Evidence for At Least Four Antigenic Sites on Type O Foot-and-Mouth Disease Virus Involved in Neutralization; Identification by Single and Multiple Site Monoclonal Antibody-resistant Mutants

By D. McCahon,1† J. R. Crowther,1 G. J. Belsham,1* J. D. A. Kitson,1 M. Duchesne,2 P. Have,3 R. H. Meloen,4 D. O. Morgan5 and F. De Simone6

1AFRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, U.K., 2Laboratoire Roger Bellon, 18 rue de Montbazon, 37260 Monts, France, 3State Veterinary Institute for Virus Research, Lindholm, 4771 Kalvehaves, Denmark, 4Central Veterinary Institute, Virology Department, Hontribweg 39, 8221 RA Lelystad, The Netherlands, 5Plum Island Animal Disease Center, United States Department of Agriculture, P.O. Box 848, Greenport, New York 11944, U.S.A. and 6Istituto Zooprofilattico Sperimentale, Brescia, Italy

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SUMMARY

Neutralizing monoclonal antibodies raised against type O foot-and-mouth disease virus have been characterized on the basis of their reactivity with a panel of single site monoclonal antibody-resistant mutants which had defined three antigenic sites. Five antibodies neutralized all these mutants, but by selecting further single site mutants with one of these antibodies it was possible to define a fourth site involved in virus neutralization. Two monoclonal antibodies still neutralized these mutants and all multiple site resistant mutants. One multiple site resistant mutant was resistant to neutralization at each of four antigenic sites but was still efficiently neutralized by type O convalescent cattle sera. The relationship between sites recognized by different monoclonal antibodies generated in different laboratories is discussed.

INTRODUCTION

Studies on the antigenic structure of two picornaviruses, poliovirus and rhinovirus, have been considerably enhanced by the solving of their three-dimensional crystal structure (Hogle et al., 1985; Rossmann et al., 1985) and the isolation and sequence analysis of monoclonal antibody (MAb) escape mutants (Minor et al., 1986; Sherry et al., 1986). Crystallographic studies on foot-and-mouth disease virus (FMDV), another picornavirus, are also in progress (Fox et al., 1987) and a number of laboratories have generated MAbs capable of neutralizing type O virus. Xie et al. (1987) characterized some 30 MAb-resistant mutants of the O1 Kaufbeuren strain of FMDV isolated using five different neutralizing MAbs. Three distinct antigenic sites were involved in virus neutralization. In this context the term antigenic site is used to describe an area of the virus surface which may contain several MAb epitopes; if one epitope is changed and this affects the ability of a second MAb to neutralize the virus then it is considered that the second MAb recognizes an epitope in the same antigenic site as that changed.

Recently Stave et al. (1988) have used different MAbs to define three sites of neutralization. In this communication we now use the panel of mutants generated by Xie et al. (1987) to map the binding sites of MAbs generated in different laboratories and then use these antibodies to show the existence of further sites involved in the neutralization of type O FMDV. We have also generated multiple site mutants which are resistant to neutralization at each of two, three or four sites.

† Present address: The Millmead Centre, Millmead, Guildford, Surrey, U.K.
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**METHODS**

**MAbs.** The neutralizing MAbs [B2, D9, 1C6 (C6), 4C9 (C9), 3C8 (C8)] used to generate the mutants described by Xie et al. (1987) were produced by Drs F. De Simone and E. Brocchi using the O1 Switzerland 1965 strain of FMDV, and have been characterized by Crowther et al. (1984). MAbs 04, 07, 010, 011, 013 and 014 were produced using O1 BFS 1860 and characterized by Meloen et al. (1983) (as MAb 4, 7, 10 etc.). MAb 14EH9.1.1 (14EH9) raised against O1 Brugge was described by Stave et al. (1988), MAb D7 was raised against O1 Aulendorf by Duchesne et al. (1984) and LMK1 (LMK) was prepared from mice inoculated with O1 Denmark 1982 (P. Have, unpublished). All MAbs used in this study efficiently neutralized the parental O1 Kaufbeuren virus used in generating all the mutants described in this study and by Xie et al. (1987).

**Isolation of mutants.** Single site mutants were isolated as described by Xie et al. (1987) and multiple site mutants were generated by using the same procedure successively with other MAbs.

**ELISA.** The trapping ELISA was performed as described in McCullough et al. (1985).

**Neutralization assays.** Neutralization assays were performed either as described by Xie et al. (1987) on BHK cells or using a microneutralization assay in which antibody dilutions and virus (10 TCD50 units) were added directly to IBRS-2 cells (3 x 10^4) in suspension and plated into microtitre wells. After 40 h wells were stained with methylene blue and scored for c.p.e. Comparative experiments indicated that equivalent results were obtained in each assay.

**RESULTS**

The studies of Xie et al. (1987) characterized a set of 30 MAb-resistant mutants isolated using five different neutralizing antibodies (B2, D9, C6, C9 and C8). Initial screening of the nine additional MAbs used a neutralization assay against representatives of the previously characterized MAb-resistant mutants. These studies (see Fig. 1) indicated that MAb D7 recognized site 1. The apparent resistance of mutant 559 (selected with a site 2 MAb) to neutralization by D7 (and to some extent by B2) is not indicative of a link between sites 1 and 2.

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**Fig. 1.** Grouping of monoclonal antibody epitopes into four epitopes by cross-neutralization assays. O, Complete resistance to neutralization; O, partial resistance; ●, sensitivity. Resistant viruses are defined as showing <50% reduction in titre at 10^-2 antibody dilution (highest concentration used); sensitive viruses showed >95% reduction in titre under these conditions and partially resistant viruses fell between these two groups. Part of these data has been published (Xie et al., 1985) and is shown for completeness.
since it has become clear that this mutant is an adventitious double mutant. Of 17 mutants selected with MAbs C6 or C9 the mutant 559 alone showed resistance to neutralization or loss of binding of antibody in ELISA with site 1 MAb B2 (data not shown). Furthermore all 16 mutants selected with B2 or D9 (site 1 MAbs) were fully neutralized by C6 or C9 and all tested (10 of these) were also fully neutralized by C8 (site 3). Sequence analysis has shown that mutant 559 contains a mutation at residue 144 of VP1 [capable of conferring resistance at site 1 (Xie et al., 1987)] in addition to a change at amino acid 72 of VP2 which alone is capable of conferring resistance to the site 2 MAbs (J. D. A. Kitson & G. J. Belsham, unpublished results). MAbs 04, 010 and LMK recognized site 3 as defined previously (see Fig. 1). These additional MAbs recognizing site 3 were particularly helpful since this site had previously only been defined by one antibody (C8). Five MAbs (07, 011, 013, 014 and 14EH9) were capable of neutralizing all of the single site MAb-resistant mutants. They also neutralized two independent multiple site mutants (861 and 871) which had been successively isolated using MAbs defining sites 1, 2 and 3 (see Fig. 2), thus indicating the existence of further antigenic sites involved in virus neutralization.

To confirm the assignment of MAb LMK to site 3 it was necessary to isolate mutants resistant to this MAb and to perform further cross-neutralization studies. The LMK-selected mutants 58 and 59 were still sensitive to neutralization by each of the other site 3 MAbs and showed some resistance to neutralization by MAbs that recognized sites 1 and 4; however, since mutant 624 (C8-resistant) was also fully resistant to LMK we feel that this antibody is correctly assigned. Furthermore subsequent sequence analysis (J. D. A. Kitson & G. J. Belsham, unpublished) showed that mutant 58 selected with LMK is altered at residue 45 in VP1 whereas mutants selected with C8 are altered at residue 43 or 44 in this protein. These changes are independent of those found previously in site 1 mutants (Xie et al., 1987) and from the change in VP3 found in site 4 mutants.

MAb 14EH9 was also used to isolate mutants (16 and 63) from the parental virus and also from the multiple site resistant mutant 871 (67). These 14EH9-resistant mutants allowed a clear subdivision of the residual five MAbs. MAbs 07 and 014 were no longer capable of fully neutralizing either the single site mutants 16 and 63 or the multiple site resistant mutant 67 and hence map to the same site as 14EH9. Furthermore, the 14EH9 MAb-resistant mutants were
still fully susceptible to neutralization by all of the site 1, 2 and 3 MAbs so it seems clear that these viruses do define a fourth site for neutralization. However, two MAbs (011 and 013) still efficiently neutralized all the viruses and so presumably recognized at least one further site; the isolation of resistant mutants is needed to confirm this but preliminary attempts to isolate such mutants have been unsuccessful (J. D. A. Kitson, unpublished results).

The same mutants studied in the cross-neutralization experiments have also been used in assays of antibody binding as detected in a trapped ELISA. Fig. 3 shows the results obtained and the subdivision obtained from the cross-neutralization data (see above). Four double resistant mutants and the triple and quadruple site mutants generated were also examined in the ELISA and confirmed the results obtained with the single site mutants. In general there was a correlation between the inability to neutralize a particular virus and lack of binding in the ELISA. Furthermore, most mutants which were still effectively neutralized showed efficient binding of the MAb in ELISA. However, a considerable number of mutants selected at one site showed diminished binding of MAbs that recognize other antigenic sites, e.g. mutants selected with the antibodies LMK and 14EH9. Rather surprisingly mutants selected with LMK still bound the LMK MAb efficiently in the ELISA, a phenomenon similar to one reported previously with poliovirus type 1 (Blondel et al., 1986).

The absence of correlation between the binding of antibody as defined by the ELISA and the ability of the antibody to neutralize a virus is also clearly seen from the initial panel of 32 mutants resistant to either B2, D9, C6, C9 or C8. We observe that four of six partially resistant mutants

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Fig. 3. Grouping of monoclonal antibody epitopes by trapped ELISA. $A_{492}$ values obtained with a polyclonal serum were used to standardize for differences in antigen content and $A_{492}$ obtained with a monoclonal antibody calculated as a percentage of the polyclonal antibody value; $\bullet$ indicates a ratio of <20%, $\circ$ indicates a ratio >50% and $\circ$ is intermediate.
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Fig. 4. Titration of the parental virus (○), a partially resistant site 1 mutant, 471 (□), and a fully resistant site 1 mutant (●) against two site 1 MAbs, B2 (a) and D9 (b). Both mutants were selected with D9.

Fig. 5. Neutralization of multiple site mutants 871 (□, triple resistant) and 67 (▲, quadruple resistant) and parental O, Kaufbeuren virus (●) by convalescent cattle serum from an O, FMDV-infected animal.

mutants also showed high antibody binding while some eight of 26 completely resistant mutants still measurably bound the antibody; hence in only 20 of 32 cases does a strict parallel occur between these properties. One very clear example of poor correlation is shown in Fig. 4. The binding of the selecting antibody D9 to the partially resistant mutant 471 appeared almost
identical to that seen with the parental virus, whereas the binding of another antibody, B2, which recognizes the same antigenic site was more significantly reduced; neither antibody bound to the fully resistant mutant 480 (also selected with D9).

It is desirable that the antigenic sites that we identify using mouse MAbs are relevant not only to mice but also to cattle. The parental virus, a triple and a quadruple resistant virus were treated with a range of dilutions of serum from cattle previously infected with type O FMDV and neutralization was determined by plaque assay. The mutants were neutralized at least as efficiently as the parental virus (Fig. 5). Since we have already shown that two monoclonal antibodies (011 and 013) still efficiently neutralize these viruses, it is likely that further sites involved in neutralization must be present on the virus surface and would need to be modified to confer resistance to polyclonal cattle sera.

**DISCUSSION**

The isolation and characterization of MAb-resistant mutants on the basis of virus neutralization and antibody binding in the ELISA has allowed the identification of at least four antigenic sites involved in virus neutralization. The antigenic sites identified need to be defined at the molecular level, from both sequence analysis and a knowledge of the three-dimensional structure of the virus. Already certain sites are becoming defined. Xie et al. (1987) showed that mutants resistant to antibodies B2 and D9 (site 1) have sequence alterations at amino acids 144, 148 and 154 of VP1 and will bind to the peptide corresponding to the 140 to 160 region of VP1 as well as isolated VP1. The antibody D7 also binds to isolated VP1 (Duchesne et al., 1984) and has now been shown to map into the same site. Recently Stave et al. (1988) showed that two further MAbs (12FE9 and 13DD2) which also bind to isolated VP1 allowed the selection of mutants altered at residues 148 or 138 and 144 respectively. Monoclonal antibody 13DB1, although failing to bind isolated VP1 does appear to recognize the same antigenic site as 13DD2 and 12FE9 since it fails to neutralize mutants selected with these two antibodies. It is therefore likely that these MAbs also recognize site 1. Furthermore we have obtained data from the ELISA (not shown) that 13DB1 and 13DD2 failed to bind mutant 480 (D9-selected) but did bind to 618 (C8-selected) and 487 (C9-selected) thus confirming this suggestion. Sequence analysis of site 1 mutants suggests that this site corresponds with site NImlI of rhinovirus 14. Sequence analysis of other mutants is in progress and will indicate whether the antigenic sites of different picornaviruses are in equivalent positions. Recent analysis of these mutants does indeed show that two distinct regions of VP1 and residues in VP2 and VP3 independently contribute to the four sites we have defined (J. D. A. Kitson & G. J. Belsham, unpublished results).

We do not find an exact parallel between the ability of antibody to bind virus as defined by the ELISA and its ability to neutralize, and such correlation would probably be different again in vivo since it is likely that virus bound to non-neutralizing antibody would be cleared in the absence of virus neutralization. It seems that the virus-MAb interaction that occurs in the mutant selection and in virus neutralization assays is constrained differently from this interaction in the ELISA. The extent of correlation may depend on the affinity of an antibody for the virus and the mechanism by which the antibody neutralizes the virus [different numbers of antibody molecules may need to be bound to achieve neutralization (see McCullough et al., 1987)].

It has been disappointing that we have not succeeded in generating mutants of the virus that can be distinguished by convalescent cattle sera. However, these mutants may prove useful starting points in the selection of mutants resistant to neutralization by such polyclonal sera. It is also becoming apparent that the antigenic status of type O FMDV may be rather more complex than in other serotypes of this virus. Rowlands et al. (1983) showed that in A12 some natural variants were altered at residues 148 and 153 (site 1) and were recognized differently by convalescent guinea-pig sera suggesting that this site was immunodominant. Although only limited sequencing of these A12 variants has been reported, further sequencing of the whole P1 region has failed to find other differences between these variants (S. E. Newton & D. J. Rowlands, personal communication). No clear immunodominance seems apparent in mice with FMDV type O since several MAbs have been found for each of the sites and the mutants
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(including those with changes at site 1) are still recognized and neutralized by the type O convalescent cattle sera.

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**REFERENCES**


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