Neutralization of Foot-and-Mouth Disease Virus Can Be Mediated Through Any of at least Three Separate Antigenic Sites

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SUMMARY

Seven neutralizing monoclonal antibodies were used to characterize 30 escape mutants of a type O foot-and-mouth disease (FMD) virus (O1 Kaufbeuren) selected with the five most active antibodies. Three non-overlapping antigenic sites were found by ELISA and cross-neutralization studies. Within two of the sites the epitopes of two or more monoclonal antibodies overlapped. Two of the sites were conformation-dependent and could not be detected on virus subunits or isolated denatured polypeptides. The third site was less conformation-dependent since the appropriate monoclonal antibodies were able to bind to 12S subunits, isolated VP1 protein and a synthetic peptide containing residues 141 to 160 of VP1 in ELISA. Electrofocusing of mutants of that site showed a high frequency of electrophoretic alterations in VP1. The sequence of most or all of the VP1 coding region of 10 escape mutants of that site plus three parental isolates was determined by primer extension sequencing. At least five amino acids were found to be involved but in only one case (residue 148 of VP1) did a change at that residue produce complete resistance to neutralization. Partial resistance was produced by changes at residues 144, 154 or 208 of VP1 or another residue(s), as yet undefined, that is probably in one of the other capsid polypeptides. Thus the site defined by these mutants was made up of at least three regions, the region involving residues 144 to 154 of VP1, the region encompassing residue 208 from the COOH terminus of VP1, plus a region, probably of VP2 or VP3, encompassing the undefined residue(s).

INTRODUCTION

Foot-and-mouth disease (FMD) virus is a picornavirus. Previous studies with FMD virus have identified fragments of isolated VP1 protein (Strohmaier et al., 1982) and synthetic peptides from VP1 (Bittle et al., 1982; Pfaff et al., 1982) which stimulate antibody production, albeit of poor neutralizing activity, or are recognized by anti-virus antibodies (Geysen et al., 1984, 1985). Such studies identified several regions within the C-terminal half of VP1 which had antigenic activity but the most significant was the region bounded by residues 141 to 160 with perhaps a minor contribution from residues 200 to 213 at the extreme COOH terminus. Two types of study using different strains of virus gave slightly differing answers as to the key amino acids involved in the antigenicity of the 141 to 160 peptide. Antibody recognition studies by Geysen et al. (1985) on synthetic hexapeptides identified two leucine residues (positions 148 and 151) as being important. Sequencing of three naturally occurring antigenic variants of A12 virus by Rowlands et al. (1983) showed two amino acid residues (positions 148 and 153) were altered in each variant, suggesting that both these residues were important.

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Recently the three-dimensional structure of two other members of the picornaviridae, poliovirus type 1 and rhinovirus 14, have been determined to atomic resolution by X-ray crystallography (Rossman et al., 1985; Hogle et al., 1985) and this work has greatly increased our understanding of the nature of the antigenic sites on those viruses. For example, in rhinovirus 14, sequencing studies on neutralization-resistant mutants have identified a number of different amino acid residues which contribute to the four neutralizing antigenic sites (Sherry et al., 1986). In some cases the residues involved in a site were well separated on the linear sequence of the polypeptide and in others more than one polypeptide was involved. However, the crystallographic studies showed that for each site the residues were clustered in three dimensions on the surface of the particle. None of the residues identified by sequencing mutants occurred outside these sites irrespective of their location on the linear sequence. A similar situation was found for the three sites on the poliovirus type 1 particle (Hogle et al., 1985).

We have now made use of the strategy employed previously with rhinovirus and poliovirus, namely the isolation and characterization of neutralization-resistant mutants. This has revealed the presence of three distinct sites on the capsid of type O FMD virus that are involved in the neutralization of virus infectivity. Sequencing studies on the mutants of one site showed that it corresponded approximately to the VP1 site defined by the earlier biochemical studies but it was more complex and more conformation-dependent than those studies could suggest. The other two sites were even more dependent on conformation and their location on the capsid has yet to be determined.

METHODS

Viruses. All viruses were supplied as primary bovine thyroid (BTY) passage material by the World Reference Laboratory of this Institute.

Monoclonal antibodies. The seven neutralizing monoclonal antibodies used in this study were a generous gift from Drs F. De Simone and E. Brocchi of the Istituto Zooprofilattico, Brescia, Italy who prepared them against the O1, Switzerland 1965 strain of FMD virus and carried out the preliminary characterization (Brocchi et al., 1983; De Simone et al., 1983). Further characterization of these antibodies has been described by Crowther et al. (1984). O1 Kaufbeuren 1967 was used as the parental virus in this study because its sequence was available through the work of Fors et al. (1984). It was related epidemiologically to the virus used to produce the antibodies and it was neutralized by all seven antibodies.

Isolation of mutants. Parental virus, O1, Kaufbeuren 1967, was first tissue culture passage material from primary BTY cells (Snowdon, 1966). This material was plaque-purified three times in a BHK cell suspension assay (McCahon & Slade, 1981) and on the last occasion 15 plaques were used to generate the 15 independent seed stocks in 5 × 10⁶ BHK cells from which the mutants were isolated. Approximately 10⁶ p.f.u. of parental virus in 250 μl was mixed with 5 μl of an appropriate dilution of monoclonal antibody and incubated at 37 °C for 1 h. This was followed by the addition of 25 μl rabbit anti-mouse IgG (37 °C for 1 h), 25 μl of a 2% solution of Staphylococcus aureus ghosts (Pansorbin, Calbiochem) (37 °C for 10 min) and then centrifugation to remove the majority of the bound and free monoclonal antibody. Finally the supernatant was assayed in a BHK cell suspension assay and typically a reduction in titre of 10³ ⁵- to 10⁴ ⁵-fold was seen. Well isolated plaques were picked and plaque in a further cell suspension assay and these plaques were then used to prepare seed stock and working stocks in BHK cells. For sequencing, working stocks were prepared from the seed stock in primary bovine thryoid cells.

Neutralization assays. Approximately 10⁴ p.f.u. in 400 μl were reacted with 50 μl of one or more dilutions of antibody for 1 h at 37 °C and then overnight at 4 °C. Finally the mixtures were diluted and assayed on BHK cell monolayers.

ELISA. Both direct and indirect ELISA were used as described in McCullough et al. (1985). In direct ELISA the antigen was bound directly to the plate in an alkaline buffer. In the indirect ELISA a polyclonal rabbit virus antisera was used to trap the antigen on the plate. The monoclonal antibody was reacted with the trapped antigen for a standard time and bound antibody was then detected with a peroxidase-conjugated anti-mouse preparation. In the large experiment, described in Fig. 2(b), two replicate wells of each antigen were reacted with a single dilution of previously titrated monoclonal antibody. The antigen was unpurified virus stock and was used at a dilution that gave an A₄₉₂ of 1 to 1.5 with a standard polyclonal serum. The polyclonal antibody values were used to correct for differences in antigen content between mutants and parents. These corrected values were then expressed as a percentage of the parental virus value for the same antibody. Six parental viruses were included in the test and a standard deviation estimated for the A₄₉₂ obtained with each antibody. Mutant values which were more than 50% of the parental value were considered sensitive (+), values between 10% and 50% of the parental value were considered partially resistant (±) and values of less than 10% of the parental value (always outside 2
standard deviations of the mean parental value) were considered resistant (−). In more detailed experiments a similar approach was used but a range of antibody dilutions was tested.

**Isoelectric focusing of proteins.** BHK cell cytoplasmic extracts containing the virus-induced polypeptides labelled with $^{35}$S-methionine were analysed on both non-equilibrium gels containing pH 3.5 to pH 10 Ampholines and a higher resolution equilibrium gel for the region pH 5 to pH 7 (King et al., 1982; McCahon et al., 1985).

**Primers.** Four synthetic primers encompassing the whole of the VP1 coding region were synthesized on an Applied Biosystems (Foster City, Ca., U.S.A.) 381 A machine and used directly without further purification. The sequences of the primers and their corresponding locations on the viral genome were dACGTCTCCGCAACCTT (nucleotides 3631 to 3647), dTTGAAGGAGGTAGGC (3453 to 3467), dTCCAACGCCCTTTCCG (3258 to 3272) and dCTTCACAAATCTGGTC (3084 to 3100).

**Viral RNA.** RNA was extracted from infected primary BTY cells by a modification of the method of La Torre et al. (1982). Seventy-five cm$^2$ flasks containing BTY cell monolayers were preincubated for 30 min at 37 °C in medium containing actinomycin D (2 μg/μl), then infected with a high multiplicity of virus and incubated at 37 °C in the presence of actinomycin D until the first signs of cytopathic effect were observed (usually 2-5 to 3 h). At that point the cultures were transferred to an ice bath, washed gently with ice-cold phosphate-buffered saline and resuspended with a single 2 to 3 min treatment of the monolayer with 4 ml TNE (10 mM-Tris–HCl, 100 mM-NaCl, 1 mM-EDTA pH 7.5) containing 0-1% NP40. After centrifugation of the extract at 4 °C to remove nuclei and other cell debris, the supernatant was transferred to a fresh container containing 400 μl 20% SDS and extracted with 4.5 ml phenol:chloroform:isoamyl alcohol (25:25:1 mixture). The RNA was precipitated from the aqueous layer of that extraction by the addition of 5 M-NaCl to 200 mM followed by 2-5 volumes of absolute ethanol and transfer to −20 °C overnight. This usually gave a clearly visible precipitate which was then washed once with 70% ethanol and resuspended in 200 μl TNE and stored in small aliquots at −70 °C. Such preparations contained approximately 2 μg/μl of total RNA based on the A$_{260}$.

**Sequencing reactions.** The sequencing method used was principally that of Zimmerm & Kaesberg (1978) as modified by Palmenberg et al. (1984). Primer and RNA were annealed in a 10 μl reaction mixture containing 5 μl RNA (2 μg/μl), 2 μl primer (appropriate dilution determined previously as that giving maximum specific and minimum non-specific priming, usually about 0.05 μg/μl), 1 μl RNasin (final concentration 100 units/ml) and 2 μl 5 x annealing buffer (250 mM-Tris–HCl pH 7.4, 50 mM-MgCl$_2$, 250 mM-NaCl and 50 mM-dithiothreitol). This reaction mixture was placed at 67 °C for 3 min and then cooled slowly (1 °C/min) to 42 °C and used in the sequencing reaction. Dideoxynucleoside triphosphate mixtures were prepared in advance and stored at −20 °C. Their compositions were as follows: for ddA, 100 μM-dCTP, 100 μM-dGTP, 100 μM-dTTP and 2 μM-ddATP; for ddC, 50 μM-dCTP, 100 μM-dGTP, 100 μM-dTTP and 10 μM-ddTTP; for ddG, 100 μM-dCTP, 50 μM-dGTP, 100 μM-dTTP and 10 μM-ddGTP and for ddT, 100 μM-dCTP, 100 μM-dGTP, 50 μM-dTTP and 10 μM-ddTTP. Sequencing reactions were performed in 10 μl reaction mixtures containing 2 μl primer/template mix, 2.5 μl 4 x reverse transcriptase buffer (200 mM-Tris–HCl pH 8.0, 200 mM-KCl, 20 mM-MgCl$_2$, 40 mM-dithiothreitol), 1 μl appropriate dideoxynucleoside triphosphate mix, 1 μl reverse transcriptase (3 units/reaction), 1 μl RNasin (final concentration 100 units/ml) and 0.3 to 0.5 μl $^{32}$P]dATP (3 μCi, 10 pmol). After incubation for 1 to 2 h at 42 °C, 1 μl of 0.5 mM solution of unlabelled dideoxynucleoside triphosphates was added and the incubation continued for 30 min. Finally, 6 μl of 0.05% xylene cyanol FF, 0.05% bromophenol blue, 8 μl urea, 10 mM-Tris–HCl pH 7.4, 2 mM-EDTA were added and the mixture was heated at 90 °C to 100 °C for 3 min, then cooled on ice and loaded onto a 6% polyacrylamide, 0-25% bisacrylamide, 8 μl-urea gel and electrophoresed for approximately 1.5 h at 1400 V. After electrophoresis the gel was dried and autoradiographed at −70 °C.

**Materials.** RNasin, placental ribonuclease inhibitor was obtained from Anglia Biotechnology Ltd., U.K.; reverse transcriptase, XL grade (catalogue no. DME 006) was obtained from P & S Biochemical Ltd., Liverpool, U.K. and $^{32}$P]dATP (400 Ci/mm) was supplied by Amersham.

**RESULTS**

**Isolation of mutants**

Although all seven of the available monoclonal antibodies neutralized the O1 Kaufbeuren strain, only the five most active (B2, C6, C8, C9 and D9) were used to isolate mutants. There were two important features in the isolation procedure (see Methods); (i) mutants were isolated from a selection of independent parental stocks to minimize the isolation of sister mutants and (ii) the mutants were not grown at any time in the presence of antibody and thus partially resistant mutants were obtained which proved useful in defining the YP1 site. Out of a total of 68 plaques picked, 43 were subsequently found to be completely resistant to their isolating antibody, 14 were partially resistant and 11 were still sensitive to neutralization by the antibody used in their selection. All the partially resistant (illustrated in Fig. 1) and sensitive isolates
resulted from treatment with two antibodies B2 and D9 which were subsequently shown to be directed against the same VP1 site. All the isolates selected with the other three antibodies (C6, C8 and C9) were completely resistant to neutralization. The degree of resistance to neutralization correlated well with the binding of antibody as judged by ELISA (Fig. 2) but there were some important exceptions e.g. the five partially resistant mutants with alterations at residue 144 still bound the monoclonal antibody in ELISA (Table 3). This binding of antibody by some partially resistant viruses was confirmed by examining the binding of various dilutions of B2 and D9 antibody in a fixed time to a standard dose of antigen. Two partially resistant mutants (R450 and R471) were examined in parallel with parental virus and a selection of other mutants. The parental virus and representative mutants of the other two sites (R487 and R618) bound both B2 and D9 antibodies equally well. One of the partially resistant mutants (R450) and three mutants completely resistant to D9 (R435, R449 and R480) failed to bind B2 or D9 antibody except to a small extent (approx. 10% of parental virus value) at high concentrations (1/50 dilution of ascites fluid). The remaining partially resistant mutant (R471, Fig. 1) bound D9 antibody at 85% of the parental virus value over a wide range of antibody concentrations but bound B2 antibody at 60% of the parental virus value only at high concentrations (1/50 dilution of ascites fluid).

Out of the 57 isolates (43 resistant and 14 partially resistant to neutralization) 30 distinct mutants were identified on the basis of their isolation plus their reactivity in neutralization and ELISA assays (Fig. 2) and from the analysis of their induced proteins in infected cells by electrofocusing (Table 2).

Demonstration of three antigenic sites

Twenty-five of the 30 mutants were examined for neutralization by the five high activity monoclonal antibodies (Fig. 2a). All five antibodies recognized a different epitope but the
epitopes of some were apparently within the same antigenic site; i.e. D9 overlapped B2, C9 overlapped C6, and C8 was separate from all the others. Examination of all 30 mutants against all seven available monoclonal antibodies by ELISA (Fig. 2b) showed essentially the same pattern of three non-overlapping groups. The two additional antibodies (G5 and C3), which were not sufficiently active for selecting mutants, had epitopes within the same antigenic site as C6 and C9. Some minor overlaps occurred between the groups with three mutants (R480, R450 and R559) but the significance of these reactions is unknown. We conclude that there are at least three non-overlapping antigenic sites involved in virus neutralization that can be defined with these antibodies. This is in agreement with the earlier studies of Crowther et al. (1984) and McCullough et al. (1987) who also found the same three groups, i.e. B2/D9, C6/C9 and C8 on the basis of competition between the antibodies binding to the virus particle in ELISA.
Table 1. Reactivity of monoclonal antibodies with virus and its components in direct ELISA

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>146S</th>
<th>12S</th>
<th>VP1</th>
<th>VP2</th>
<th>VP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>5-1†</td>
<td>5-1</td>
<td>4-5</td>
<td>≤1-0</td>
<td>≤1-0</td>
</tr>
<tr>
<td>D9</td>
<td>5-2</td>
<td>5-1</td>
<td>4-5</td>
<td>≤1-0</td>
<td>≤1-0</td>
</tr>
<tr>
<td>1C6</td>
<td>4-0</td>
<td>2-6</td>
<td>≤1-0</td>
<td>≤1-0</td>
<td>≤1-0</td>
</tr>
<tr>
<td>4C9</td>
<td>5-1</td>
<td>1-5</td>
<td>≤1-0</td>
<td>≤1-0</td>
<td>≤1-0</td>
</tr>
<tr>
<td>3C8</td>
<td>3-2</td>
<td>1-5</td>
<td>≤1-0</td>
<td>≤1-0</td>
<td>≤1-0</td>
</tr>
<tr>
<td>Rabbit polyclonal‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-VP1</td>
<td>5-1</td>
<td>5-1</td>
<td>5-0</td>
<td>1-7</td>
<td>2-0</td>
</tr>
<tr>
<td>Anti-VP2</td>
<td>5-5</td>
<td>5-1</td>
<td>≤1-0</td>
<td>5-3</td>
<td>2-0</td>
</tr>
<tr>
<td>Anti-VP3</td>
<td>5-1</td>
<td>5-1</td>
<td>≤1-0</td>
<td>1-5</td>
<td>5-0</td>
</tr>
<tr>
<td>Mouse polyclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-virus</td>
<td>4-5</td>
<td>3-9</td>
<td>2-3</td>
<td>1-7</td>
<td>1-7</td>
</tr>
</tbody>
</table>

* O1 Kaufbeuren parent virus grown in BHK cells was purified by differential centrifugation followed by sucrose gradient analysis. The 12S subunits were prepared by heating a sample of the purified virus at 56 °C for 40 min at pH 6-2 and then fractionated on a sucrose gradient in parallel with marker proteins. The isolated polypeptides were prepared by electrofocusing a sample of the purified virus on a pH 3 to pH 10 Ampholine gel and then eluting the protein from appropriate portions (King et al., 1982).

† Reciprocal of endpoint dilution of antibody (log10) producing detectable A492 over background.

‡ Rabbit hyperimmune antisera prepared against virus polypeptides isolated by electrofocusing (Haresnap & Mc Cahon, 1983).

Nature of the antigenic sites

Information on the nature of the three sites was obtained by examining the reactivity of the five high activity antibodies with the virus particle and breakdown products of the particle in a direct ELISA (Table 1). All the antibodies reacted with the intact particle but only B2 and D9 reacted strongly with any sub-viral component including isolated polypeptide VP1. The reactivity of B2 and D9 with isolated VP1 suggested the possibility that they were reacting with the well known site on FMD virus which has been shown by direct biochemical methods to be between amino acids 140 and 160 of VP1 (see Introduction). This was confirmed by examining the reactivity of the antibodies in ELISA with a synthetic peptide containing the amino acids 140 to 160 of VP1 of O Kaufbeuren strain (Fig. 3). Only B2 and D9 reacted with the synthetic peptide. Therefore we conclude that two of the sites (i.e. those defined by C6, C9 and C8) are principally conformational since these antibodies bind only to the intact virus whereas the third site (i.e. that defined by antibodies B2 and D9) is less dependent on conformation and is probably principally contained within amino acids 140 to 160 of VP1.

Approximate localization of the sites by electrofocusing

Electrofocusing was used to examine the induced polypeptides of a selection of the mutants and the results were consistent with the analysis of the nature of the antigenic site (Table 2). Only the B2- and D9-selected mutants showed a clear pattern of mutation; four of nine mutants had electrophoretic alterations in VP1 suggesting that these epitopes were at least partially within VP1. None of the other mutants showed any clear pattern of electrophoretic mutation that might give a clue to the location of their mutations. This is in marked contrast to the results obtained with rhinovirus neutralization-resistant mutants where between 30% and 95% of mutants at the four neutralization sites showed electrophoretic shifts in individual capsid proteins (Sherry et al., 1986).

Sequence location of the B2/D9 site

The use of four synthetic oligonucleotide primers provided most of the sequence for the VP1 coding region of ten distinguishable mutants isolated with B2 and D9 antibodies and three parental seed stocks. All three parental stocks gave sequences identical to the published
Fig. 3. Titration of the binding of monoclonal antibodies to a synthetic peptide (residues 141 to 160) of VP1 in a direct ELISA. ■, B2; ○, D9; ●, 1C6; ▲, 3C8; □, 4C9.

Table 2. Electrophoretic alterations in the capsid proteins of mutants*

<table>
<thead>
<tr>
<th>Mutants resistant to MAb</th>
<th>Polypeptide</th>
<th>B2/D9</th>
<th>1C6/4C9</th>
<th>3C8</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>4/9†</td>
<td>0/9</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>VP3</td>
<td>0/9</td>
<td>0/9</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>VP0 (VP2 + VP4)</td>
<td>1/9</td>
<td>1/9</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

* Electrofocusing of the induced polypeptides of the mutants and parental viruses was performed as described in Methods.
† Ratio of mutants showing alterations in that polypeptide to the total number of mutants of that type examined. A few mutants showed electrophoretic alterations in non-structural proteins, but these were considered to be irrelevant to their antigenic structure.

sequence for O1 Kaufbeuren (Forss et al., 1984). Nine of the ten mutants had sequence changes and all were within the 3' third of VP1 (Table 3). All the changes resulted in amino acid substitutions and the changes were consistent with the electrofocusing data. One mutant (R606) showed no change despite sequencing of all of the VP1 coding region except that encoding the last eight amino acids at the N terminus. Since that region is highly conserved even between serotypes (Cheung et al., 1983) it seems more likely that an amino acid residue from VP2 or VP3 contributes to the site. Within VP1, four different residues were identified as definitely contributing to the site, residues 144, 148, 154 and 208. Five different mutants had the same T to
Table 3. Sequence changes found in VP1 of B2/D9-resistant mutants

<table>
<thead>
<tr>
<th>Change</th>
<th>Mutants</th>
<th>Neutralization</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAb Mutant</td>
<td>B2 D9</td>
<td>B2 D9</td>
</tr>
<tr>
<td>No change</td>
<td>B2 R606</td>
<td>± +</td>
<td>± +</td>
</tr>
<tr>
<td>Residue 144</td>
<td>T → C</td>
<td>± +</td>
<td>± +</td>
</tr>
<tr>
<td></td>
<td>Residue 148</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T → G</td>
<td>± +</td>
<td>± +</td>
</tr>
<tr>
<td></td>
<td>Residue 154</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A → T</td>
<td>± +</td>
<td>± +</td>
</tr>
</tbody>
</table>

* -, Complete resistance to neutralization or complete failure to bind detectable antibody in ELISA; ±, partial resistance or binding; +, sensitivity or binding indistinguishable from that seen with parental virus. Criteria for these definitions are given for the ELISA in Methods and for neutralization in Fig. 1 footnotes.

† Additional mutations: R457, residue 171, Thr → Ile; R471, residue 152, Ala → Val; R449, residue 208, Pro → Leu.
‡ ND, Not determined.

C change resulting in a change from leucine to serine at residue 144. All five mutants showed the same phenotype in antibody binding by ELISA but they varied slightly in their neutralization phenotype. Since all showed some degree of resistance to neutralization by B2 and/or D9 and none was completely resistant to D9, they were classified as being partially resistant at that site. Two of the mutants (R457 and R471) had an additional mutation which was not present in their parental viruses. These additional mutations apparently did not produce a significant difference in phenotype from those three mutants which only had the 144 change. Therefore, we conclude that the mutation at residue 144 was primarily responsible for producing the partial resistance observed with these mutants. The contribution of residue 148 was clearly demonstrated by two mutants (R435 and R480) which had the same single change, and this produced complete resistance to neutralization and prevented binding by both antibodies. The situation with residues 154 and 208 was more complex, in that whereas the change at 154 conferred partial resistance on R450, an additional change at residue 208 in mutant R449 produced a completely resistant virus (see Fig. 1). Therefore we conclude that both the 154 and 208 residues can contribute to the site.

Epidemiological significance of sites

A potential problem with the use of mouse monoclonal antibodies to identify antigenic sites is that the mouse immune system may recognize different epitopes from those recognized by animals susceptible to FMD virus. Therefore we examined a selection of isolates of type O virus obtained either in the field from animals having no history of disease and no antibody from vaccination, or from populations in which the disease was endemic and/or vaccination had occurred (Table 4). The first group of samples contained isolates obtained from different host animals (cows, pigs and sheep), during the course of two outbreaks in fully susceptible animals (Denmark 1982 and U.K. 1967 to 1968). The Danish outbreak was very small, involving only 22 farms and approximately 4000 animals and the 15 samples tested were from different farms. The British outbreak was much larger, lasting 32 weeks, involving 2346 farms and approximately 400 000 animals. The 12 samples tested were a selection separated in time and geography during the course of the outbreak. All of these isolates from susceptible animals were sensitive at all three sites and showed no indication of any development of resistance to neutralization by the
Table 4. Variation at the antigenic sites in field isolates*

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Monoclonal antibody reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Related isolates from susceptible animals</td>
<td></td>
</tr>
<tr>
<td>Denmark 1982 (15 samples)</td>
<td>D9  C9  C8</td>
</tr>
<tr>
<td>U.K. 1967–1968 (12 samples)</td>
<td>+†  +  +</td>
</tr>
<tr>
<td>(b) Related isolates originating from an endemic area (South America)</td>
<td></td>
</tr>
<tr>
<td>Pacheco (Uruguay, 1962)</td>
<td>−   +  −</td>
</tr>
<tr>
<td>BFS 1781 (Northumberland, U.K., 1966)</td>
<td>+   −  −</td>
</tr>
<tr>
<td>BFS 1810 (Hampshire, U.K., 1967)</td>
<td>−   +  +</td>
</tr>
<tr>
<td>BFS 1837 (Warwickshire, U.K., 1967)</td>
<td>±   +  +</td>
</tr>
<tr>
<td>BFS 1848 (Shropshire, U.K., 1967)</td>
<td>+   +  +</td>
</tr>
</tbody>
</table>

* All isolates were grown and tested in a plaque neutralization assay in primary tissue culture, either calf kidney or BTY cells (Snowdon, 1966) as illustrated in Fig. 1.
† −, Resistance to neutralization; ±, partial resistance and +, sensitivity similar to that of the 1967 parental virus used to produce the monoclonal antibodies. Exact criteria are given in Fig. 1 footnote.

antibodies. By contrast the isolates obtained from endemic areas or originating from endemic areas did show variation at all three sites. The four U.K. samples were from epidemiologically distinct outbreaks between 1966 and 1967 which were thought to have been caused by virus originating from South America. Since at that period the disease was endemic in much of South America and Europe but not the U.K. and vaccination was beginning to be used in the endemic areas, the virus was undoubtedly exposed to immunological pressures. Although the exact relationship between the various isolates is unknown there is reasonable evidence that they are related, namely (i) all the viruses shared at least one of the epitopes recognized by the monoclonal antibodies, (ii) all the viruses were classed as subtype O1 in complement fixation tests by the World Reference Laboratory at Pirbright and (iii) all the viruses were indistinguishable in polypeptides VP4, VP2, VP3, P2C and P3C by electrofocusing (data not shown). Since variation at the antigenic sites was only observed when immune selection could reasonably have been expected to be occurring we conclude that the sites are recognized by the immune system of the natural host and therefore the sites are epidemiologically significant.

**DISCUSSION**

Using seven neutralizing monoclonal antibodies we have demonstrated the existence of three separate antigenic sites on the FMD virus particle that are involved in virus neutralization. More recently a further 19 neutralizing antibodies from four other laboratories became available to us; 14 of these reacted strongly in ELISA with representatives of all three groups of neutralization-resistant mutants and also with two triple resistant mutants isolated by successive selection with antibodies directed against the three sites (data not shown). Therefore we conclude that there is at least one more epitope, if not another antigenic site, that is involved in virus neutralization beyond those defined by the seven monoclonal antibodies used in this study.

The relative importance in virus neutralization of the three sites described here is difficult to determine. On the basis of the number of different monoclonal antibodies raised against each site in the total of 26 available to us, there is no clear dominance of any one site. One site (B2/D9) clearly corresponds to the one frequently referred to as the major immunogenic site but in reality this may reflect the ease with which that site is detected by direct examination of the virus polypeptides and not necessarily its importance. The only evidence for its immunodominance is the finding by Rowlands et al. (1983) that antiviral polyclonal sera were able to detect differences between plaque isolates known to be altered at that site. However, we could find no evidence of immunodominance for any of the sites when representative mutants of each site were examined in neutralization assays with a cattle convalescent serum (data not shown). The variation observed in field samples suggests that all the antigenic sites are not only recognized by the mouse but also by the natural host and are therefore epidemiologically significant.
Two of the three sites identified in the study are apparently dependent on conformation and disappointingly the electrofocusing analysis gave no indication of their polypeptide location. That must await sequencing. By contrast the third site appears to be less dependent on conformation and its approximate location was readily identified both from the electrofocusing studies and the ELISAs as being at least partially contained within amino acids 140 to 160 of VP1 and thus corresponding to the well known site in that region defined by systematic analysis of the virus peptides by a succession of workers (see Introduction). Sequencing of the RNA from a selection of ten mutants has shown at least five amino acid residues contribute to the site, four within VP1 (residues 144, 148, 154 and 208) and one undefined residue probably outside VP1, in one of the other capsid proteins. Only at one residue (148) did change confer complete resistance to both monoclonal antibodies (B2 and D9) as judged by neutralization and ELISA. Change at any one of the other four residues produced a partial resistance phenotype. Perhaps significantly, only residue 148 was detected in the previous studies on overlapping peptides (Geysen et al., 1984, 1985) and sequencing of spontaneous antigenic variants of A12 virus (Rowlands et al., 1983). Those earlier studies also suggested other residues were important, namely residues 149, 151 and 152 in the peptide studies and residue 153 in the A12 mutant study. One mutant in the present work (R471) did have a change at residue 152 but it was in addition to a change at 144 that was capable of conferring resistance on its own in three other mutants. Therefore we conclude that the change at 152, like that at 171 in another mutant (R457) is not essential for conferring antibody resistance in our strain of virus. Presumably the differences between the studies reflect both strain or type differences in the viruses used and, especially in the case of the peptide work, the different method of detecting the relative importance of residues.

The intensive studies on monoclonal antibody-resistant mutants of type A influenza viruses (Webster et al., 1983), polioviruses (Minor et al., 1986) and rhinovirus type 14 (Sherry et al., 1986) coupled with knowledge of the three-dimensional structure of those viruses or their subunits has clearly demonstrated the validity of mutational analysis as a tool for mapping dominant immunogens. With only a few exceptions of apparently random second mutations, all the residues of one site have been shown to be clustered together on the surface of the peptide even though particular residues may be well separated in their linear sequence or even found on different polypeptides. Little evidence has been found for amino acid changes acting at a distance to alter the conformation of a site. Hence it seems reasonable to conclude that the amino acid residues that we have identified as affecting the B2/D9 site are actually part of that site. Thus these results bring together into one antigenic site the two important regions of VP1 identified by analysis of the polypeptide, namely residues 140 to 160 and 200 to 213, plus a possible contribution from one of the other capsid polypeptides. Thus it is a conformational site as has been suspected from the low immunogenicity of isolated peptides and the improved immunogenicity of a larger peptide made up of the two VP1 regions (Di Marchi et al., 1986). The physical location of the site must await the appearance of crystallographic studies on this virus. However the original suggestion by Rossman et al. (1985), based on their rhinovirus crystallographic studies, that this FMD virus site equates to the Nim II site of rhinovirus and is in close association with VP2 and/or VP3, still seems valid.

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