Foot-and-mouth disease (FMD) is a severe, clinically acute vesicular disease of cloven-hoofed domestic and wild animals and is present in all continents except Australasia and North America. The causative agent of FMD is Foot-and-mouth disease virus (FMDV), the prototype member of the genus Aphthovirus in the family Picornaviridae. There are seven distinct serotypes of FMDV (O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3) and multiple strains, indicating significant antigenic variability. Currently, immunization with conventional inactivated whole-virus vaccine is the main method of FMD control, but strain composition of vaccines must be selected with care. The humoral immune response has been generally accepted as the most important factor in conferring vaccine-induced protection against the disease, as a strong correlation has been reported between the levels of virus-neutralizing antibody produced after vaccination and subsequent protection of cattle, one of the main target species (Pay & Hingley, 1987). The mechanism by which antibodies protect against FMDV is poorly understood, and different factors may be involved in vivo compared with the situation measured in vitro (Dunn et al., 1998; McCullough et al., 1992). Several studies have been carried out in the last two decades to identify the targets on the virus surface for the binding of neutralizing antibodies, and mAbs have been powerful tools for identifying the amino acid footprint of different antigenic sites, mainly by sequencing mAb-resistant (mar) mutants. This approach has been used successfully to delineate the neutralizing antigenic sites of viruses representing serotypes O (Aktas & Samuel, 2000; Barnett et al., 1998; Crowther et al., 1993b), A (Baxt et al., 1989; Bolwell et al., 1989; Mahapatra et al., 2011; Thomas et al., 1988a), C (Mateu et al., 1990), Asia-1 (Grazioli et al., 2003) and SAT (Crowther et al., 1993a; Grazioli et al., 2006).

FMDV serotype O is the most prevalent globally and has been studied most extensively. Five neutralizing antigenic sites (1–5), involving three of the capsid proteins (VP1–3), have been identified on the surface of serotype O FMDV (Aktas & Samuel, 2000; Barnett et al., 1998; Crowther et al., 1993b; Kitson et al., 1990; McCahon et al., 1989; Xie et al., 1987). Site 1 is linear and trypsin-sensitive, whereas the rest of the identified antigenic sites have been reported to be conformation-dependent and trypsin-resistant. The most prominent surface projection is formed by the βG–βH (G–H) loop of VP1, which, together with the C terminus of VP1, contributes to antigenic site 1 with critical residues at positions 144, 148, 154 and 208. Amino acid residues at positions 70–73, 75, 77 and 131 of VP2 contribute to antigenic site 2, whereas residues 43 and 44 of the βB–βC (B–C) loop of VP1 constitute site 3. Amino acid residues at positions 56 and 58 of VP3 have been reported to be critical for antigenic site 4, whereas site 5, characterized by an amino acid at position 149 of VP1, is probably formed by interaction of the VP1 G–H loop region with other surface-located amino acids.

Historically, antigenic site 1 has been considered to be immunodominant and its linear structure has made it an obvious target for the development of peptide vaccines. Guinea pigs and cattle immunized with peptides corresponding to parts of VP1 (aa 141–160 or 141–158 and 200–213) conferred neutralizing antibody and protection (Bittle et al., 1982; DiMarchi et al., 1986; Pfaff et al., 1982). However, these peptide vaccines have not been fully effective in cattle (Taboga et al., 1997). Using a mAb-based competition ELISA (cELISA), Samuel (1997) reported the presence of relatively higher titres of site 2-specific antibodies in cattle. In contrast, using a similar assay, Aggarwal & Barnett (2002) reported that none of
antigenic sites 1–3 can be considered as immunodominant in bovine polyclonal sera. However, a potential problem with this approach is that antibody bound to one site may interfere with a competitive ligand targeted elsewhere on the virus surface, due to the small size of the virion and close vicinity of different antigenic sites. Other studies involving serotype C (Mateu et al., 1995) and serotype A (Thomas et al., 1988b) also indicated the participation of other antigenic sites in addition to site 1 in generation of an immune response following either natural infection or vaccination.

The relative importance of the different neutralizing antigenic sites in FMDV vaccine-induced protection has been poorly studied. We have addressed this question by using site-specific mar-mutant viruses to quantify the relative amount of antibodies against the known neutralizing antigenic sites of FMDV in the polyclonal sera of three different target species: cattle, pigs and sheep. Most of the animals exhibited significantly higher levels of antibodies to antigenic site 2. This knowledge may help in vaccine strain selection, as well as the design of novel vaccines.

Mar mutants of the O1 Kaufbeuren (O1K) strain of FMDV, representing each of five antigenic sites, were used in this study (Crowther et al., 1993b; Table 1). Prior to the commencement of binding studies, the presence of the expected mutations was confirmed by capsid sequencing of all mutant viruses. The inactivated FMDV vaccines used to vaccinate animals were either made in house or obtained from a commercial source (Merial), and were either O1K, O1 Lausanne or O1 BFS. All of these virus strains belong to the European–South American topotype. The capsid sequences of O1 Lausanne and O1 BFS are 99.6 and 99.2 % identical to that of O1K, respectively, at the amino acid level and have residues identical to those in O1K at the positions of change in the mar mutants. All of the animals received a comparable amount of antigen and the polyclonal antisera were collected either 21 or 28 days post-vaccination. The polyclonal sera were initially tested for antibodies against FMDV O1K using a virus-neutralization (VN) test and samples with a titre of >2 log_{10} were selected for further study. In total, 110 serum samples were from cattle vaccinated with O1K (n=5), O1 Lausanne (n=18) or O1 BFS (n=34), whereas the samples from pigs and sheep were from animals vaccinated with the O1 BFS vaccine.

Titres of neutralizing antibodies against the O1K virus and various mar-mutant viruses were measured by a microneutralization assay essentially as described by Rweyemamu (1984). The VN titres were calculated as log_{10} of the reciprocal antibody dilution required for 50 % neutralization of 100 TCID_{50} virus. All tests were carried out in duplicate and at least twice, and the mean of the results was used for further analysis. Pre-immune and several control sera from each species were obtained and analysed, and no anti-FMDV antibodies were detected. Statistical analyses were performed on serum titres obtained from the neutralization test using Stata/SE 11.2 (StataCorp LP). Pairwise comparisons of individual groups (parent and various mar mutants) were carried out using the Bonferroni-adjusted t-test. For the analysis, the parent group was considered as the reference, and the variability in the VNT log_{10} response for each of the mar mutants was calculated for each species. Furthermore, differences between each pair of means for each group were also assessed. Graphs were constructed using the ggplot2 package (Wickham, 2009) for R 2.13.0 (http://www.R-project.org).

The mar-mutant viruses used in this study are single mutants, in which the critical residue(s) of one of the neutralizing epitopes has been mutated, thereby abrogating the binding to the corresponding mAb (Kitson et al., 1990). These were used for the first time to quantify the relative amount of antibodies produced against each epitope in response to FMDV vaccination. Our hypothesis is that antibodies produced against an intact or native epitope will not recognize the mutated epitope, as mutations on antigenic sites have been shown to completely destroy binding with relevant virus-specific antibodies in FMDV (Dunn et al., 1998) and in poliovirus (Rezapkin et al., 2010). Indeed, Crowther et al. (1993a) reported a 15 % drop in neutralizing-antibody titre in post-vaccination cattle sera following a single mutation (G67 to G67+C3 mutant). Therefore, the reduction in the neutralizing-antibody titre for a particular mar mutant will indicate the presence of the relative amount of the antibodies against that particular epitope in the polyclonal serum, which in turn reflects the immunodominance of that epitope.

The titre reductions of neutralizing antibodies measured against various mar-mutant viruses compared with the parental virus O1K are shown in Fig. 1(a, b). Reduced neutralization was observed with mutants for all five antigenic sites in all three target species studied, albeit to various degrees (Fig. 1a) with significantly greater reductions (P<0.01) against antigenic site 2. Similarly significant reductions against antigenic site 1 (P<0.001) were observed only in cattle and pigs, and the BFS vaccine group. Differences between species were found to be statistically not significant (data not shown).

Table 1. Critical residues of FMDV serotype O mAbs

Numbers in parentheses indicate mar-mutant number (Crowther et al., 1993a; McCahon et al., 1989; Kitson et al., 1990).

<table>
<thead>
<tr>
<th>mAb</th>
<th>Virus strain</th>
<th>Site</th>
<th>Critical residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9 (480)</td>
<td>O1, Swiss 1965</td>
<td>1</td>
<td>VP1–148</td>
</tr>
<tr>
<td>C6 (599)</td>
<td>O1, Swiss 1965</td>
<td>2</td>
<td>VP2–73</td>
</tr>
<tr>
<td>C8 (597)</td>
<td>O1, Swiss 1965</td>
<td>3</td>
<td>VP1–44</td>
</tr>
<tr>
<td>14EH9 (63)</td>
<td>O1, Brugge</td>
<td>4</td>
<td>VP3–58</td>
</tr>
<tr>
<td>C3 (O1KC3)</td>
<td>O1, Caseros</td>
<td>5</td>
<td>VP1–149</td>
</tr>
</tbody>
</table>
Sera from 57 vaccinated cattle were used in this study and the results are shown in Fig. 1(a). Antibodies to all five antigenic sites, although to various degrees, were detected in these animals. Significantly higher (P<0.001) levels of antibodies were found to be directed against antigenic site 2, followed by site 1. The majority of the animals used in this study (76%) had more antibodies directed against antigenic site 2, whereas about 15% of the animals had more antibodies against antigenic site 1 (Fig. 2a). There were 6% of animals that had approximately equal levels of antibodies against sites 1 and 2 (Fig. 2a). The 57 serotype O sera tested in this study comprised samples from animals vaccinated with O1 BFS, O1 Lausanne or O1K vaccine. When the results were analysed for each vaccine, a consistent picture emerged, with more antibodies directed against antigenic site 2 followed by site 1 in most cattle (BFS, 62%; Lausanne, 78%; Kaufbeuren, 100%) (Fig. 2b).

The amount of antibodies produced against other sites (sites 1, 3, 4, and 5) was variable among the various vaccine strains. Interestingly, the lowest amount of antibody against site 1 was detected in O1K-vaccinated cattle; this could be due to the small sample size for this vaccine or because these five cattle were the only animals that received a booster dose on day 21 following vaccination. Herremans et al. (2000) also observed a change in site-specific dominance associated with booster vaccination, finding a relative rise in the level of antibody to site 3 in poliovirus. In the case of the O1 BFS group, 9% of animals had approximately equal levels of antibodies against sites 1 and 2 (Fig. 2b).

Sera from 20 vaccinated sheep were used for this study and the results are depicted in Fig. 1(a). As in cattle, a varied immune response was observed in these animals; however, overall, significantly more antibodies (P<0.001) were directed against site 2. The levels of neutralizing antibodies against antigenic sites 1, 4, 5 and 3 were not statistically significant. About 65% of animals had more antibodies against site 2, which is consistent with the results from the cattle study.
against site 2, followed by site 1 (20 %) and site 4 (15 %) (Fig. 2b).

Sera from 33 vaccinated pigs were used for this study and the results are shown in Fig. 1(a). Although a varied immune response was observed in these animals, as a whole, significantly higher \((P<0.0001)\) levels of antibodies were directed against site 2, followed by site 1. The majority of the animals used in this study (58 %) had more antibodies against antigenic site 2, whereas about 18 % had more antibodies against antigenic site 1 (Fig. 2a). In addition, 15 % of animals had approximately equal levels of antibodies against sites 1 and 2 (Fig. 2a).

Although a three- or four-site mutant was neutralized using FMDV post-infection sera (McCahon et al., 1989; Crowther et al., 1993a), use of post-vaccination sera showed a different pattern. A neutralization test involving multiple mutant G67 (a four-site mutant) and FMDV post-vaccination sera demonstrated the group mean residual percentage titre to be 15 % (presented as a percentage of the titre found against the parent O 1K virus). However, when the fifth site was mutated (G67 + C3, a five-site mutant), the mean residual percentage titre decreased to an undetectable level, indicating that the drop was due to the loss of neutralization against the fifth site (Crowther et al., 1993a). It was only a single mutation at position 149 of VP1 that resulted in about 15 % drop in neutralizing-antibody titre.

In this study, we have quantified the relative amounts of antibody produced against each antigenic site in response to vaccination against serotype O FMDV using a panel of mar mutants. The results indicate that most of the antibodies produced in response to FMDV vaccination in cattle, sheep and pigs are against antigenic site 2, followed by site 1. Using a serum immunoglobulin-fractionation study involving serotype C FMDV, Mateu et al. (1995) also reported that about 27 % of virus-neutralizing activity from vaccinated pigs corresponded to antigenic site 1, whereas the majority of the antibodies were directed against other antigenic sites. Other reports are also consistent with our findings: (i) mAbs produced following immunization with serotype O FMDV vaccines are often found to be against antigenic site 2 (Barnett et al., 1998; M..fig. 2. Percentage of animals showing highest antibody titres against various neutralizing antigenic sites by species (a) and by vaccination (b). Error bars represent 95 % confidence intervals.

http://vir.sgmjournals.org
Mahapatra, unpublished data), indicating that this site is often recognized by antibody-producing cells. (ii) The VP1 G–H loop (site 1) is not essential for protection in natural hosts against FMDV infection (Fowler et al., 2008, 2010; Rieder et al., 1994), and vaccination with VP1 G–H loop peptide vaccines has not been fully successful in eliciting complete protection (Krebs et al., 1993; Rodriguez et al., 2003; Taboga et al., 1997). However, our results contradict those of Aggarwal & Barnett (2002), who used a mAb-based cELISA involving mAbs to antigenic sites 1–3, some homologous and some heterologous, to determine the relative importance of various antigenic sites in polyclonal responses to serotype O FMDV. Their results indicated that none of these antigenic sites can be considered as immunodominant. Interestingly, they also reported that pigs did not respond to epitopes on the C-terminal end of VP1 as efficiently as ruminants. Although a cELISA-based approach has been used successfully to define the epitopes of FMDV (Barnett et al., 1998; McCullough et al., 1987; Thomas et al., 1988b) and poliovirus (Rezapkin et al., 2005, 2010), competition has been reported to be dependent on the affinity of the mAbs (Thomas et al., 1988b) and, sometimes, competition might be observed due to steric interference because of the large size of the antibody molecule and the relative proximity of the antigenic sites on the surface of FMDV.

In summary, it appears that, following vaccination, the predominant antibody response in the case of serotype O FMDV is against antigenic site 2, followed by site 1. This trend was consistent in all of the three different target species studied (cattle, pigs and sheep), and also with three different vaccine formulations (BFS, Lausanne and Kaufbeuren). The antibody profile induced in humans following vaccination with inactivated and live-attenuated oral polio vaccine was found to be significantly different (Rezapkin et al., 2010). Crowther et al. (1993a) also reported a significantly higher VN titre in post-infection sera than in post-vaccination sera using a G67 (four-site mutant) virus. Hence, it is possible that the antibody profile following FMDV infection could also be different from that following vaccination.

Acknowledgements

We would like to thank B. Haas, B. Statham, P. Barnett, Y. Li, N. Ferris, A. Dekker and A. Samuel for making some of the reagents available for this study and A. Di-Nardo for help in statistical analysis of the data. This work was supported financially by DEFRA grants SE2922 and SE2937. D. J. P. is a Jenner Institute Investigator and is funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

References


Kimpton, J. D., McCahon, D. & Belsham, G. J. (1990). Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth...
disease virus: evidence for the involvement of the three surface exposed capsid proteins in four antigenic sites. *Virology* 179, 26–34.


