Identification of a fifth neutralizable site on type O foot-and-mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants

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A monoclonal antibody (C3) produced against foot-and-mouth disease virus type O1Caseros was found to neutralize quadrivalent monoclonal antibody escape mutant (G67) of foot-and-mouth disease virus type O1Kautbeuren. This mutant had been characterized at the sequence level as having distinct changes affecting four non-overlapping neutralizable sites. The C3 monoclonal antibody was used to prepare a quintuple escape mutant from the G67 and a single escape mutant from the parental O1Kautbeuren viruses. Polyclonal post-vaccinated and infected cattle sera as well as polyclonal mouse and guinea-pig sera, which neutralized the quadrivalent mutant, no longer neutralized the quintuple mutant, indicating that a fifth site had been identified and that changing the fifth site eliminated all neutralization. The site was characterized using serological techniques and found to be conformationally dependent, trypsin-sensitive and independent of sites previously characterized by monoclonal antibodies. Amino acid sequencing comparing parental, single C3 and quintuple mutants showed that a single change from a glutamine to a histidine, at amino acid 149 in the structural protein VP1, (1D) characterized the C3 mutation. The fifth site probably represents a conformational epitope which is formed due to the interaction of the VP1 loop region with other surface amino acids.

Introduction

Previous studies on foot-and-mouth disease (FMD) virus (FMDV) have indicated that there are at least four independent antigenic sites on the capsid involved in virus neutralization (Haresnape et al., 1983; Xie et al., 1987; McCullough et al., 1987; Thomas et al., 1988; Pfaff et al., 1988; McCahon et al., 1989; Kitson et al., 1990). Such studies involved the sequencing of escape mutants produced by selected monoclonal antibodies (MAbs) mainly for type O and A viruses. Xie et al. (1987) reported the sequence variation in O1Kautbeuren (O1K) mutants selected using seven neutralizing MAbs produced against type O1/Swiss/1965 virus. Four non-overlapping sites were found by ELISA and neutralization assays, two sites were conformational and the third was less conformationally dependent. Four antigenic sites were proposed, of which site 1 was associated with MAbs recognizing a synthetic peptide (140 to 160) of VP1 but which did not recognize trypsin-treated virus. Sequencing of the VP1 region of these mutants identified substitutions at residues 1144, 1148 and 1154. The critical amino acid substitution at 1144 was identified by the study of a range of mutants isolated after pressure from two similar MAbs. Antigenic site 2 (isolated after pressure from C6 or C9 MAbs), identified substitutions in VP2 at 2070, 2071, 2073 and 2031 for C6 and 2071, 2072, 2075 and 2077 for C9. Variations in the resistance to neutralization by the individual MAbs of the various single mutations were also observed. This site was dependent on conformation. Antigenic site 3 (C8 MAb) involves residues 1043 and 1044. The site was dependent on conformation. Antigenic site 4 was identified by mutants isolated after pressure from a single MAb (14EH9). Residue 3058 was identified in the two mutants examined and the site was assessed as being dependent on conformation.

Multiple site mutants were obtained by successive single step selection with neutralizing MAbs (McCahon et al., 1989). Sequence analysis of the viruses isolated showed that they had accumulated mutations which had been identified separately as conferring resistance at individual sites. The viruses and substitutions are shown in Table 1.

These findings and the influence of antigenic variation may have relevance for vaccine selection and in under-
standing the evolution of viruses. The quadruple mutant G67 and the triple mutants 861 and 871 are non-infectious in guinea-pigs (J. R. Crowther, unpublished). However, guinea-pigs inoculated with inactivated triple and quadruple mutants as vaccine were protected against challenge with parental O1K. This indicates that there is at least one other site involved in neutralization, a point substantiated by the fact that polyclonal sera produced against parental virus neutralize the quadruple mutant. This study investigates the reaction of a virus after a further selection with a MAb (C3) which was found to neutralize the quadruple mutant 67.

**Methods**

**Viruses.** Type O viruses OIK, single mutants 480, 597 and 599, triple mutants 861 and 871, and the quadruple mutant G67, were obtained from the Division of Molecular Biology, at the AFRC Institute.

**Antisera.** Polyclonal antisera were obtained from cattle vaccinated with O1BFS vaccine after 21 days. Cattle sera were obtained 10 days after infection with the same virus. Rabbit and guinea-pig antisera against type O FMDV were obtained from the World Reference Laboratory for FMD (WRL) at the AFRC Institute. The sera have been produced against purified O1BFS virus by multiple vaccination with inactivated virus and were used in the ELISAs described below.

**Monoclonal antibodies.** Neutralizing MAbs B2, C6, C8, C9 and D9 were from the Istituto Zooprofilattico, Brescia, Italy (as described by Xie et al., 1987). MAb C3 (tissue culture sample) against type O1Caseros was produced by one of the authors, S. Farias, at the Department of Physiology, Biotechnology Centre, Federal University, Porto Alegre, Brazil. Other MAbs used in the characterization are described in the Results.

**Production of new single and quintuple mutants.** The parental and G67 quadruple mutant of O1K were titrated in a microtitre plate assay in BHK-21 cells by dilution of virus in Eagle’s medium in 50 µl volumes plus addition of 50 µl of BHK-21 cells (5 x 10⁵ ml) in Eagle’s medium containing 8% normal bovine serum. The plates were covered, incubated for 2 days and then examined microscopically for c.p.e. One ml of virus G67 at 10⁶ TCID₅₀ was added to 50 µl of MAB C3. After mixing and incubation at 37 °C for 20 min the mixture was pipetted onto a monolayer of washed (with PBS) BHK-21 cells (25 ml flask). Ten ml of Eagle’s medium was then added containing MAB C3 at a 1/50 dilution and the cells were incubated at 37 °C. After 24 h, the infected cell debris was removed by low speed centrifugation and 1 ml of the supernatant was processed as above by the addition of MAB and inclusion of MAB at 1/50 in the Eagle’s medium. One ml was also processed in the same way, except that the 10 ml of medium did not have MAB added. A further 1 ml was added without addition of MAB to either stage. The virus contained in supernatant fluids was examined in a sandwich ELISA (antigenic profiling) as described by Samuel et al. (1991), where MAB panels were reacted with virus captured on ELISA plates coated with rabbit polyclonal antisera. The reaction of the MAbs with the viruses was compared to their reaction with captured parental virus. The amount of virus available in the system was checked by determining the reaction of the captured viruses using the polyclonal guinea-pig serum follow by anti-guinea-pig conjugate. In this way the epitope profile of the mutants was determined. Thus mutants could be determined when binding of the selecting MAB was eliminated. Mutants grown in the absence of MAB selection pressure that maintained the appropriate epitope profile were then tested in virus neutralization (VN) tests. Samples were stored at −70 °C after the addition of an equal volume of sterile glycerol.

**Preparation of trypsin-treated virus.** To 100 µg of purified OIK from a sucrose gradient preparation was added 50 µl of trypsin solution at 2 mg/ml in 0.1 M-phosphate buffer pH 7.4. The mixture was incubated at 37 °C for 15 min. The treated virus was then diluted to the assay concentrations in the relevant buffers.

**Preparation of denatured virus.** To 1 ml of purified OIK at 50 µg/ml was added 10 mg of SDS and 20 µl of 2-mercaptoethanol, and the mixture was heated in boiling water for 3 min.

**Characterization of MAbs using mutants.** The sandwich ELISA was used to screen the reactivity of MAbs obtained from sources worldwide against mutant viruses captured using the rabbit antiserum-coated plates. The various MAbs were added as triplicate samples at a concentration that was found to give a plateau maximum absorbance when titrated against the parental virus in the sandwich ELISA. After incubation and washing, the reaction was determined by addition of an anti-mouse horseradish peroxidase system and o-phenylenediamine (OPD)/H₂O₂ substrate solution. The reactions of the MAbs were standardized with reference to the concentrations of the captured viruses; these were determined using a polyclonal guinea-pig antibody system.

**Examination of trypsin-treated virus.** The sandwich and direct ELISAs were used to examine the effect of trypsinization of the virus. In the direct ELISA purified and trypsin-treated OIK virus was added directly to PVC plates in PBS at various concentrations (5 µg/ml, 2.5 µg/ml and 1.25 µg/ml). The plates were incubated at 37 °C for 2 h with rotation, and wells were then washed with PBS. MAbs B2, D9, C6, C8 and C3 were then added as dilution ranges diluted in blocking buffer (5% skimmed milk/0.1% Tween 20 in PBS). Plates were incubated for 1 h as above and washed. The system was then developed by addition of anti-mouse horseradish peroxidase diluted in blocking buffer,
incubation for 1 h as above and then addition of OPD/H₂O₂ substrate. Colour development was stopped at 10 min.

A similar procedure was used for the examination of trypsin-treated virus in the sandwich ELISA except that the virus was captured via rabbit antibody attached to PVC plate wells as described elsewhere in this paper.

**Examination of denatured virus.** Denatured and non-denatured O₁K was added to PVC plate wells (50 µl) diluted in PBS from 20 µg/ml in a two-fold range over 11 wells. Plates were incubated at 37 °C for 2 h then washed. MAbs B2, D9, C3, C6 and C8, as well as a polyclonal mouse antisemur to O₁K, diluted in blocking buffer (50 µl in triplicate wells) at twice the concentration that gave a plateau maximum colour from previous titrations in the non-capture ELISA using parental virus were then added. After incubation for 1 h at 37 °C and washing, bound mouse antibodies were detected using the anti-mouse conjugate system as described already.

**Virus neutralization tests.** The mutant virus samples grown under different conditions were titrated in microtitre plates (as octuplicates) as described above to determine their TCID₅₀ titres. The various MAbs and polyclonal sera (as described in results) were diluted in quadruplicate in Eagle's medium across a microtitre plate. The various viruses were added diluted to 50 TCID₅₀ in Eagle's medium. The mixture was incubated at 37 °C for 2 h then washed. MAbs B2, D9, C3 and C8, as well as a polyclonal mouse antisemur to O₁K, diluted in blocking buffer (50 µl in triplicate wells) at twice the concentration that gave a plateau maximum colour from previous titrations in the non-capture ELISA using parental virus were then added. After incubation for 1 h at 37 °C and washing, bound mouse antibodies were detected using the anti-mouse conjugate system as described already.

**Table 1.**

<table>
<thead>
<tr>
<th>Competitors</th>
<th>MAb B2</th>
<th>MAb D9</th>
<th>MAb C3</th>
<th>MAb B2</th>
<th>MAb D9</th>
<th>MAb C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₁K virus</td>
<td>38</td>
<td>30</td>
<td>*-</td>
<td>80</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>(O₁K) peptide</td>
<td>24</td>
<td>10</td>
<td>10</td>
<td>80</td>
<td>20</td>
<td>250</td>
</tr>
<tr>
<td>(G67) peptide</td>
<td>-</td>
<td>350</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>250</td>
</tr>
<tr>
<td>(G67+C3) peptide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O₁K virus</td>
<td>0.6</td>
<td>2</td>
<td>0.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>O₆ + C3 virus</td>
<td>3.7</td>
<td>5.6</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G67 virus</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G67 + C3 virus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* - No competition for MAb.
† ND, Not done.

A similar competition assay was done using O₁K virus captured on the PVC plates using only the peptides as competitors. Plates were coated with rabbit anti-type O serum in 0.05 M-carbonate/bicarbonate buffer pH 9.6, and were then incubated overnight at 4 °C. Next, the plates were washed and purified type O₁K was added, diluted in blocking buffer (as for indirect ELISA) in 50 µl per well at 2 µg/ml. Plates were incubated for 1 h at 37 °C and then washed. The procedure described above for the competition assay against virus directly attached to plates was followed. Data were plotted relating the weight of peptide added against the inhibition of the MAb reaction. Competition curves were plotted and the amount of peptide giving 50% competition was calculated. The results are shown in Table 2. Fig. 5 shows the results where the amounts of the peptides showing 50% inhibition of the different MAbs have been expressed as a reciprocal and multiplied by 1000. Fig. 6 shows the same analysis where the purified viruses are the competitors.

**Results**

Table 1 gives the mutants used in this study and the amino acid residues involved, as determined from sequence analysis of the mutants. Fig. 1 shows the ELISA data for the analysis of O₁K mutants with different neutralizing MAbs produced against type O FMD viruses and illustrates one use of established MAbs to characterize MAbs with unknown reactivity rapidly. The profiles shown are illustrative data taken from analysis of 54 MAbs from all over the world. Thus MAbs 48 and 99 react with the epitope identified by MAbs 2, 4 and react with a C6/C9-like epitope, reactions that have been confirmed subsequently by other methods. The reaction of MAb 23 is identical to that of C3. All of the MAbs used were able to neutralize the parental isolate, but because MAbs that do not neutralize are able to cross-react with all the mutants, care is needed in assessing results based on ELISA data alone. VN testing of the MAbs with the mutants confirmed the picture shown in Fig. 1 where binding in
Fig. 1. ELISA reactions of MAbs against parent and mutant viruses G67 (quadruple) and G67+C3 (quintuple). The specificity of MAbs for MAb escape mutants is shown, and mutants 480, 598, 599, 861, and 871 are included for comparison. MAbs 23, 48, 99, 11, and L4 illustrate the use of escape mutants for rapid screening of MAb specificity. ■, Same reaction as with homologous virus; □, no reaction.

The ELISA correlated with neutralization of virus (results not shown).

Use of purified trypsin-treated virus in the sandwich and non-capture ELISAs showed that the MAb against C3 no longer reacted with O1K after treatment in either system. The test also confirmed the trypsin sensitivity of the sites recognized by B2 and D9 and the lack of sensitivity of sites C6, C8, and C9.

When the parental virus was denatured, C3 MAb no longer bound with the antigen directly bound to the wells. The test also confirmed that MAbs B2 and D9 were not affected when virus is denatured, whereas C6, C8, and C9 were. The results of VN tests using polyclonal sera against the parental virus and some of the mutants are shown in Fig. 2. Fig. 3 shows the VN titre obtained against G67, plotted as a percentage of the titre found against the parental virus. The group mean residual percentage titre against G67 for the post-vaccinated animals was 15 (s.d. = 5) and this was significantly different (P = 0.01) to the group mean titre of the post-infected animals (44, s.d. = 32).

Nucleotide sequence analysis is incomplete. Most of the VP1 gene has been sequenced and a deduced single amino acid substitution has been found at residue 149 on both the G67+C3 (quintuple mutant) and the O1K parent virus with the single C3 mutation. There is a change from a glutamine (Q) to histidine (H) in both cases. The sequences of approximately half of the 3’ end of the VP2 gene has been determined and no changes are evident. Approximately 30% of the 3’ end of the VP3 gene has been sequenced and again no substitutions have been found for the G67+C3 virus. Fig. 4 shows the peptides synthesized based on the changes noted for the sequencing of the parent and escape mutants.

The results showing the competition data for the peptides competing for O1K virus directly coated on

Fig. 2. VN titres of post-infection (a) and post-vaccination (b) cattle sera reacting with parental virus and quadruple and quintuple MAb escape mutants. No neutralization was observed for the quintuple mutant. ■, O1K; ☐, G67; □, G67+C3.
Fig. 3. VN titres of individual post-infection and post-vaccination cattle sera against G67 quadruple mutant expressed as a percentage of their VN reaction for patient virus O\textsubscript{K}.

Fig. 4. Amino acid sequences of synthetic peptides produced, residues 141 to 152 of O\textsubscript{K} VP1. Sequences are based on those changes found in MAb escape mutants G67, G67+C3 and parent+C3.

Competing peptide

Parent+C3 9 [Non-captured]
Parent 10
G67 11
G67+C3 12
Parent+C3 9 [Captured]
Parent 10
G67 11
G67+C3 12

Fig. 5. Comparison of amount of peptides causing 50% inhibition of the reaction of captured and non-captured virus with MAbs. High values indicate good competition (low amount of peptide achieved competition) and high affinity. MAbs: ■, B2; □, D9; □, C3.

Fig. 6. Comparison of amount of viruses causing 50% inhibition of the reaction of non-captured virus with MAbs. High values indicate good competition (low amount of virus achieved competition) and high affinity. MAbs as in Fig. 5.

competitors for the particular systems. The difference in scales in Fig. 5 and 6 should be noted.

There are qualitative and quantitative differences in
the results for the peptides between the non-capture and capture systems. In the non-capture system, the peptide with the parental structure competes for all three of the MAbs to a similar degree. Peptide 9 (parent + C3) competes similarly for B2 and D9 but not for C3 as might be expected, and only peptide 11 (G67) is able to compete for C3 binding. None of the MAbs are inhibited using the G67 + C3 peptide structure. In the capture system, more peptides are needed to achieve 50% competition. This probably reflects an increased affinity of the MAbs for non-captured parental virus. This is most marked when comparing the reactions of peptides 10 (parental) and 11 (G67) for MAB C3. Data obtained for the competition of the purified viruses shows that there is a large decrease in the amount of competitor needed to achieve 50% competition for the parental virus as compared to the peptide data.

Discussion

The existence of at least one additional antigenic site involved in neutralization of type OIK FMDV was indicated by the ability of polyclonal antisera from various species, particularly cattle sera, to neutralize multiple site mutants selected by four MAbs. These MAbs have been shown to characterize four non-overlapping neutralizable antigenic sites and comparisons with poliovirus and human rhinovirus 14 (HRV14) showed that essentially the same elements within the virus capsid proteins were recognizable. Thus, for each of the picornaviruses, one site is located around the fivefold axis (site 3 of OIK, NIM-I/IB of HRV14 and site 1 of poivovirus). A second site is located in the centre of the GH loop (site 1 of OIK, NIM-II of HRV14 and site 3 of poliovirus), and a third site is located close to the two- and threefold axes (sites 2 and 4 of OIK, NIM-III of HRV14 and sites 3 and 4 of poliovirus).

Screening by ELISA identified MAbs that bound to the quadrivalent mutant and VN tests confirmed that the two antibodies (C3 and 28) neutralized this virus. The use of available MAB escape mutants should be stressed since it rapidly identifies MAbs defining new epitopes. The epitopes defined by C3 was highly conserved in type OIK FMDV since all the field isolates profiled by ELISA with C3 were reactive. An amino acid change at 149 on the loop region of VP1 from glutamine to histidine was critical for the C3-defined epitope. It is interesting that the histidine at 149 is highly conserved in isolates of type O for which sequence data are available. The site was trypsin-sensitive and probably conformation-dependent since the MAb reaction was eliminated when virus was denatured, unlike the site 1-reactive MAbs (B2 and D9) which are trypsin-sensitive but do react with denatured virus and isolated VP1. Mutants isolated using MAbs B2 and D9, which recognize linear epitopes on the loop of residues 140 to 160, still bound MAB C3, and MAbs B2 and D9 still bound to the OIK+C3 mutant. This is evidence that the C3 binding site is a distinct neutralizable epitope which, although involving a component of the 140–160 loop, has a conformation-dependent interaction on the virion surface. The C3 site may be formed through a particular orientation of the VP1 (GH) loop i.e. when the loop is in a ‘down’ position and lying close to other surface residues (Parry et al., 1990).

Examination of the quintuple (G67 quadruple + C3) escape mutant showed that alteration of a fifth determinant eliminated the ability of polyclonal cattle sera to neutralize the virus. The change elicited by C3 selection on the parental virus did not affect the VN titre of polyclonal sera since there are four other sites through which neutralization can be mediated. There was a significant difference in the titres of sera in the post-vaccinated and infected cattle reacting with the quadrivalent mutant as compared to the reaction with parental virus. The neutralization titre against the G67 mutant can be considered to be the result of interaction of antibodies with the ‘fifth’ neutralizable epitope, since alteration of the site eliminates neutralization. Thus there was a qualitative difference in the specific antibody produced in animals receiving inactivated vaccine as compared to live replicating virus. Further work to examine a larger number of sera is needed to obtain more confidence in this result, but it may be a reflection of the fifth site’s susceptibility to alteration throughout the vaccine manufacturing process and/or it may be affected by the virus becoming adsorbed to the solid-phase in vaccines, such as alhydrogel.

The fact that neutralization was eliminated with the quintuple mutant also gives some confidence that the epitopes recognized in the mouse are the same as those in cattle. This has implications for the use of MAbs as reagents in the rapid characterization of field and vaccine strains in that the epitopes examined are probably relevant in cattle. Other studies (results not included) have shown that virus neutralization of the quintuple mutant by polyclonal guinea-pig and mouse sera is eliminated. Examination of the ability of specific peptides to compete in the liquid phase for the interaction of MAbs with parental virus is interesting. The capture system required more peptide to achieve 50% competition and this possibly reflects the increased affinity of the MAbs for the non-captured virus. This was pronounced for competition of peptides 10 and 11 for the C3 MAb where there was a 30- and 40-fold difference in the amount giving 50% inhibition between the non-capture and capture systems, respectively. The effect was not so marked for the competition of peptides 9 and 10.
for B2 and D9. Thus, the reaction of C3 MAb (hence the C3 epitope) appears to be altered when the parental virus is attached directly to the solid phase. Attachment of virus directly to wells has been shown to affect the binding of MAbs due to conformational changes in antigenic sites on the virion (McCullough et al., 1985; Ouldridge et al., 1984). The reaction with the peptides and C3 is contrary to the ELISA evidence that the MAb is against a wholly conformational site, but large amounts of the peptides were needed to achieve this competition as compared to the mutant viruses. The parental virus competed with all three MAbs, with a 70-, 50- and 70-fold reduction in the amount of virus needed for 50% competition, respectively, compared to the peptides. The data also show that O,K+C3 competes only for B2 and D9 MAbs (with a 100- and 120-fold reduction in weight of virus compared to peptide) and that G67 only competes for C3 MAb (125-fold reduction). Thus the relative affinity for the MAbs and liquid phase-competing viruses is greatly increased as compared to that of the peptides. The relative affinities are increased further when considering the amount of peptide contained in the viruses, which would represent approximately 1/100 of the total weight of virion protein. Thus the viruses compete 100 times better than the values in Fig. 6 suggest.

This paper demonstrates that selection of a quintuple MAb escape mutant (G67+C3) eliminates neutralization by polyclonal antibodies. This is the first example of this phenomenon and we now have a set of MAbs that represent five distinct neutralizable epitopes. Assessing the likelihood that a field virus is a threat to vaccinated animals will be made easier if MAbs that represent each of the five neutralizing sites are used in a panel for antigenic profiling, especially if the mass of the antibody elicited against each site can be quantified. Paratope profiling of immune sera from cattle is possible using the set of mutant viruses that have successive mutations at the different neutralizing antigenic sites. Similarly, the amount of non-neutralizing antibody for a particular serum could be estimated and the effects of competition between neutralizing and non-neutralizing antibodies investigated. The structure of the virion in the quintuple (G67+C3) and single (O,K+C3) mutants is being examined using X-ray crystallographic analysis. The C3 MAb is also being used to prepare further mutants to examine whether other amino acid residues can be identified in other surface structural proteins that affect the same site, in an attempt to examine where interaction of the VP1 loop and other surface amino acids may take place.

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References


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