Monoclonal antibodies, against O₁ serotype foot-and-mouth disease virus, from a natural bovine host, recognize similar antigenic features to those defined by the mouse


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Eight neutralizing and two non-neutralizing anti-foot-and-mouth disease virus (FMDV) bovine IgG1 and IgG2 monoclonal antibodies (BMAbs) recognize conformationally dependent epitopes. The majority of those shown to neutralize virus passively protected mice from virus challenge, regardless of isotype. Well-characterized anti-FMDV mouse MAbs, representing five independent neutralizing epitopes on O₁ serotype virus, were examined with each of the ten BMAbs in a competition-based ELISA. Five of the neutralizing BMAbs (C48, C65, C74, C83 and MH6) were shown to be directed against epitopes on, or in close proximity to, that previously defined as neutralizing antigenic site 2. Another neutralizing BMAb, MH5, bound to an epitope on, or in close proximity to antigenic site 3. Two neutralizing BMAbs (C2 and C96) simultaneously abrogated the binding of mouse antibodies to sites 2 and 4, contesting the autonomous nature of these two regions. None of the BMAbs were shown to be directed towards the immunodominant antigenic site 1. Sequence analyses of neutralization escape mutants supported the competition ELISA results, and included changes at site 2 (VP2 aa C78Y), site 3 (VP1 N46S) and site 4 (VP3 E58K). Additionally, a substitution at a previously unrecorded location (VP2 aa T188I) prevented the binding of site 2 (C48) and sites 2 and 4 (C2) directed BMAbs. Although the bovine and murine anti-FMDV repertoires may not be identical these results support the recognition of similar antigenic features. This is the first report characterizing anti-FMDV MAbs produced from a natural target host, the cow.

Introduction

The advent of monoclonal antibody (MAb) technology in 1975 provided tools with a wide variety of practical applications. Arguably, the greatest contribution of this technology has been towards a better understanding of antigenic structure and, particularly, the important epitopes involved in the immune responses against infectious diseases. This is especially true of studies involving members of the Picornaviridae, small non-enveloped icosahedral viruses containing single-stranded, positive-sense RNA, since a large body of evidence demonstrates that the humoral response is the major effector in protection against many of its representatives (see review by Mateu, 1995).

Foot-and-mouth disease, for example, is a highly contagious and economically important virus disease of cloven-hoofed livestock whose causative agent, foot-and-mouth disease virus (FMDV), is an aphthovirus. The 22–30 nm diameter capsid, possesses 5:3:2 symmetry and is made up of 60 protomer copies of each of four proteins, VP1–4, which surround an RNA molecule. Proteins VP1–3 have the same basic folding arrangement: a ‘conserved core’ consisting of an eight-stranded antiparallel β-barrel, with flanking sequences and loops that connect the strands. Protein VP4 is smaller and confined to the interior of the capsid. These viruses occur as seven distinct serotypes – O, A, C, Asia1 and South African Territories 1–3 – within which there is extensive antigenic diversity. Such antigenic variability impedes the effectiveness of vaccination as a control measure and has thus stimulated many studies, including those using MAbs, to identify important neutralizing epitopes and deduce the molecular basis for such diversity. As a result, several antigenic sites have been located in serotypes O, A and C following the sequencing of...
MAb-resistant mutants (see review by Mateu, 1995). The understanding of their topography has been greatly aided by high-resolution three-dimensional structure determinations of this genus using X-ray crystallography (Acharya et al., 1989; Lea et al., 1994; Curry et al., 1997). Predictably, these have highlighted the antigenic sites as structural protrusions on the virus surface, formed mainly from the loops connecting the β-barrel strands of the conserved cores.

For O1 serotype strains, five functionally independent neutralizing antibody sites have been identified (Kitson et al., 1990; Crowther et al., 1993b), and their location on the surface of the capsid has been clarified by the structural resolution of the related strain O1 BFS (Acharya et al., 1989). Site 1 encompasses the G–H loop of VP1, which had previously been identified as immunodominant by the use of peptides (Bittle et al., 1982; Pfaff et al., 1982), and its C terminus, and includes residues 1144, 1148 and 1154. Site 2 includes VP2 amino acids 2031, 2070–2073, 2075 and 2077. Site 3 involves residues 1043 and 1044 of VP1 and finally residue 3058 of VP3 representing site 4. Only site 1 appears to be conformationally independent. More recently, a fifth functionally independent neutralizing epitope has been identified despite encompassing part of site 1 (Crowther et al., 1993b). Some of the structural elements encompassing these critical residues, namely the G–H loop and C terminus of VP1, the B–C loop of VP2 and part of VP3 have also been found to be important in other FMDV serotypes (see review by Mateu, 1995) including SAT2 (Crowther et al., 1993a). However, the relative importance of each of these individual neutralizing antigenic sites is still undefined.

As the antibodies employed in these previous studies were derived from immunized mice, it is entirely possible that they are of a different specificity to those elicited by the virus in one of its natural hosts, namely the bovine. For FMDV and other picornaviruses, however, a number of lines of evidence tend to argue against such a postulate. For example, the strong correlation between surface accessibility and antigenicity (Acharya et al., 1990; Arnold & Rossmann, 1990; Lea et al., 1994; Novotny et al., 1986), the immunodominance of the same site in different animal species (Icenhogle et al., 1986; Brown, 1990; Ping & Lemon, 1992; Mateu et al., 1995a), the effective blocking of MAb binding to virus by polyclonal antibodies from different animal species (Icenhogle et al., 1986; Thomas et al., 1988) and the identification of the same critical residues for both MAbS and antibodies from the natural hosts (Mateu et al., 1990, 1995a, b; Martínez et al., 1991; Ping & Lemon, 1992). Perhaps the most direct evidence to date is the limited study undertaken by Garmendia et al. (1989) using anti-idiotypic antibodies, which showed that the major immunodeterminants defined by mouse MAbS against FMDV serotype A12 were also recognized by bovine and swine.

In an attempt to establish the existence of comparable bovine antibodies to those defined by the mouse model we have produced a number of bovine (B)MAbS by a similar method to that outlined by Groves et al. (1987) using an aminopterin-sensitive bovine × mouse heterohybridoma fusion partner. This technique of back-fusing lymphocytes from the species of interest to an already generated, aminopterin-sensitive, heterohybridoma has already met with some success (Ostberg & Pursch, 1983; Tucker et al., 1984; Anderson et al., 1987; Groves et al., 1987, 1988; Flynn et al., 1989). Beside their value for defining antigenic sites and investigating the relative importance of individual FMDV epitopes in a natural host, such antibodies might also be used to explore more fully the humoral role of disease intervention. Not excluding the roles of different isotypes which may or may not vary in accordance with their protective ability (Mulcahy et al., 1990) such antibodies may offer a route to a clearer understanding of other functionally important cell processes, such as opsonization and phagocytosis, which may ultimately lead to host protection (McCullough et al., 1988).

Methods

(a) Animal immunizations. The cell populations isolated for fusions and subsequent cloning were derived from Friesian cattle used in a longitudinal experiment on the cellular recognition of FMDV and its subunit VP1 by bovine peripheral blood T lymphocytes (Garcia-Valcarcel et al., 1990). Briefly, cattle were vaccinated, both intramuscularly and subcutaneously, with either a total of 0.5 mg of fusion VP1 (animals SG42, 43 and 44) or a total of 0.25 mg of recombinant VP1 (animals SG48, 49 and 50) mixed 50:50 with oil adjuvant. These animals were then re-vaccinated 38 days later by the same regimen. Cows SG63 and 65 were vaccinated once, both intramuscularly and subcutaneously, with a total of 7 µg of inactivated O1 Lausanne virus mixed 50:50 with incomplete oil. Thirty-five days after the final vaccination all the animals were given an intradermal challenge of 105 infectious bovine doses of virus. After approximately 5 months convalescence all the animals were injected with a single bovine dose (4–1 µg) of inactivated O1 Lausanne virus, within the proximity of the pre-scrapular lymph nodes, at 4, 3 and 2 days prior to euthanasia.

(b) Harvesting of lymphocytes. Following euthanasia the pre-scrapular lymph nodes were excised and the excess connective tissue removed. Single cell suspensions were prepared by cutting the lymph nodes into small 1 cm3 pieces and processing the tissue in a Griffith-type glass homogenizer using cold, sterile PBS. After the suspension on ice for a few minutes to allow larger fragments to settle out, the cell suspension was centrifuged, washed and then re-centrifuged at 250 g for 5 min. Red blood cells were then removed from the pellets by briefly treating with 0.85% ammonium chloride. The final cell suspension (107/ml) was stored at −70 °C in 90% foetal calf serum (FCS) and 10% DMSO.

(c) Fusion and cloning procedure. The fusion partner, BMT(s)B, was cloned from the heteromyeloma cell-line B/MT.AA.17.H5.A5 (supplied by B. A. Morris, University of Surrey), which was derived in a similar manner to that designated B/MF-2 (Groves et al., 1987). Frozen bovine lymphoblasts were thawed quickly at 37 °C, resuspended in 5 ml cold FCS and left on ice for 3 min, to reduce cell flocculation, before centrifugation. Both cell populations were washed twice in cold serum-free growth medium before mixing the bovine lymphocytes and BMT(s)B heteromyeloma cells at a ratio of 10:1 respectively and again pelleted.
by centrifugation. This pellet was resuspended and the cells were fused using 1 ml of 50% (v/v) PEG in serum-free Iscove's modified Eagle's medium (IMEM) for 1 min at 4 °C. Cells were then left a further minute before diluting with 20 ml of pre-warmed (37 °C) serum-free IMEM over an additional 5 min. The cell suspension was then left at 37 °C for 15 min prior to centrifugation and resuspension in 50 ml IMEM containing 20% FCS and HAT supplements. The cells were then distributed between two 75 cm² tissue culture flasks and left at 37 °C in a gassed (5% CO₂) incubator for 4 days. They were centrifuged and the pellets either cryostored or cloned immediately by resuspending in 5¢×10⁶/v cellulose and dispensing onto 3¢×10⁶/v macrophage feeder cells. Fused cells were titrated in each plate to give individual colonies were then screened for 10–14 days at 37 °C and individual colonies were picked and transferred to mouse macrophage feeders in 96-well plates. Individual colonies were then screened after 4 days for isotype class and anti-FMDV activity by ELISA.

(d) Monoclonal cell storage and antibody harvesting. Colonies from wells containing specific antibody were passaged to high cell density and stored over liquid nitrogen. Small volume pools of the tissue culture fluid from these monoclonal cell lines were used for characterization studies either in a concentrated or unconcentrated form.

■ Isotyping. Antibody class and subclass were determined by ELISA, and by the Ouchterlony double immunodiffusion technique using a commercially available kit (The Binding Site Ltd, Birmingham, UK).

■ Mouse MAbbs. MAbbs representing the five neutralizable sites on type O FMDV were obtained from several sources. MAbbs B2, D9, C6, C8 were supplied by Emiliana Brochii of the Instituto Zoolo-profilattico, Brescia, Italy (Xie et al., 1987). MAb C3 was supplied by Sandra Farias of the Department of Physiology, Biotechnology Centre, Federal University, Porto Alegre, Brazil (Crowther et al., 1993b). MAb 14EH9 was supplied by Barry Baxt of the Plum Island Animal Disease Center, Greenport, NY 11944, USA.

■ ELISA techniques. Anti-virus activity was determined by an indirect ELISA, and an indirect sandwich ELISA. In the indirect assay, virus, diluted to 2 µg/ml in a carbonate–bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6), and applied at 100 µl per well, was used to coat micro-plates (Nunc Immunoplates). This was followed by successive additions of test sample and peroxidase-conjugated anti-bovine antibody (Dakopatts), using PBS which contained 5% (v/v) methylcellulose and dispensing onto 3¢×10⁶/v plates pretreated with mouse macrophage feeder cells. Fused cells were titrated in each plate to give approximately 60, 30 or 10% of the original cloning mixture. Plates were then incubated for 10–14 days at 37 °C in a 5% CO₂ atmosphere and individual colonies were picked and transferred to mouse macrophage feeders in 96-well plates. Individual colonies were then screened after 4 days for isotype class and anti-FMDV activity by ELISA.

■ Virusese. The following strains of FMDV were prepared from infected baby hamster kidney (BHK)-21 cell monolayers: O1 Laussanne and O1 Kaufbeuren. The virus stocks used for the micro-neutralization tests were stored as clarified tissue culture harvest material at −20 °C in 50% glycerol. For other immunoassays, virus was purified by the method of Brown & Cartwright (1963) and stored at −70 °C. For passive protection experiments O1 Kaufbeuren was obtained from mouse-adapted stocks kept as glycerinated preparations. Dilutions for inoculation were made in 0.25 M phosphate buffer, pH 7.2.

■ Antibody selected virus mutants. These were produced by a modified method of that previously described by Crowther et al. (1993b). Briefly, 1 ml of high titre parental virus O1/Kaufbeuren/FRG/66 was mixed with 50 µl of a selected BMAb and the mixture incubated for 30 min at 37 °C. This mixture was then pipetted onto a monolayer of washed IB-RS2 cells (25 cm² flask) together with 10 ml of Eagle's medium containing a 1/50 dilution of the same bovine antibody, and incubated for 24 h at 37 °C. The cell debris resulting from virus infectivity was removed by centrifugation and a 1 µl aliquot of this clarified suspension was then reprocessed by the same procedure. The existence of BMAB-resistant virus was established by the lack of binding to the appropriate antibody in a sandwich ELISA (Samuel et al., 1991). Individual BMAB-resistant mutants were then isolated by standard plaque purification techniques.

■ RNA extraction and sequencing of BMAb selected virus mutants

(a) RNA extraction. Viral RNA was extracted from the clarified supernatant of a high titre FMDV tissue culture flask using a RNeasy kit (Qiagen) according to the manufacturer's protocol. Samples were stored at −20 °C until required.

(b) Reverse transcription of vRNA. Extracted viral RNA was used as a template for reverse transcription (RT) using primer NK61 and the resultant cDNA used as a target for PCR amplification, as described by Höfner et al. (1993) with the modifications of Carpenter et al. (1996).

(c) PCR amplification. The region of the cDNA corresponding to the capsid coding region of the viral genome was amplified in two fragments (1301 and 1260 bp) by the method of Höfner et al. (1993), using primer pairs pNK61/pARS4 and pARS3/L-463F respectively. The presence of PCR products was checked by subjecting a 5 µl sample to electrophoresis on a 2% agarose–TBE gel and visualizing with UV-light. Post-PCR purification was carried out using Wizard Preps (Promega) according to the manufacturer's protocol and products were then stored at −20 °C.

(d) Cycle sequencing for ALF Express Automated Sequencer. An fmol DNA sequencing kit (Promega), based on the technique of Murray (1989) and as described by Carpenter et al. (1996), was used. As a modification to the manufacturer's protocol, 20 pmol of one of the Cy5 amidite-labelled primers was added in place of the [γ³²P]ATP-radio-labelled primer. The reaction mixtures were heated to 95 °C for 3 min prior to loading 8 µl samples, in the order A, C, G, T, to the appropriate lane on an ALF DNA Sequencer (Pharmacia). The software AM V3.01 (Pharmacia) was used to process the data, which were then exported as an ASCII text file and aligned manually before analysis using the program SEQUPROG (N. J. Knowles, IAH, Pirbright). The se-
Table 1. Frequency, isotype and virus neutralizing ability of BMAbs selected from FMDV serotype O1 immunized donors

Lymphocytes from eight individual cows were fused with cell line BMT(s)B. Colonies were arbitrarily selected, grown and tested for reactivity with virus, IgG isotype and virus neutralizing activity (details in Methods).

<table>
<thead>
<tr>
<th>Primary immunogen</th>
<th>Cow no.</th>
<th>Fusion partner</th>
<th>No. of colonies picked</th>
<th>No. of colonies virus specific</th>
<th>IgG1</th>
<th>IgG2</th>
<th>Neutralizing$\S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-VP1*</td>
<td>SG42</td>
<td>BMT(s)B</td>
<td>384</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F-VP1</td>
<td>SG43</td>
<td>BMT(s)B</td>
<td>144</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>F-VP1</td>
<td>SG44</td>
<td>BMT(s)B</td>
<td>288</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-VP1†</td>
<td>SG48</td>
<td>BMT(s)B</td>
<td>288</td>
<td>56</td>
<td>4</td>
<td>52</td>
<td>24 (13 IgG1, 11 IgG2)</td>
</tr>
<tr>
<td>R-VP1</td>
<td>SG49</td>
<td>BMT(s)B</td>
<td>384</td>
<td>74</td>
<td>50</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>R-VP1</td>
<td>SG50</td>
<td>BMT(s)B</td>
<td>288</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Inactivated vaccine‡</td>
<td>SG63</td>
<td>BMT(s)B</td>
<td>192</td>
<td>60</td>
<td>60</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>SG65</td>
<td>BMT(s)B</td>
<td>216</td>
<td>133</td>
<td>26</td>
<td>107</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Fusion protein VP1, † recombinant protein VP1 and ‡ inactivated vaccine were prepared as described by Garcia-Valcarcel et al. (1996).
$\S$ Determined by microtitre assay in which a 1/2 dilution of the prerequisite antibody is mixed with an equal volume of virus (100 TCID\textsubscript{50}) and residual infectivity is detected by BHK-21 cells.
ND, Not determined.

Table 2. Specificity and virus neutralizing activity, \textit{in vivo} and \textit{in vitro}, of anti-FMDV O1 BMAbs

Selected BMAbs neutralizing virus \textit{in vitro} and recognizing antibody-captured virus (‘sandwich’ ELISA) were further characterized by an additional ‘indirect’ ELISA (virus directly bound to a solid phase) and by a mouse protection test, measuring virus neutralization \textit{in vivo}.

<table>
<thead>
<tr>
<th>Cow/immunogen</th>
<th>MAb</th>
<th>Class</th>
<th>Indirect ELISA</th>
<th>Sandwich ELISA*</th>
<th>Neutralizing†</th>
<th>Protective‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG63/virus</td>
<td>C34</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·7</td>
<td>- ve &lt; 0·3</td>
<td>ND</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C37</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 1·9</td>
<td>- ve &lt; 0·3</td>
<td>ND</td>
</tr>
<tr>
<td>SG50/R-VP1</td>
<td>C2</td>
<td>IgG2</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·0</td>
<td>+ ve 1·38</td>
<td>- ve 0·4</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C48</td>
<td>IgG2</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·6</td>
<td>+ ve 1·38</td>
<td>+ ve &gt; 2·5</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C65</td>
<td>IgG2</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·6</td>
<td>+ ve 1·2</td>
<td>+ ve 3·0</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C74</td>
<td>IgG2</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·7</td>
<td>+ ve 1·5</td>
<td>+ ve 2·4</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C83</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·2</td>
<td>+ ve 1·68</td>
<td>+ ve 1·6</td>
</tr>
<tr>
<td>SG48/R-VP1</td>
<td>C96</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·3</td>
<td>+ ve 1·2</td>
<td>+ ve 2·0</td>
</tr>
<tr>
<td>SG63/virus</td>
<td>MH5</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·1</td>
<td>+ ve 0·9</td>
<td>+ ve 2·4</td>
</tr>
<tr>
<td>SG63/virus</td>
<td>MH6</td>
<td>IgG1</td>
<td>- ve &lt; 1/1</td>
<td>+ ve 2·1</td>
<td>+ ve 0·9</td>
<td>+ ve 2·4</td>
</tr>
</tbody>
</table>

* log\textsubscript{10} reciprocal antibody dilution giving 0·5 A\textsubscript{492} unit.
† Reciprocal antibody dilution giving 50% neutralization of 100 tissue culture infective doses of virus (log\textsubscript{10} SN\textsubscript{50}/100 TCID\textsubscript{50}).
‡ Protective antibody titre (T) calculated from the log\textsubscript{10} reduction of the ID\textsubscript{50} virus titre compared to that observed in the absence of antibody as described by Mulcahy et al. (1990).
Bovine monoclonal antibodies against FMDV O

sequence of the complete capsid coding region was determined for each BMAb selected virus mutant.

- **Neutralization assay.** This was performed using a two-dimensional micro-neutralization technique previously described by Barnett *et al.* (1996).

- **Mouse protection test.** This was carried out as described by Mulcahy *et al.* (1990). Test antibody was deemed protective if the reduction in virus titre was $\geq 1.5$.

**Results**

Eight fusions, each utilizing lymphocytes from a different animal, produced a total of 2184 colonies of which 332 were specific to FMDV O1 Lausanne (Table 1). Of these FMDV-specific colonies, 145 were of the isotype IgG1 and 187 were of the isotype IgG2 and at least 43 colonies from both isotype groups secreted antibodies that neutralized virus *in vitro*. Interestingly, the fusions using cells from individual animals that underwent the same vaccination regime produced differing IgG1:IgG2 ratios.

**Specificity and virus neutralizing activity of selected BMABs**

Ten of these FMDV-specific colonies, eight neutralizing (three IgG1 and five IgG2) and two non-neutralizing (IgG1), were selected for further characterization (Table 2). In contrast to their recognition of antibody-captured virus in the sandwich ELISA, none of the BMABs bound to virus directly adsorbed to the solid phase in the indirect ELISA. With the exception of BMAB C2 seven of the eight neutralizing BMABs, including three that were IgG1, were also effective at passively protecting mice from virus challenge.

**Competitive inhibition of mouse MAbs by BMABs**

In another series of experiments the epitope recognition profiles of the neutralizing and non-neutralizing BMABs were examined using competition-based ELISAs and murine MAbs defining the five independent neutralizing antigenic sites (Fig. 1). The two non-neutralizing BMABs, C34 and C37, did not compete with any of the murine MAbs used. BMABs C48, C65, C74, C83 and MH6 impaired the binding of site 2 directed murine MAb C6. BMAB MH5 competed against site 3 directed murine MAb C8. The two remaining bovine antibodies, C2 and C96, were each capable of blocking both C6 and 14EH9 murine MAbs representing antigenic sites 2 and 4 respectively. However, the degree of blocking shown against site 2 murine MAb C6 was considerably greater using BMAB C96 than that observed with BMAB C2.

**Sequence determination of BMAB-derived neutralization escape mutants**

BMABs MH5, C48, C2 and C96, representing each of the different epitope specificities as elucidated by competition ELISA, were used to derive neutralization escape mutants from the parent O1 Kaufbeuren strain. Sequence changes detected in these mutants by PCR methodology are shown in Table 3. The site 3 directed BMAB MH5 produced a mutational change at VP1 aa N46S. Site 2 directed BMAB C48 produced a mutational change in a previously unobserved position, VP2 aa T188I. The same residue change was also seen in mutants produced from the site 4/site 2 directed BMAB C2. BMAB C96, which had also indicated a dual specificity to sites 2 and 4 by the competition ELISA, produced escape mutants.
Table 3. Amino acid substitutions in O1 Kaufbeuren/FRG/66 escape mutants generated by four BMAbs

<table>
<thead>
<tr>
<th>BMAb</th>
<th>Locality of binding footprint*</th>
<th>Residue change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>Site 4/2</td>
<td>VP2 amino acid T188I (threonine to isoleucine)</td>
</tr>
<tr>
<td>C48</td>
<td>Site 2</td>
<td>VP2 amino acid T188I (threonine to isoleucine)</td>
</tr>
<tr>
<td>C96</td>
<td>Site 2/4</td>
<td>VP3 amino acid E58K (glutamic acid to lysine) VP2 amino acid C78Y (cysteine to tyrosine)</td>
</tr>
<tr>
<td>MH5</td>
<td>Site 3</td>
<td>VP1 amino acid N46S (asparagine to serine)</td>
</tr>
</tbody>
</table>

* Location of the antigenic site directed binding, according to competition ELISA results, using site specific mouse MAbs.

encompassing two residue changes that indicated the involvement of both these regions. The replacement of glutamic acid with lysine at residue position 58 of VP3 mimicked the change previously observed with site 4 directed murine MAb 14EH9. This result also supported the previous finding that BMAb C96 was unable to bind to a mouse MAb escape mutant containing this same residue change (data not shown). The second residue change, VP2 aa C78Y, was also at a location some five to seven residues downstream to those amino acids previously identified by murine MAbs as being part of antigenic site 2, namely V70G, T71N and D73H.

Reactivities of selected BMAbs with BMAb-derived neutralization escape mutants

An indirect sandwich ELISA, used to antigenically profile each of these BMAb escape mutants with the other seven neutralizing BMAbs, confirmed the competition data (Fig. 2). For example, the critical residue change, VP2 aa T188I, defined by BMAbs C2 and C48, was also important in the binding ability of BMAbs C65, C74 and C83. BMAb MH6 was unaffected by this residue change. Finally, the reactivities of the panel of neutralizing BMAbs to the escape mutants produced by C96 and MH5 endorsed the individuality of these two other epitopes.

Discussion

The primary aim of this study was to ascertain the proportion and biological activities of anti-FMDV IgG1 and IgG2 antibodies synthesized by a natural host of the disease. Thus bovine heterohybridoma technology was developed, and anti-FMDV MAbs recovered from fusions using lymphocytes from immunized cattle were analysed for their isotype and ability to neutralize virus in vitro and in vivo.

A total of 2184 hybridoma colonies were isolated of which 332 or 15% were specific to FMDV O1 Lausanne. Of these anti-FMDV colonies, around 44% were of the IgG1 isotype and 56% were of the IgG2 isotype, and some 13% of these specific colonies, from both isotype groups, neutralized virus in vitro. Differences in the ratios of IgG1 and IgG2 antibodies following the immunization of cattle with either synthetic peptide or whole virus have previously been reported (Mulcahy et al., 1990). Thus a predominance of IgG1 antibodies over IgG2 antibodies was seen in animals receiving infectious or inactivated virus, whereas animals immunized with synthetic peptide produced significantly less IgG1 in comparison to IgG2. It was proposed that these differences in isotype profiles might, in part, account for the lower virus protective index of the peptide in cattle. Assuming the fusion event that produces a secreting clone is random, and that the B cell lymphocytes produce antibodies at similar rates in vivo, then the proportion of IgG1 and IgG2 colonies isolated from an individual animal may be systemically representative of the overall ratio of these isotypes. However, one vaccinee that received multiple doses of infectious and inactivated FMD virus produced exclusively IgG1 antibodies following fusion and another, similarly vaccinated animal, produced a predominance of IgG2 antibodies. This variability in IgG1 and IgG2 clone ratios was also observed with other individual animals whose primary immunization consisted of a recombinant FMDV VP1 subunit vaccine followed by repeated immunizations of whole virus. Given that these animals were consecutively boosted with both infectious and inactivated whole virus, there appears to be no evidence from these observations to suggest that one isotype predominates in respect of the immunizing antigen.

Functional activity and isotype of an antibody are known to be interrelated (McGuire et al., 1979; Sellei, 1984; Plumas-Marty et al., 1993; Xu et al., 1994; Spira et al., 1996), and the class or subclass induced by a particular antigen is correlated with the appropriate protective mechanism (Mulcahy et al., 1990). Having ascertained the reproducibility of this hybridoma methodology, ten FMDV-specific colonies, five of the IgG1 isotype and five of the IgG2 isotype, were selected.
Bovine monoclonal antibodies against FMDV O1

Fig. 2. Histograms showing percentage reactivity of the neutralizing BMAbs with the bovine antibody resistant mutants as compared to that of the parental virus O1 Kaufbeuren/FRG/66.

for further characterization. None of these antibodies bound to virus directly adsorbed onto a solid phase, in contrast to their positive reactivity in an ELISA using antibody-captured virus. Given that virus particles may be distorted after adsorption onto a solid phase (McCullough et al., 1985), this suggests that these BMAbs recognize conformational-dependent discontinuous sites rather than linear epitopes. Eight of these antibodies, including three IgG4, neutralized homologous virus in vitro at various levels, and nearly all were effective at passively protecting suckling mice against virus challenge, indicating that neither in vitro neutralization nor protection is governed by the antibody’s isotype. BMAb C2 demonstrated a low protective T value of 0.4, which was confirmed using higher doses of antibody. This passive immunity test has been shown to be a more accurate indicator of protection in the target species than the conventional serum neutralization assays (Mulcahy et al., 1990), but the reason for this is unclear. Higher ratios of C2-like antibodies in some polyclonal responses might be one interpretation. This could also explain why some FMDV-vaccinated animals, and animals vaccinated with FMDV synthetic peptides (DiMarchi et al., 1986; Mulcahy et al., 1990), still succumb to disease following homologous challenge, despite having neutralizing antibody titres at levels normally considered to be protective. Furthermore, this example of a neutralizing but non-protective antibody may help to define some of the parameters of protection.

As an alternative approach towards defining the antigenic epitopes recognized by the BMAbs, the ability of the neutralizing and non-neutralizing bovine MAbs to block the binding of mouse MAbs representing each of the five independent antigenic sites was examined by competitive ELISA. The two non-neutralizing BMAbs, C34 and C37, did not compete with any of the murine monoclonals, and therefore may not be directed to sites on, or adjacent to, the five independent neutralizing sites, but elsewhere on the surface of the capsid. All of the remaining eight neutralizing BMAbs were capable of competing against at least one of the representative panel of murine MAbs. BMAbs C48, C65, C74, C83 and MH6 only impaired the binding of murine MAb C6, suggesting that these BMAbs are directed to sites at or close to that already identified as antigenic site 2. Conversely, BMAb MH5 only competed with murine MAb C8, indicating that this antibody is directed to an epitope encompassing or adjacent to site 3. Surprisingly, the two remaining antibodies, C2 and C96, were each capable of blocking both C6 and 14EH9 murine antibodies, suggesting that their epitopes may encompass two sites previously regarded as independent of each other.

In an attempt to more precisely locate the epitopes to which some of these bovine antibodies are directed, BMAbs MH5, C48, C2 and C96, representing each of the different antigenic specificities as defined by competition ELISA, were used to produce neutralization escape mutants which were
Fig. 3. For legend see facing page.
subsequently sequenced by PCR methodology. Using RASMOl images (Sayle & Milner-White, 1995), ribbon and spacefill plots (Fig. 3) of resulting substitutions (yellow) were then highlighted on the molecular structure of O\textsubscript{1}/BF\textsubscript{5}/1860. For comparison, mouse MAb-derived substitutions (white), representing the five neutralizing antigenic sites (Kitson \textit{et al.}, 1990; Crowther \textit{et al.}, 1993\textit{b}), were also included. As a prerequisite to this it was established by ELISA that these BMAbs were able to react with trypsin-treated FMDV (results not shown). Hydrolysis of the virus with trypsin cleaves the major antigenic sites encompassing amino acids 135 and 160 and the C terminus of VP1 and thereby confirmed the absence of any site 1 directed antibodies in this panel. The site 3 directed BMAb MH5 produced a mutational change at VP1 aa N46S, wholly consistent with site 3 directed murine MAbs which had previously produced escape mutants with amino acid changes two to three amino acids N-terminal of this location. Site 2 directed BMAb C48 produced a mutational change, VP2 aa T188I, in an entirely new location. This residue change was considerably further towards the C terminus than previously identified amino acid changes with site 2 directed murine MAbs, e.g. VP2 V70G, T71N, D73H and S131P. Nonetheless, when this amino acid change is highlighted on the known molecular structure of the O\textsubscript{1} FMDV capsid its locality remains close to those changes previously identifying site 2. Given the novelty of this mutational change, it was surprising to see the same change in mutants produced from the site 4 and site 2 directed BMAb C2, particularly as the competitiveness of the C2 antibody was more towards site 4 than 2. It is unclear why there is such a discrepancy between the competition results of BMAbs C2 and C48, considering the same antibody derived mutational change has been revealed by both. One possibility might be the orientation of these antibodies when bound to the surface of the capsid. This is pertinent if we assume that the new mutational location is independent of both site 2 and site 4 and that BMAb C2 binding is monovalent. In the case of BMAb C2 the antibody’s binding footprint encompasses VP2 residue 188, close to the threefold axis, but is oriented in the direction of site 4 (Fig. 4\textit{a}), thus blocking the ability of other site 4 directed antibodies from binding to this region. It is interesting to note that such an orientation would also ‘mask’ the heparan sulfate binding site (E. Fry and others, unpublished observations). The interaction of FMDV with this particular sulfated glycan is functionally important for efficient infection of cells in culture (Jackson \textit{et al.}, 1996) and thereby might explain why the non-protective BMAb C2 can neutralize virus \textit{in vitro}. However BMAb C48, although binding at a similar location, is oriented away from site 4 towards the site 2 region, thus impairing the binding of other site 2 directed antibodies (Fig. 4\textit{b}). In this latter case, bivalent binding might occur across the virus twofold axis of symmetry in a similar manner to that described for human rhinovirus type 14 (Smith \textit{et al.}, 1996). Interestingly BMAb C96, whose binding appeared to be directed towards an epitope that encompassed both site 2 and site 4 (disputing the autonomous nature of these two regions in FMDV type O), produced escape mutants with substitutions at both site 4 (VP3 E58K) and site 2 (VP2 C78Y) sim-
ultaneously. This is the first evidence for type O FMDV that these sites are linked in a similar manner to that of antigenic site D of FMDV type C, which comprises residues analogous to sites 2 and 4 of type O (Lea et al., 1994).

Antigenic profiling of these selected BMAb escape mutants with the other bovine antibodies supported the earlier competition data and showed that C2, C48, C65, C74 and C83 are recognizing the same (or overlapping) epitopes and that C96 and MH5 are each recognizing other, different epitopes. BMAb MH6 was capable of recognizing all the BMAb escape mutants produced. Since the competition ELISA results indicated that this antibody was also directed to antigenic site 2, this may imply the involvement of other critical residues.

These preliminary studies have revealed a reliable method for the production of MABs from a natural host of FMDV and provided the opportunity to study a more relevant immunological repertoire in greater detail. Neutralizing and protective, neutralizing and non-protective, and non-neutralizing bovine antibodies have been shown to be directed to conformational epitopes and those that neutralize virus are not dictated by antibody isotype. The binding specificities of some of these bovine antibodies appear to be similar to those previously defined by mouse MABs and encompassed independent antigenic sites 2, 3 and 4. Sites 2 and 4 may not be regarded as antigenically independent in view of the importance of both regions to the binding of C96. Surprisingly, none of the neutralizing BMAbs were shown to be directed towards antigenic site 1 (Strohmaier et al., 1982; Bittle et al., 1982). The lack of an antibody directed to immunodominant site 1 and the dominance of site 2 directed antibody in this limited panel of BMAbs suggests that site 2 may play a more important role in the immunological intervention of the disease in the natural host. Further production and characterization of bovine antibodies in vivo should confirm and extend these preliminary observations on the nature of the protective serological response to FMDV in the natural bovine host.

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References


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