Analysis of Neutralizing Epitopes on Foot-and-Mouth Disease Virus

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For the investigation of the antigenic determinant structure of foot-and-mouth disease virus (FMDV), neutralizing monoclonal antibodies (MAbs) against complete virus were characterized by Western blot (immunoblot), enzyme immunoassay, and competition experiments with a synthetic peptide, isolated coat protein VP1, and viral particles as antigens. Two of the four MAbs reacted with each of these antigens, while the other two MAbs recognized only complete viral particles and reacted only very poorly with the peptide. The four MAbs showed different neutralization patterns with a panel of 11 different FMDV strains. cDNA-derived VP1 protein sequences of the different strains were compared to find correlations between the primary structure of the protein and the ability of virus to be neutralized. Based on this analysis, it appears that the first two MAbs recognized overlapping sequential epitopes in the known antigenic site represented by the peptide, whereas the other two MAbs recognized conformational epitopes. These conclusions were supported and extended by structural analyses of FMDV mutants resistant to neutralization by an MAb specific for a conformational epitope. These results demonstrate that no amino acid exchanges have occurred in the primary antigenic site of VP1 but instead in the other coat proteins VP2 and VP3, which by themselves do not induce neutralizing antibodies.

Foot-and-mouth disease virus (FMDV) belongs to the aphthovirus genus of the family Picornaviridae. Infection with this virus causes a severe, although rarely fatal, disease of cloven-hooved animals. Like other picornaviruses, FMDV possesses a single-stranded positive RNA of about 8,500 nucleotides with a small protein (VPg) covalently bound to its 5' end, an internal poly(C) tract, and a poly(A) sequence at the 3' end (for reviews, see references 1 and 31). The viral RNA is encapsidated by the four structural proteins VP1, VP2, VP3, and VP4, which are produced by posttranslational cleavage of a common precursor (32). Of these, VP1 is of particular interest because immunization studies have shown that this protein harbors the main antigenic determinant for virus neutralization (2, 19). This major neutralizing antigenic determinant on the virion has been identified as a hexadecapeptide in VP1 (35). Synthetic peptides containing this hexadecapeptide sequence were able to induce neutralizing antibodies with an efficacy similar to that of VP1 and 12S capsomers (7, 25). In comparison to whole virions (146S particles), the ability of isolated VP1, 12S subunits, or synthetic peptides to induce neutralizing antibodies is very low. Immunization studies of guinea pigs and cattle with synthetic peptides as antigens have shown that they are fully protective in guinea pigs but not in cattle (7, 11, 26, 27). To compare the immune response against the synthetic peptides with that against whole virus, neutralizing monoclonal antibodies (MAbs) elicited against viral particles were used for characterization of epitopes important for virus neutralization.

In this report we describe four neutralizing MAbs directed against this major determinant. Two of these recognize continuous epitopes, while the other two recognize conformational epitopes.

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For the competition ELISA, the 1:100 dilutions of the...
ascites fluids were incubated with different amounts of synthetic peptides (0.1 to 100 μg), coat proteins VP1, VP2, and VP3 (0.1 to 50 μg), and 146S particles (0.1 to 5 μg) and then reacted with 146S particles bound to the wells.

**Plaque reduction assay.** Plaque reduction assays were performed by the method of Baxt et al. (3). The titer is expressed as log_{10} of the serum dilution required to neutralize 50% of the virus in monolayers of BHK cells.

**Western blot (immunoblot).** FMDV 146S particles were denatured in 2.5% sodium dodecyl sulfate (SDS)-5% dithiothreitol in 100 mM Tris hydrochloride, pH 6.8, for 5 min at 100°C. Coat proteins were separated on 12.5% SDS-polyacrylamide gels (17) and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, FRG) at 60 V overnight in 25 mM Tris (pH 8.3)-192 mM glycine-20% (vol/vol) methanol (36). The filters were saturated with 2% BSA dissolved in PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.5) for 2 h to prevent non-specific protein binding, followed by incubation with a 1:1,000 dilution of ascites fluid or antisera (2% BSA and 0.05% NP40 in PBS) for at least 12 h. Parallel incubations with antisera or ascites, also diluted 1:1,000, which were previously allowed to react with an excess of the synthetic peptide (10 μg), were also carried out. After two washes, the binding of specific antibodies was monitored by incubation with 125I-labeled rabbit antiserum or goat anti-rabbit immunoglobulins (both from Amersham-Buchler, Braunschweig, FRG) for 2 h. The immune reaction was visualized by autoradiography.

**Isolation of neutralization-resistant mutants.** Antigenic variants of O,K, 64th passage, pretreated with antibody, were selected on BHK cells by plaque formation under a methylcellulose overlay which also contained the antibody. Putative mutants were subjected to another cycle of selection.

**Isolation of viral RNA, cDNA synthesis, and sequencing analysis of the coat protein region.** Virus preparations were grown in BHK cells and purified by the method of Strohmaier et al. (35). The viral proteins were extracted twice with neutralized 80% (wt/wt) phenol and once with chloroform-isooamyl alcohol (24:1), and the RNA was precipitated with 2 volumes of ethanol and stored in aliquots at −70°C. Eight primer oligonucleotides were synthesized by the phosphoramidite method (9) with an automated oligonucleotide synthesiser (Applied Biosystems 380A). The selected oligonucleotides were complementary to sequences most conserved between different serotypes and correspond to positions in the O,K coat protein region 238 to 260, 549 to 568, 914 to 932, 1143 to 1163, 1350 to 1372, 1723 to 1746, and 2006 to 2027 and in addition a 20-mer oligonucleotide starting 6 nucleotides past the 3’ end of the VP1 gene. Synthesis of cDNA and the sequence analysis were performed as described (6). In addition, direct RNA sequencing was performed for a few primers by the method of Zimmer and Kaesberg (38) as modified by Palmenberg et al. (23).

**RESULTS**

**Immunological characterization of the Mabs.** Neutralizing Mabs were raised against intact FMDV strain O,K (146S particles). Four Mabs with high neutralizing titers were obtained and tested in an indirect ELISA for their ability to bind to whole virus, 12S subunits, isolated VP1, VP2, and VP3, and synthetic peptides A (amino acids aa) 144 to 159, G1-21 (aa 140 to 160), and G1-32 (aa 129 to 160) (Fig. 1). The Mabs used and their immunological characteristics are shown in Fig. 2. Mabs 7 and 48 recognized only intact 146S particles and to a minor extent the shorter synthetic peptides A and G1-21, whereas Mabs 75 and 99 reacted with intact 146S particles as well as with the 12S subunit, isolated VP1, and the three synthetic peptides. In no case was a reaction observed with VP2 or VP3.

The reactivity of the Mabs against the four denatured viral capsid proteins was analyzed in a Western blot (Fig. 3). Polyclonal rabbit antisera elicited by isolated viral proteins VP1, VP2, and VP3 and by synthetic peptide A were used as positive controls. Mabs 7 and 48 did not react with any of the viral proteins, whereas Mabs 75 and 99 recognized the VP1 protein (Fig. 3). The specificity of this immune reaction was confirmed by the fact that binding of the antibodies could be inhibited by preincubation with synthetic peptide G1-21, which is part of VP1.

Similar results were obtained in a competition ELISA with the viral coat proteins and the synthetic peptide used as competitors (Fig. 4). In this assay, the antibody binding of Mabs 7 and 48 could also be measured, because intact viral particles were used as an antigen. The antibodies were preincubated with the different coat proteins or synthetic peptides, and the remaining binding capacity was measured. As a control, intact viral particles were used as the competing antigen.

The results (Fig. 4) demonstrate that VP1 and peptide A clearly competed with 146S particles for binding to Mabs 75 and 99. This is in accord with the data from the Western blots and localizes the antibody-binding sites for these two Mabs between nn144 and 159. In the case of Mabs 7 and 48, no significant competition was observed with the isolated coat proteins VP1 (Fig. 4) or VP2 and VP3 (data not shown). Only large amounts of peptide A (10 μg, which on a weight basis was 10-fold over intact virus) led to a significant decrease in binding of the Mabs. Even when 100 μg of peptide was used, only a 25% competition was observed. This indicates that Mabs 7 and 48 also recognized to some extent the sequence of peptide A. However, amino acids outside of peptide A (in coat protein VP1 or in other coat proteins) and only available for binding in intact virus must participate in the epitope structures recognized by Mabs 7 and 48.

**Characterization of the Mabs by plaque reduction assay.**

![Diagram](http://jvi.asm.org/Downloaded/from/http://jvi.asm.org/)
The four MABS elicited by and able to neutralize FMDV O1K virions were characterized further in plaque reduction assays with FMDV strains of different serotypes and subtypes and less well characterized field isolates. According to the results of these assays (Table 1), MABS 75 and 99 neutralized all O strains tested. In addition, MAB 75 also cross-neutralized C1Oberbayern (C1O), which belongs to a different serotype. This cross-neutralization was also observed with a polyclonal anti-peptide A antiserum (Table 1) (25). The two other neutralizing MABS, 7 and 48, were highly

<table>
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<th>Strain</th>
<th>MAb 7</th>
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<th>MAb 75</th>
<th>MAb 99</th>
<th>Polyclonal antiserum</th>
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* Titters are expressed as log10 values of the serum dilution required to neutralize 50% of the virus in monolayers of BHK cells.

ND, Not determined.
specific for certain O isolates but showed distinct differences. MAb 7 neutralized its homologous strain O1K and also O1BFS and O Israel, which is presently not classified into a subtype. It did not neutralize O1Lausanne (O1L), O1Murchin, or O1Dänemark. In contrast, MAb 48 neutralized O1K, O1BFS, O1L, O1Murchin, and O1Dänemark, but not O Israel. From these neutralization patterns, it appears that these MAb s recognize four different epitopes on the virus surface.

Sequence comparison of FMDV strains tested in the plaque reduction assay. We compared changes in the cDNA-derived amino acid sequence of coat protein VP1 of all strains tested to correlate the different binding specificities of the MAb s with the amino acid sequence of coat protein VP1. A list of the amino acid sequences of all O strains tested (4, 6, 17, 20, 29) indicates that the five isolates O1K, O1BFS, O1L, O1Murchin, and O1Dänemark showed only minor differences in their sequences (Fig. 5A). For example, one (O1L, O1Murchin, O1Dänemark) or two (O1BFS) amino acid exchanges were found in positions 134 and 137, but these exchanges occurred outside the known antigenic site between aa 144 and 159. Within this site, three exchanges occurred in O Israel, four in O2Normandie, and five in O Wuppertal. Despite these changes within the antigenic site, MAb 75 and 99 reacted with all FMDV O isolates, whereas these changes were noticed by MAb 48, since it neutralized only FMDV O1 isolates. However, MAb 7 did not neutralize O1L among the O1 strains but neutralized O Israel, although this strain differed by three amino acids within the antigenic site from the O1 strains. Inclusion of other FMDV serotypes (C1Oberbayern and A1Westfeld) into such a sequence analysis indicated one highly conserved R-G-D triplet among all strains examined (Fig. 5B). It is interesting that MAb 75 was distinguished from MAb 99 by its ability to neutralize all strains that contained the R-G-D-L tetrapeptide.

Sequence analysis of the whole coat protein region of O1L. Because MAb 7 could differentiate between O1 strains that contained an identical major antigenic site, we sequenced the complete coat protein region of O1L by cDNA sequencing and compared this sequence with the published amino acid sequence of O1K (5) (for details, see Materials and Methods). Thirteen nucleotide exchanges were found in the P1 coding sequence; four of these resulted in amino acid exchanges, two in VP2 (aa 200, Y → C; aa 260, N → D) and two in VP3 (aa 411, V → I; aa 461, G → D) (Fig. 6).

Selection and analysis of FMDV mutants resistant to MAb 7. To analyze in more detail the changes leading to the lack of neutralization of some O1 strains by MAb 7, we selected mutants resistant to MAb 7 from an O1K virus pool (for details, see Materials and Methods) and analyzed the mutants for amino acid exchanges in their coat proteins. Four virus clones were isolated and characterized further by a neutralization assay with the four MAb s and a polyclonal anti-peptide A antiserum. As demonstrated (Table 1), all four mutants were neutralized by MAb 7, 75, and 99 but not by MAb 7. In contrast, neutralization with the polyclonal anti-peptide A serum was equally effective with the isolated FMDV mutants, field isolate O1L, and the original FMD
FIG. 5. (A) Comparison of the VP1 amino acid sequence of the different FMDV O strains tested. The O1K sequence is shown completely. For the other sequences, only differences from the O1K sequence are specified. The amino acid sequence of O1Danemark was identical to that of O1Murchin. The amino acid sequence of O Austria was identical to that of O Wuppertal. (B) Comparison of the antigenic determinant amino acid sequence of all strains tested. The O1K sequence is shown completely. For the other sequences, only differences from the O1K sequence are specified. Dashes represent deletions used to optimize alignment of the sequences. The conserved sequence is underlined.
O1K virus pool. Thus, the four mutants selected from FMDV O1K showed a neutralization pattern identical to that of FMDV O1L. For two of these mutants, the relevant changes leading to an escape from MAb 7 neutralization were characterized in detail by cDNA sequencing of the coat protein P1 coding region except for the first 50 amino acids of VP4. Both mutants had the same nucleotide sequence. By comparison of the deduced amino acid sequence with that of the original strain O1K (5), no differences were detected in VP1 or the sequenced part of VP4, but two exchanges in VP2 (aa 200, Y → C; aa 207, L → Q) and three in VP3 (aa 435, W → R; aa 436, D → Y; aa 461, G → D) had occurred, as shown for one mutant in Fig. 6. Two of these, Tyr → Cys in VP2 and Gly → Asp in VP3, were also present in the FMDV O1L sequence. A partial sequence analysis of the coat protein region of the parent strain used for the selection of the antibody-resistant mutants showed that in the majority of the virions, a cysteine was present at aa 200. Therefore it seems that normally a cysteine is present in this position in all O1 subtypes and that the cloned and sequenced O1K genome (5) represents only a minority of the FMDV virions in the parent strain.

**DISCUSSION**

By characterizing four MAbs elicited by whole FMDV, we provide evidence for the presence of four different epitopes on the viral surface that are involved in virus neutralization. Two of these epitopes are linear and are part of the previously identified major antigenic site, a hexadecapeptide between aa 144 and 159 in coat protein VP1 which is able to induce neutralizing antibodies in animals (7, 11, 25–27). The other two are noncontiguous or conformational determinants that must include additional amino acids outside the hexadecapeptide of VP1. This conclusion is based on our observation that MAbs 7 and 48 reacted most strongly with intact viral particles and only to a minor extent with peptide A and not at all with denatured VP1 or 125 subunits. In contrast, MAbs 75 and 99 reacted well with all four types of antigens.

Based on competition experiments with peptide A, the contiguous epitopes recognized by MAbs 75 and 99 were mapped within aa 144 to 159 of coat protein VP1. This result was confirmed by direct binding studies with the synthetic peptides A, G1-21, and G1-32 (Fig. 1). However, the latter two MAbs differed in their neutralization activities against different FMDV serotypes and subtypes. A comparison of the amino acid sequences revealed a homologous stretch of five amino acids (R-G-D-L-Q) that was present in all strains neutralized by MAbs 75 and 99. Four of these five amino acids (R, G, D, and L) were also conserved in the otherwise extensively different sequence of strain C10, which was also neutralized by MAb 75 but not by MAb 99. This indicates that the sequence R-G-D-L is important for neutralization by MAb 75, and strains containing only the tripeptide R-G-D are resistant. This finding is also supported by the finding of Rowlands et al. (29), Ouldridge et al. (22), and Xie et al. (37) that aa 148 (Fig. 5B) seems to play an important role in virus neutralization.

The structure of the epitope recognized by MAb 99 may be more complex. This antibody also recognized R-G-D-L-containing peptides but needed an additional glutamine (Q) for neutralization. However, if the antigenic site is presented in an α-helical conformation (25), it is evident that
almost all amino acid exchanges within the FMDV O serotype are located on the hydrophobic part of the helix (Fig. 7). Such exchanges had no influence on the efficacy of neutralization by MAb 99, in contrast to exchanges on the hydrophilic side, which affected neutralization by MAb 99 but not by MAb 75. Therefore, the possibility that the secondary structure of VP1 influences the MAb 99 epitope cannot be excluded.

Geyser et al. (13) tested 207 overlapping synthetic peptides representing the total VP1 sequence for their reactivity with hyperimmune sera from animals. The only peptide showing reactivity contained the amino acid sequence R-G-D, which is conserved in all FMDV strains except A10 (in which only G-D is present). It is interesting that this amino acid sequence plays a central role in binding of the glycoprotein fibronectin to the cell surface of many cells (for a review, see reference 30). Analysis of the cell attachment site of several other proteins led to the further conclusion that the core tripeptide R-G-D may provide a general binding sequence of various proteins to their target cells. This sequence also seems to play a role in the attachment of animal viruses (e.g., yellow fever virus) to host cells or in the adhesion of Escherichia coli to host tissue. For FMDV the sequence R-G-D may also be involved in cell binding, which is the first required step in virus infection. Thus, MAb 75 could function in virus neutralization by blocking the binding site on FMDV.

MAbs 7 and 48 recognized conformation-dependent epitopes formed by aa 144 to 159 and other amino acids outside this sequence. This suggestion is supported by the results of the ELISA (Fig. 2), in which the short peptides A and G1-21 were recognized by MAbs 7 and 48 but peptide G1-32 and coat protein VP1 were not. This observation is probably related to the loss of conformational flexibility in the longer polypeptides.

Sequence analyses of the coat proteins VP1, VP2, and VP3 and a part of VP4 of O1L and O1K mutants resistant to MAb 7 led to the conclusion that both of the other major coat proteins, VP2 and VP3, are most likely involved in the formation of the epitope structure recognized by MAb 7. Amino acid exchanges correlated with antibody resistance were found only in VP2 and VP3 and within the same region or even in the same position in the sequenced strain O1L and the mutants. A computer-derived three-dimensional model for FMDV that is based on the known structure of the human rhinovirus type 14 (HRV-14) indicates that the regions in VP2 and VP3 in which amino acid exchanges occurred are physically close to the major antigenic site in VP1 (R. Meloen, personal communication).

The epitope recognized by MAb 48 is different from the MAb 7 epitope, since only the latter antibody neutralized FMDV strain O Israel. In this case, lack of neutralization by MAb 48 is probably related to the two amino acid exchanges in positions 144 and 155. At the same position (aa 144), Xie et al. (37) found a Leu → Ser exchange when they analyzed five mutants selected with neutralizing MAbs. This position and the second exchange at position 155 (Val → Ala) are located next to each other in the suggested α-helix (Fig. 7). Thus, the amino acid in position 144 or 155 is important for the epitope recognized by MAb 48.

As shown previously for FMDV and other picornaviruses, conformation-dependent epitopes are important for virus neutralization (3, 12, 14, 15, 21, 24, 34). In the cases for HRV-14 and poliovirus type 1, binding sites of neutralizing antibodies have also been mapped by inhibition with synthetic peptides and sequence analysis of neutralization-resistant mutants selected by neutralizing MAbs and involve capsid proteins VP1, VP2, and VP3 or combinations of these polypeptides (8, 10, 33). These results have been confirmed by the three-dimensional structure of HRV-14 (28) and poliovirus type 1 (16).

In summary, we have shown for the first time that structural proteins other than VP1 are involved in neutralization of FMDV. The information obtained from mapping the different epitopes involved in FMDV neutralization could be used to gain an understanding of the humoral and cellular immune response mounted against FMDV infection and to formulate potential new vaccines from either synthetic peptides or recombinant proteins. The neutralizing epitopes mapped with murine MAbs should be verified with MAbs produced from bovine spleen cells, because, as has been shown for poliovirus, epitopes recognized by the murine immune system are not necessarily recognized by the natural host's immune system (10).

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LITERATURE CITED