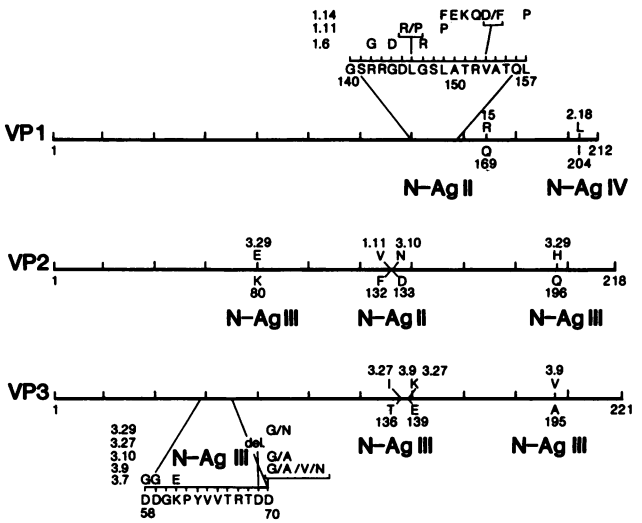

FIG. 2. Antigenic architecture of FMDV A10. All mutations are shown. Below the line, the wild-type amino acid is given; above the line, the changed amino acid and the MAb used in the isolation of this variant are given. Double mutations in one variant are given in Table 3. The antigenic-site nomenclature is from Wimmer et al. (28). Classification of mutation VP1 169 is discussed in the text. Abbreviation: del. (VP3 69), deleted.

MAb1.14 bound to the peptide ARV (VP1 151 to 153). Maximal binding occurred to AARVATQ (VP1 150 to 156) (R. Meloen, personal communication). The location of the mutations of MAb1.14 variants in VP1 149 to 157 (Table 3) agreed with these ELISA data.

Complete resistance of FMDV O1K to neutralization by MAb D9 correlated to the L-to-R-change at VP1 148, a residue corresponding to VP1 146 in FMDV A10. In contrast, other mutations in this site led to only partial resistance (29). The analogous L-to-R MAb1.11 mutation in FMDV A10 also resulted in complete resistance to neutralization (Table 1).

(ii) Group 2. Nonneutralizable variants of MAb2.18 were very difficult to isolate, because even at a high ascites concentration (10^{-1}), MAb2.18 was unable to prevent replication of parental virus (data not shown). MAb2.18 has previously been studied in detail, and its binding to the C-terminal part of VP1 has been documented (17). The only MAb2.18 mutant from which RNA could be sequenced showed a nucleotide change in VP1 204 (I to L; Table 3, Fig. 2). In ELISA studies with synthetic peptides, VP1 204 I could be exchanged only for V, and no antibody bound when I was replaced by L (17).

(iii) Group 3. Variants of group 3 MAbs were charge altered in VP3; only MAb3.29 mutants were changed in VP2. Some of the MAb3.29 mutants were changed in VP3 as well (not shown). MAb3.9 variants were changed in VP3 70, VP3 139, and VP3 195 (Table 3): three residues in the lower part of the 12S protomer (20). All MAb3.10 mutations (10 independently isolated variants) involved only VP3 70. This site corresponds to N-Ag III of poliovirus type 1 (10, 18, 28) and Nim III for HRV14 (20, 22).

Some group 3 mutants were sequenced in the VP1 140 to 160 region to verify that the phenotype of group 3 variants was not caused by an amino acid change in the highly variable VP1 140 to 160 sequence. In accordance with serological data (Tables 1 and 2), no amino acids were changed.

Although residues VP3 58 to 60 in poliovirus type 1 are part of N-Ag III (containing VP3 70) and although MAb3.7 variants were changed in VP3 58, VP3 59, and VP3 61 (Table 3), the MAb3.7 variants were neutralized by MAb3.9 (Table 1). This discrepancy might be eliminated by raising MAbs that would cover part of the MAb3.7 and MAb3.9 epitopes. Unfortunately, no firm link could be established between MAb3.7 and group 3 MAbs. The proximity of VP3 58 to 61 and VP3 70 on the viral surface is too great to regard these amino acids as belonging to independent antigenic sites (10, 20).

The double mutation in one MAb3.10 variant (VP2 133 and VP3 70; Table 3) cannot be explained. Neutralization of other MAb3.10 variants showed that mutation of VP3 70 sufficed for resistance.

Remarkably, although MAb3.27 and MAb3.29 variants reacted similarly with MAb3.9, MAb3.10, and MAb3.17, the epitopes of MAb3.27 and MAb3.29 did not seem to overlap, because MAb3.27 variants were still neutralized by MAb3.29 and vice versa (Table 2). This indicates either that the viral amino acids contributing to MAb binding are different for both MAbs or that the antigenic site to which they bind is large enough to accommodate two independent epitopes.

The mutations in MAb3.27 variants showed that VP3 139 and VP3 69 (or VP3 70, both triplets being GAC) were involved in the epitope of MAb3.27 (Table 3; Fig. 2). Surprisingly, one of the variants had a deletion of an amino acid residue, a phenomenon not yet described for picornaviral mutants resistant to neutralization. One variant was changed at two neighboring positions (VP3 136 and VP3 139), which suggests that both residues may belong to the epitope of MAb3.27.

A thrice-cloned MAb3.29 variant was mutated at VP2 80; this residue verged upon the VP1 140 to 160 sequence and the VP2 132 residue (20; Palmenberg, personal communication). Two single-cloned variants were changed at two and three positions: the first at VP2 80 and VP3 70 and the second at the same positions plus VP2 196 (Table 3). Because these variants were changed at more positions, conclusions cannot be based on results with these variants. VP2 196 lies directly opposite VP3 70, however, close to the threefold axis (20).

All three MAb3.29 variants were resistant to MAb3.9. If the distance on the viral surface between VP2 80 (MAb3.29) and VP3 70, VP3 139, and VP3 195 (MAb3.9) was spanned by one MAb molecule, the epitope bridged two protomers. In addition, MAb3.9 and MAb3.10 bound to residues near the protomer border. The location of group 3 epitopes near the protomer boundary explains the absence of reactivity of most MAb3s with 12S subunits in ELISA (2; R. Meloen and S. J. Barteling, unpublished results).

Resistance to neutralization to some group 1 MAbs of MAb3.29-resistant mutants (Table 1) agrees with the VP2 80 mutation. This residue is located near the site of group 1 MAbs (20; Palmenberg, personal communication). Results suggest that the locations of MAb1.5 and MAb1.14 are closer to VP2 than are the locations of MAb1.4, MAb1.6, and MAb1.11.

(iv) MAb15. After mapping the mutations found in the MAb15 variants (Table 3), we understood our failure to classify this MAb. VP1 169 belongs to a distinct loop, apparently not involved in other sites. This site was barely antigenic in poliovirus type 1 (10, 28), but was present in HRV14 and was identified as Nim IB (22). The alignment of HRV14 with the FMDV sequence (Palmenberg, personal communication) suggests that the MAb15 site is similar to Nim IB.

DISCUSSION

In this study, VP1, VP2, and VP3 of the genome of FMDV A10-Holland were sequenced (Fig. 1). Mutants that were resistant to neutralization by a specific MAb were isolated, and their genomes were compared with that of the parent virus (Table 3). In this way, we found amino acid residues involved in MAb binding and located epitopes by aligning amino acid sequences of picornaviruses (Palmenberg, personal communication) and by comparing those sequences with the three-dimensional structure of picornaviruses (10, 13, 20). This is a well-established procedure for locating epitopes (6, 10, 18, 20, 22, 23). The alignment and the comparison with three-dimensional structures strongly suggest that all altered amino acids were located on the viral surface.

The antigenic sites of poliovirus type 1, HRV14, and FMDV A10 are compared in Table 4. Antigenic sites are designated N-Ag for poliovirus (28) and Nim for HRV (22). The three picornaviruses have N-Ag II (Nim II) and N-Ag III in common (Fig. 2). N-Ag II contains VP1 residues in FMDV (except for a double-mutation VP1 146 and VP2 132 for one MAb1.11 variant) and only VP2 residues in HRV 14 (23) (Table 2). Poliovirus combines both sequences in one antigenic site (18, 28). Both sequences adjoin in the picornavirus

TABLE 4. Comparison of antigenic sites in three picornaviruses

Location[a]	Antigent sites in:						Proposed designation[a]
	Poliovirus type 1[b]		HRV14[c]		FMDV A-10 Holland[d]		
VP1 bB-bC	N-Ag I	VP1 93–103	Nim IA	VP1 91, 95			1 B * C
VP1 bG-bH	N-Ag II	VP1 220–222	Nim II		Group 1	VP1 142–157	1 G * H
VP2 bE-aB		VP2 166, 169, 170		VP2 136, 158, 159, 161, 162		VP2 132	2 E * aB
VP2 C-term.		VP2 270					2 I * t
VP1 C-term.	N-Ag III		Nim III	VP1 287	Group 3		1 I * t
VP2 bB-bC		VP2 72					2 B * C
VP2 bC-aA						VP2 80	2 C * aA
VP2 bH-bI						VP2 196	2 H * I
VP3 bB-knob		VP3 58–60				VP3 58–61	3 B * B
VP3 bB-bC		VP3 71–73		VP3 72, 75, 78		VP3 69, 70	3 B * C
VP3 bE-aB						VP3 136, 139	3 E * aB
VP3 bH-bI						VP3 195	3 H * I
VP1 C-term.	N-Ag IV	VP1 271, 295			Group 2	VP1 200–212	1 I * t
VP1 bB-bC			Nim IB	VP1 83–85	MAb15		1 B * C
VP1 bD-bE				VP1 138–139			1 D * E
VP1 bH-bI						VP1 169	1 H * I

[a] The amino acid sequences between beta-sheets (b) or between beta-sheet and alpha-helix (a) (Fig. 3) are indicated. Use was made of the alignment of amino acid sequences of picornaviruses by Palmenberg (personal communication). The designation indicates the capsid protein (1, 2, or 3), the peptide sequence involved in MAb binding (*), protruding from the beta-sheets (capital letters, as used by Rossmann et al. [20]), or the alpha-helix (capitals, preceded by the letter a). C-terminal sequences extend from the beta-sheet I and are designated b I * t (for terminus). 3 B * B is the VP3 puff, a peptide loop between two parts of beta-sheet B.

[b] References 6, 18, and 28.

[c] References 20, 22, and 23.

[d] This study.

and are therefore regarded as belonging to one antigenic site (18, 20, 28).

N-Ag III is composed of residues from many different loops (Table 4). However, these residues make up one antigenic site (Tables 1 and 2). The C terminus of VP1 was included in this antigenic site in HRV14. Despite a predicted relationship based on the picornaviral structure, no relationship could be established between the C terminus and N-Ag III for FMDV and poliovirus 1 (10, 20). N-Ag IV (the C terminus of VP1) seems unimportant in FMDV A10, since the three MAbs binding to this site (MAb2.13, MAb2.18, and MAb2.24) do not compete with polyclonal sera in ELISA (25a) (Table 2). Within the detection limits of the competition ELISA, the data suggest that there are no neutralizing antibodies directed against the C terminus of VP1 in sera from susceptible animals. Moreover, the neutralizing capacity of group 2 MAbs is limited (Table 2) (A. Thomas, unpublished data). The poor ability of the C terminus of VP1 in the complete virus to generate protecting antibodies has been previously discussed (14).

The absence in FMDV of N-Ag I (or Nim IA), a strongly immunodominant site in poliovirus type 3 (18), has been previously noted (28).

Competition of MAb15 with convalescent bovine (or pig) serum antibodies for binding to FMDV in ELISA showed that MAb15 competed less successfully than most of the MAbs from groups 1 and 3 (results not shown). Furthermore, MAb15 was the only MAb from our panel that recognized the antigenic site that corresponded to Nim IB (Table 1). We conclude, therefore, that MAb15 binds to an antigenic site of minor importance in FMDV.

Peptides mimicking the epitope of MAb15 were identified by a trial-and-error method with synthetic peptide ELISA (9). Two peptide elements were identified: WQM and HS or HT. When connected by glycine or β-alanine, these elements formed a "mimotope peptide" that bound strongly to MAb15 (9). The amino acid that is changed in the two MAb15 variants (Q VP1 169; Table 3) is part of a peptide sequence close to the loop containing the tripeptide MHT (20). Therefore, the results of synthetic peptide ELISA support the location of the MAb15 epitope.

Table 4 shows a nomenclature for peptide sequences involved in each antigenic site. VP1, VP2, and VP3 are approximately equal in size, and their structures are similar, consisting of antiparallel beta-sheets connected by loops (Fig. 3). Since the basic picornavirus structure is constant (10, 13, 20), beta-sheets (bA-bI) and alpha-helices (aA and aB) can be uniformly designated (20); thus, antigenic peptide sequences extending from these sheets and helices can likewise be uniformly designated (Table 4). For example, the well-studied FMDV loop VP1 140 to 160 (Fig. 3, top right) can be designated 1 G * H, because it is located in VP1 and connects the beta-sheets G and H. Because of the great similarity between capsid proteins 1, 2, and 3 and between picornaviruses in general, this method of naming allows a direct comparison of the antigenic sites of different picornaviruses. Furthermore, the similarity between VP1, VP2, and VP3 is immediately obvious, since, for example, antigenic sites B * C and H * I occur on all three major structural proteins.

The C terminus of VP1 cannot be definitely classified. For FMDV-A10 and poliovirus type 1 it forms a distinct antigenic site, but for HRV14 it is part of N-Ag III (22, 23). For FMDV O1 a link was made between N-Ag II and the C terminus of VP1 (29). Whether this failure to classify the C terminus of VP1 is virus dependent is unknown.

Of the HRV14 mutants, 86% underwent a charge shift, although theoretically only one-third should have experienced a change in amino acid charge (23). In this study, 78% of all mutations involved a charge shift, whereas 8 of the 12

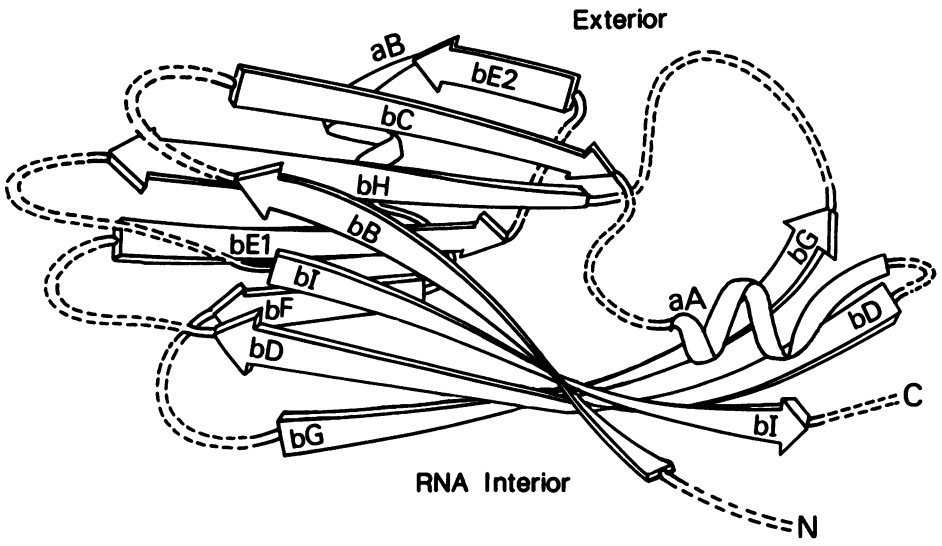

FIG. 3. Basic structure of a picornaviral structure protein. A schematic picture is given of a picornaviral structural protein, indicating the beta-sheets and alpha-helices. Adapted from reference 20, Fig. 3. Loops connecting sheets and helices are dotted; N and C termini are also dotted. The lengths of the dotted peptide sequences vary between different viruses and proteins. Beta-sheet A is, in general, absent. For further details, see the text.

charge-neutral changes were selected with MAb1.11 or MAb1.14. Apparently a charged amino acid is not favored at positions 146 to 157 in VP1. If these amino acid residues adjoin the receptor site (20), a charged amino acid could decrease cell receptor binding.

The importance of FMDV VP1 140 to 160 (1, 4, 7, 11, 25) is supported by the data in this study. However, MAbs from group 3 that bound to VP3 sequences competed with polyclonal sera from susceptible animals as well as MAbs from group 1 that bound to the VP1 140 to 160 sequence (Table 2) (25a). Furthermore, 12 of 23 MAbs bound to N-Ag III, and 7 MAbs bound to the trypsin-sensitive site VP1 140 to 160 (Tables 1 and 2). Iodination of tyrosine residues revealed that the role of VP1 in immunodominance in picornaviruses was overestimated; later studies showed that when small lysine-modifying reagents are used, VP1 to 3 of poliovirus (26), rhinovirus (12), and FMDV (19) are labeled (see also discussion in reference 19). The resolution of picornaviral structures revealed that VP2 and VP3 were also surface exposed (10, 13, 20). This study clearly shows at least two important antigenic sites on FMDV: first, the well-studied VP1 sequence 140 to 160 and, second, VP2 and VP3 residues as defined in Fig. 2. A peptide vaccine that incorporates peptides from the latter site may prove more effective than the peptide vaccines used so far.

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LITERATURE CITED

1. **Bachrach, H. L., D. M. Moore, P. D. McKercher, and J. Polatnick.** 1975. Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. J. Immunol. **115**:1636–1641.
2. **Barteling, S. J., J. Boerke, R. Woortmeijer, and A. Thomas.** 1986. Neutralising monoclonal antibodies against foot and mouth disease virus (FMDV) are directed towards antigenic sites on VP1, VP2 and VP3. Bull. Off. Int. Epizoot. **17**:141–151.
3. **Beck, E., and K. Strohmaier.** 1987. Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. J. Virol. **61**:1621–1629.
4. **Bittle, J. L., R. A. Houghten, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, and R. A. Lerner.** 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature (London) **298**:30–33.
5. **Boothroyd, J. C., T. J. R. Harris, D. J. Rowlands, and P. A. Lowe.** 1982. The nucleotide sequence of cDNA coding for the structural proteins of foot-and-mouth disease virus. Gene **17**: 153–161.
6. **Diamond, D. C., B. A. Jameson, J. Bonin, M. Kohara, S. Abe, H. Itoh, T. Komatsu, M. Avita, S. Kuge, A. Nomoto, A. D. M. E. Osterhaus, R. Crainic, and E. Wimmer.** 1985. Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229**:1090–1093.
7. **DiMarchi, R., G. Brooke, C. Gale, V. Cracknell, T. Doel, and N. Mowat.** 1986. Protection of cattle against foot-and-mouth disease by a synthetic peptide. Science **232**:639–641.
8. **Geysen, H. M., R. H. Meloen, and S. Barteling.** 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81**:3998–4002.
9. **Geysen, H. M., S. J. Rodda, and T. J. Mason.** 1986. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol. Immunol. **23**:709–715.
10. **Hogle, J. M., M. Chow, and D. J. Filman.** 1985. Three-dimensional structure of poliovirus at 2.9 Å resolution. Science **229**: 1358–1365.
11. **Laporte, J., J. Grosclaude, J. Wantyghem, S. Bernard, and P. Rouzé.** 1973. Neutralisation en culture cellulaire du pouvoir infectieux du virus de la fièvre aphteuse par des sérums provenant de porcs immunisés à l'aide d'une protéine virale purifiée. C.R. Acad. Sci. Ser. D **276**:3399–3401.
12. **Lonberg-Holm, K., and B. E. Butterworth.** 1976. Investigation of the structure of polio- and rhinovirions through the use of selective chemical reactivity. Virology **78**:207–216.
13. **Luo, M., G. Vriend, G. Kamer, I. Minor, E. Arnold, M. G. Rossmann, U. Boege, D. G. Scraba, G. M. Duke, and A. C. Palmenberg.** 1987. The atomic structure of Mengo virus at 3.0 Å

resolution. Science **235**:182–191.

14. **Meloen, R. H., and S. J. Barteling.** 1986. An epitope located at the C-terminus of isolated VP1 of foot-and-mouth disease virus type O induces neutralizing activity but poor protection. J. Gen. Virol. **67**:289–294.
15. **Meloen, R. H., and J. Briaire.** 1980. A study of the cross-reacting antigens on the intact foot-and-mouth disease virus and its 12S subunits with antisera against the structural proteins. J. Gen. Virol. **51**:107–116.
16. **Meloen, R. H., J. Briaire, R. J. Woortmeijer, and D. van Zaane.** 1983. The main antigenic determinant detected by neutralizing monoclonal antibodies on the intact foot-and-mouth disease virus particle is absent from isolated VP1. J. Gen. Virol. **64**: 1193–1198.
17. **Meloen, R. H., W. C. Puyk, D. J. A. Meijer, H. Lankhof, W. P. A. Posthumus, and W. M. M. Schaaper.** 1987. Antigenicity and immunogenicity of synthetic peptides of foot-and-mouth disease virus. J. Gen. Virol. **68**:305–314.
18. **Minor, P. D., M. Ferguson, D. M. A. Evans, J. W. Almond, and J. P. Icenogle.** 1986. Antigenic structure of polioviruses of serotypes 1, 2 and 3. J. Gen. Virol. **67**:1283–1291.
19. **Morrell, D. J., E. J. C. Mellor, D. J. Rowlands, and F. Brown.** 1987. Surface structure and RNA-protein interactions of foot-and-mouth disease virus. J. Gen. Virol. **68**:1649–1658.
20. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H.-J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317**:145–153.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
22. **Sherry, B., A. G. Mosser, R. J. Colonno, and R. R. Rueckert.** 1986. Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14. J. Virol. **57**:246–257.
23. **Sherry, B., and R. Rueckert.** 1985. Evidence for at least two dominant neutralization antigens on human rhinovirus 14. J. Virol. **53**:137–143.
24. **Smith, A. J. H.** 1980. DNA sequence analysis by primed synthesis. Methods Enzymol. **65**:560–580.
25. **Strohmaier, K., R. Franze, and K.-H. Adam.** 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. J. Gen. Virol. **59**:295–306.

25a. **Thomas, A. A. M., R. J. Woortmeijer, S. J. Barteling, and R. H. Meloen.** 1988. Evidence for more than one important, neutralizing site on foot-and-mouth disease virus. Arch. Virol. **99**:237–242.

26. **Wetz, K., and K.-O. Habermehl.** 1979. Topographical studies on poliovirus capsid proteins by chemical modification and cross-linking with bifunctional reagents. J. Gen. Virol. **44**:525–534.
27. **Wild, T. F., J. N. Burroughs, and F. Brown.** 1969. Surface structure of foot-and-mouth disease virus. J. Gen. Virol. **4**:313–320.
28. **Wimmer, E., E. A. Emini, and D. C. Diamond.** 1986. Mapping neutralization domains of viruses, p. 159–173. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis II. Springer Verlag, New York.
29. **Xie, Q.-C., D. McCahon, J. R. Crowther, G. J. Belsham, and K. C. McCullough.** 1987. Neutralization of foot-and-mouth disease virus can be mediated through any of at least three separate antigenic sites. J. Gen. Virol. **68**:1637–1647.