# PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS

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***Summary.*** **Monoclonal antibodies (MAbs) produced against foot-and-mouth disease virus (FMDV) types O, A and C are described. The preliminary characterization is discussed in terms of virus neutralization and mouse protection test results and the reactivity of MAbs in enzyme-linked immunoelectrotransfer blot assays with the denatured protein of the virus. Some MAbs which did not neutralize virus infectivity in tissue culture were able to neutralize virus infectivity in suckling mice. The MAbs designated as 7EE6 and 7JA1 which originated from $C_3$ Indaial virus cross-reacted with heterologous FMDV $O_1$ Campos and $A_{24}$ Cruzeiro viruses.**

Protection of host animals against aphthovirus infection is a complex phenomenon which may occur by a variety of different mechanisms. For instance, neutralization of virus infectivity both directly or indirectly, by antibody and phagocytosis, has been proposed as a major mechanism of immunological defense against viral invasion (*19*). The introduction of monoclonal antibody (MAb) technology has helped understand the mechanisms of viral neutralization (*20*), characterize the extensive antigenic diversification of viruses and define the antigenic sites on the viral surface (*6,7,9*).

Many groups have been using MAbs to understand the antigenic complexity of foot-and-mouth disease viruses (FMDV). Sequence analyses of MAb resistant mutants of FMDV type O (strain $O_1K$) (*12*) have identified four antigenic sites, one of which corresponds to a trypsin-sensitive region in the amino acid sequence 140-160 and the C-terminus residues 208 of $VP_1$. A second site, involving amino acids 43 and 44 has been identified also on $VP_1$, a third was found associated with the residues of the amino acid sequence 70-73, 75, 77 and 131 on $VP_2$, and the last one involved residue 58 on $VP_3$ (*12*).

For FMDV type A (strain $A_{10}$ Holland) (*23*), four antigenic sites were reported as follows: The first site was trypsin-sensitive and included the $VP_1$ 140 to 160 sequence, while the second one was trypsin-insensitive and included mainly $VP_3$. Two other minor sites were located near $VP_1$ 169 residues and on the C terminus of $VP_1$.

Current knowledge on the discontinuous epitopes of the C serotype of FMD (strain C-S8) antigenic sites which are independent of site A ($VP_1$ 140-160), has been reviewed recently (*14*). One of these sites, called site C, corresponds to the carboxy-terminal segment of $VP_1$. Although in FMDV type O , sites A and C form together a discontinuous domain (site 1), they appear as independent continuous sites in type C viruses.

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Another site on the latter virus, located close to the capsid threefold axis, is a discontinuous antigenic domain which appears as a major antigenic site, (*13*) while a third independent antigenic site is located close to the capsid fivefold axis. Furthermore, at least 14 different continuous epitopes in site A, and 2 epitopes in site C have also been described by Mateu et al. (*14,15*), using a panel of 18 neutralizing MAbs, some of which are included in the present study.

A set of MAbs directed against the different antigenic sites may be useful for studying the antigenicity of vaccines. Equally, they may constitute an important epidemiological tool to study the antigenic relationships between FMD virus vaccine strains and FMD viruses emerging in the field and assist in the design of a proper vaccine strategy.

This report describes the production of a series of MAbs against FMDV types O, A and C. Their preliminary characterization is discussed on the basis of results obtained with virus neutralization and mouse protection tests and by their reactivity with the virus proteins in enzyme-linked immunoelectrotransfer blot (EITB).

## MATERIALS AND METHODS

*Viruses:* The virus strains used in the present study were those employed for the formulation of FMDV vaccine in most South American countries: $O_1$ Campos Br/58, $A_{24}$ Cruzeiro Br/55 and $C_3$ Indaial Br/71. Viral preparations were usually inactivated with BEA (*4*) and purified in a cesium chloride gradient.

*Immunization:* Six to ten week-old male Balb/c mice were inoculated weekly by the intraperitoneal route with 40 µg of purified inactivated or non-inactivated viral preparations. For the first inoculation, virus samples were emulsified in complete Freund's adjuvant, whereas incomplete Freund's adjuvant was used for the second and the third immunizations. The fourth inoculation was administered without adjuvant, two days before the fusion.

*Monoclonal Antibodies:* Spleens removed aseptically from immunized mice were washed and macerated in Dulbecco's Modified Eagle Medium (DMEM). Cells were harvested and concentrated by centrifugation at 1000 g for 8 minutes at 4°C. The pellet was treated with ACK buffer ($NH_4Cl$ 0.15 M ; $KHCO_3$ 0.01 M; $Na_2EDTA$ 0.01 M) for 1 minute in an ice bath, and then neutralized with 40 ml of DMEM containing fetal calf serum (5%) and heparin (5 IU/ml). Subsequently, the cell suspensions were centrifuged and the pellets resuspended in DMEM.

$SP_2/0$ Ag 14 myeloma cells in the exponential grow phase were cultured in SP2 medium (DMEM with 2 mM of L-glutamine, 1 mM sodium pyruvate and supplemented with 15% fetal calf serum), and then washed and resuspended in DMEM. The spleen cells and the myeloma cells were mixed at a 4:1 proportion respectively, and centrifuged. Cell pellets were dispersed gently and the fusions were carried out by adding of 1 ml of polyethyleneglycol 1500 (NBS Biologicals*) (PEG 1500 50% w/v in DMEM and 5% dimethylsulfoxide) for every 1.8 x $10^8$ cells, stirring by rotation for the next two minutes. Immediately thereafter, 20 ml of DMEM were added slowly over a period of three minutes using a dropper. Cell suspensions were gently pipetted and incubated at 37°C for 15 minutes in a water bath.

After incubation, supernatants were transferred to fresh tubes, the volumes adjusted to 40 ml with DMEM, and centrifuged. The cell pellets were resuspended in HAT medium (SP2 medium with hypoxanthine 0.1mM, aminopterin 0.0004 mM, and thymidine 0.016 mM) with 2.5 x $10^6$ cells/ml aproximately, and distributed in 96 well plates containing 0.2 ml/well. Well plates were placed at 37°C in an incubator with 5% $CO_2$ in a humid atmosphere.

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* Mention in this paper of commercial firms or their products is for identification only and does not constitute endorsement by the authors or their organizations.

Hybridoma growth was monitored daily until approximately 300 cells were observed in the hybridoma colonies. Supernatants were collected and screened for specific antibodies by ELISA tests as described below. Positive hybridomas were cloned three times by the limiting dilution technique. Hybridomas were maintained in HT culture medium (SP2 medium with hypoxanthine 0.1mM and thymidine 0.016 mM).

*ELISA test:* Hybridoma products were assayed by ELISA tests, according to the methods described (*2*). Briefly, rabbit hyperimmune serum specific to each of the FMDV types (O, A and C) was coated to the ELISA plate (Nunc Immunoplate I*), to be used as capture antibody. FMDV homologous to each capture antibody was added to the plate and incubated. After the incubation and washing steps, supernatants from each hybridoma culture were reacted with the homologous virus for 30 minutes at 37°C.

Positive reactions were detected by adding goat anti-mouse horseradish peroxidase immunoglobulin conjugate and developing with O-phenylenediamine dihydrochloride.

*Characterization of monoclonal antibodies:* The MAb isotype was determined in agar gel immunodiffusion (AGID) tests, using antisera specific for each isotype.

MAb concentrations were determined by AGID as described elsewhere (*22*). Briefly, agarose (1%) was prepared in 10 mM Tris-HCl, 140 mM NaCl and 0.1% $NaN_3$. After agarose fusion, 90 µg of goat anti-mouse IgG were added per 12 ml of the agarose solution at 40-50°C, and poured into Petri dishes with 10 cm diameters. Seven wells were made in each plate and filled with 10 µl of the corresponding MAbs. MAb concentrations were estimated by measuring the precipitation halo and then comparing it with a standard slope based on data obtained with known MAb concentrations (0 µg; 0.2 µg; 0.4 µg; 0.8 µg).

The ability of MAbs to neutralize virus infectivity *in vitro* and *in vivo* was assayed by virus neutralization (VNT) and mouse protection (MPT) tests, respectively. For the VNT (*10*), IBRS-2 cell culture suspensions containing 3 x $10^5$ cells/ml were distributed in 0.1 ml amounts in 96 microtiter wells and incubated at 37°C in a $CO_2$ incubator for 24 hours.

Four fourfold dilutions of the positive MAbs were incubated with pre-titrated virus (100 $TCID_{50}$) at 37°C for 1 hour. The MAb-virus mixture was then distributed in 0.1 ml amounts over the cell culture monolayer. MAb titers were expressed as the $\log_{10}$ of the reciprocal of the lowest dilution of the MAb which permited any plaque formation.

The MPT procedure was performed as described (*8*). In brief, 4-6 day-old suckling mice were inoculated subcutaneously with 0.1 ml of undiluted MAb. One hour later, they were inoculated intraperitoneally with 0.05 ml of four 10-fold virus serial dilutions ($10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$). The $ID_{50}$ of the virus in the presence of MAb was calculated by the Reed Muench method, and the results were expressed in terms of the mouse protection index. Positive and negative control sera and the virus titrations were included with the VNT and MPT procedures. The results were accepted only when these controls were in agreement with the standard titers determined previously.

For determining the MAb reactivity with the structural protein of the virus, an EITB assay was developed on the basis of the procedure described in the technical manual of the Mini Trans-Blot BIO-RAD supplier*. The virus structural proteins were separated in SDS-PAGE with 10% acrylamide/bisacrylamide. For each gel, 100 µg of virus purified in a cesium chloride gradient were used and transfered to nitrocellulose membranes. A 2% solution of bovine serum albumin (BSA) in PBS (0.136 M NaCl; 0.002 M KCl; 0.01 M $Na_2HPO_4$; 0.001 M $KH_2PO_4$) was used for saturating nitrocellulose membranes overnight at 5°C. Strips from the saturated nitrocellulose membranes were reacted with each MAb supernatant diluted 1:2 in PBS with 2% BSA (diluting buffer), and incubated at 25°C for 2 hours under constant agitation. Subsequently, nitrocellulose strips were washed three times with PBS containing 0.05% Tween 20, goat anti-mouse horseradish peroxidase immunoglobulin conjugate was added, and

the preparation was allowed to incubate for 1 hour. The reaction was developed with 4-cloro-1-naphthol (*16*).

## RESULTS

*Monoclonal Antibodies:* The immunization procedure with purified and/or inactivated and live FMD viruses elicited a good immune response in mice against each FMDV strain used. However, immunization procedure using non-inactivated virus yielded higher numbers of positive hybridomas. The fusions resulted in 120 hybridoma cell lines continuously secreting antibody. Of these, 35 hybridomas were specific to $O_1$ Campos, 58 to $A_{24}$ Cruzeiro and 27 to $C_3$ Indaial.

**Table 1. Properties of MAbs for FMDV type O**

| MONO-CLONAL | ISOTYPE | CONC[a] | VNT[b] | MPT[c] | EITB[d] |
|---|---|---|---|---|---|
| 11HC10 | K/IgG3 | — | > 3.50 | 4.27 | VP1[e] |
| 11AD6 | K/IgG2a | 186 | < 1.20 | 2.35 | CONF[f] |
| 12BB5 | K/IgG3 | 162 | < 1.20 | 3.25 | CONF. |
| 12FA5 | K/— | 10 | < 1.20 | 0.88 | CONF. |
| 12DB7 | K/IgG2a | 99 | 1.90 | 2.83 | CONF. |
| 12GG11 | K/IgG2a | 118 | < 1.20 | 0.00 | CONF. |
| 12EH6 | K/IgG1 | 118 | < 1.20 | 0.06 | CONF. |
| 12FG2 | K/IgM | — | < 1.20 | 0.38 | CONF. |
| 36AF8 | K/— | — | < 1.20 | — | VP1 |
| 12CA12 | K/IgG1 | 47 | < 1.20 | — | CONF. |
| 13EG1 | K/IgG2a | 118 | < 1.20 | 0.00 | CONF. |
| 13EC5 | K/IgG1 | 108 | < 1.20 | 0.00 | CONF. |
| 13DG11 | K/IgM | — | < 1.20 | 0.31 | CONF. |
| 34CH4 | K/— | 33 | < 1.20 | 0.50 | CONF. |
| 36AC6 | K/IgM | — | > 3.60 | 2.50 | CONF. |
| 36BE8 | K/Ig2a | — | 1.90 | — | VP1 |
| 36CC2 | K/IgG2a | 129 | < 1.20 | < 1.25 | CONF. |
| 36BB11 | K/IgG1 | 108 | < 1.20 | — | CONF. |
| 36AA8 | K/IgG2a | 118 | < 1.20 | < 1.25 | CONF. |
| 36AD5 | K/IgG2a | 186 | < 1.20 | < 1.25 | CONF. |

[a] MAb concentration determinated by Agar Gel Immuno-difusion test.
[b] Titer in virus neutralization tests.
[c] Mouse protection index, determined by mouse protection tests.
[d] Enzyme-linked Immunoelectrotransfer Blot assay.
[e] Positive and [f] Negative reactions with denatured virus protein determined by EITB.
- Not determined.

**Table 2. Properties of MAbs for FMDV type A**

| MONO-CLONAL | ISOTYPE | CONC[a] | VNT[b] | MPT[c] | EITB[d] |
|---|---|---|---|---|---|
| 16EC11 | K/ – | 118 | < 1.20 | 3.00 | VP1[e] |
| 16EC2 | K/IgM | – | < 1.20 | 3.00 | CONF[f] |
| 17CA6 | K/IgG2a | 33 | < 1.20 | 1.25 | CONF. |
| 31DC7 | K/IgG3 | 140 | < 1.20 | 2.24 | VP1 |
| 31DE3 | K/ – | 20 | < 1.20 | 3.00 | VP1 |
| 31DB8 | K/ – | 162 | < 1.20 | 4.02 | VP1 |
| 31DF4 | K/IgG3 | 33 | 3.40 | 4.03 | VP1 |
| 31EF12 | K/IgG2a | 15 | 1.80 | 4.25 | CONF. |
| 31EH2 | K/ – | 47 | 1.50 | 2.75 | CONF. |
| 31ED1 | K/IgG2a | 47 | 1.40 | 4.50 | CONF. |
| 31EE8 | K/IgG2a | 63 | 1.60 | 1.50 | CONF. |
| 31EA12 | K/IgG2a | – | 1.30 | 2.00 | CONF. |
| 31EG9 | K/IgG2a | 63 | 1.30 | 4.65 | CONF |
| 31EB2 | K/IGg2a | 63 | 1.40 | 4.25 | CONF. |
| 31FE7 | K/ – | 162 | < 1.20 | 3.10 | VP1 |
| 31FA4 | K/IgG2a | 150 | < 1.20 | 3.15 | VP1 |
| 31GA6 | K/IgG3 | 70 | < 1.20 | 3.85 | CONF. |
| 31GA3 | K/IgG3 | 118 | < 1.20 | 3.35 | VP1 |
| 32AA5 | K/Ig2a | 140 | < 1.20 | 0.85 | VP1 |
| 32DH3 | K/IgG2a | 186 | < 1.20 | 4.25 | CONF. |

[a] MAb concentration determinated by Agar Gel Immuno-difusion test.
[b] Titer in virusneutralization tests.
[c] Mouse protection index, determined by mouse protection tests.
[d] Enzyme-linked Immunoelectrotransfer Blot assay.
[e] Positive and [f] Negative reactions with denatured virus protein determined by EITB.
- Not determined.

Data on twenty MAbs against each virus strain are shown on tables 1, 2 and 3. They were selected from all of the MAbs obtained in this study, on the basis of their characteristics (this report), and their lack of cross reactivity with other FMDV strains of a different serotype (data not shown).

Hybridoma colonies were subjected to the cloning process to certify the homogeneity and stability of the hybridoma cell lines. In each case the good capacity of the hybridoma cell lines to secrete antibody was confirmed.

*Characterization of Monoclonal Antibodies:* The MAb isotypes are shown in tables 1, 2 and 3. All the IgG3 isotype MAbs showed ability to neutralize virus infectivity in suckling mice. The sole excep-

tion was 7JA1 MAb, which cross reacted with O and A viruses.

The MAb concentration, as determined by AGID revealed wide variations in the MAb concentration of the supernatants from the hybridoma cultures (tables 1, 2 and 3). These differences were inherent to each hybridoma, although the management of hybridoma cultures possibly had some influence in the MAb concentration.

Crossed reactions were detected in ELISA tests. MAbs 7EE6 and 7JA1, which originated from the $C_3$ Indaial strain, also reacted to differing entents with the $O_1$ Campos and $A_{24}$ Cruzeiro viruses (figure 1). Of these two MAbs, only 7EE6 gave high titers in the homologous serum neutralization and mouse protection tests (table 3). Cross reactivity in VNT and MPT with 7EE6, resulted in titers of <1.20 and 1.52 respectively, for $O_1$ Campos, and of <1.20 and 0.55 for $A_{24}$ Cruzeiro.

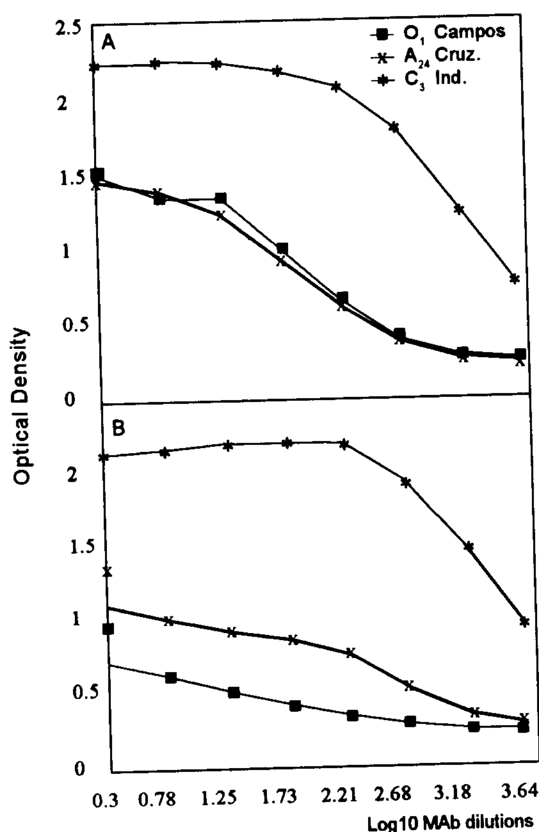


**Figure 1. Reactivity of MAbs 7EE6 (A) and 7JA1 (B) against homologous ($C_3$ Indaial) and heterologous ($O_1$ Campos and $A_{24}$ Cruzeiro) viruses in ELISA tests**

**Table 3. Properties of MAbs for FMDV type C**

| MONO-CLONAL | ISOTYPE | CONC[a] | VNT[b] | MPT[c] | EITB[d] |
|---|---|---|---|---|---|
| 1BH8 | K/ – | 26 | 1.90 | 2.22 | VP1[e] |
| 2LC9 | K/ – | – | < 1.20 | 1.70 | VP1 |
| 7AB5 | K/IgG3 | 250 | 3.50 | > 4.75 | VP1 |
| 7AH1 | K/IgG2b | 211 | < 1.20 | 0.60 | VP1 |
| 7BH11 | K/IgG2b | 236 | < 0.61 | 1.25 | CONF[f] |
| 7CA8 | K/IgG3 | 295 | > 3.60 | 3.25 | VP1 |
| 7CH1 | K/IgG1 | 200 | 1.90 | 3.75 | VP1 |
| 7CA11 | K/IgG1 | 160 | 3.50 | 3.60 | VP1 |
| 7DH4 | K/IgG2b | 165 | < 1.20 | 1.40 | CONF. |
| 7DG11 | K/IgG1 | – | < 0.61 | 0.21 | CONF. |
| 7DF10 | K/IgG1 | 120 | 1.50 | 4.32 | CONF. |
| 7EE6 | K/IgG3 | 160 | 3.40 | 3.07 | VP1 |
| 7EG3 | K/IgG1 | – | < 1.20 | 0.71 | CONF. |
| 7FC4 | K/IgG2a | 256 | < 1.20 | 0.75 | CONF. |
| 7FC12 | K/IgG1 | 104 | 3.30 | 3.00 | VP1 |
| 7JA1 | K/IgG3 | 90 | < 0.61 | 0.46 | VP1 |
| 7JD1 | K/IgG1 | 200 | > 3.61 | 3.50 | VP1 |
| 7LA5 | K/IgG3 | 90 | < 1.20 | 4.25 | CONF. |
| 7DH9 | K/IgG2a | 295 | < 1.20 | 0.60 | CONF. |
| 7LE9 | K/IgG1 | – | – | 3.58 | CONF. |

[a] MAb concentration determinated by Agar Gel Immunodifusion test.
[b] Titer in virusneutralization tests.
[c] Mouse protection index, determined by mouse protection tests.
[d] Enzyme-linked Immunoelectrotransfer Blot assay.
[e] Positive and [f] Negative reactions with denatured virus protein determined by EITB.
- Not determined.

The ability of MAbs to neutralize viral infectivity in tissue culture was tested by the VNT (tables 1, 2 and 3). The $A_{24}$ Cruzeiro virus appeared to induce mostly low-titer neutralizing MAbs. With the exception of 31DF4, all of $A_{24}$ Cruzeiro MAbs titers were 1.80 or less in VNT. In contrast, $A_{24}$ Cruzeiro virus induced a higher number of MAbs with high mouse protection index. 17 of the MAbs showed an MPT titer of 2.00 or higher (table 2). The $O_1$ Campos virus strain presented poor ability to induce MAbs with neutralizing or protective properties, particularly when compared with

the MAbs obtained from $A_{24}$ Cruzeiro and $C_3$ Indaial viruses.

Data on ability of the MAbs to protect mice in MPT are shown in tables 1, 2 and 3. Neutralization of virus infectivity in MPT showed different results from those observed in VNT. Thus, many MAbs which did not neutralize virus infectivity in tissue culture, did protect suckling mice in MPT.

Of the virus proteins defined by the MAbs in EITB (tables 1, 2 and 3), an average of 50% of the epitopes identified by the MAbs elicited by $A_{24}$ Cruzeiro and $C_3$ Indaial viruses corresponded to non-conformational epitopes in $VP_1$. In contrast, the epitopes identified by most of MAbs elicited by $O_1$ Campos virus strain, were found to be conformational. Only MAbs 11HC10, 36AF8 and 36BE8 were found to react with the $VP_1$ linear sequence in EITB.

## DISCUSSION

All of the MAbs described in this paper contained Kappa light chains and most of the heavy chains were of the IgG isotype. These characteristics reflect the facts that 95% of all mouse immunoglobulin molecules contain Kappa light chains, and that extended immunization procedures typically result in an IgG response (*11*).

The MAbs induced by $O_1$ Campos, which recognized the linear sequence of $VP_1$, were revealed only by MAbs 11HC10, 36AF8 and 36BB11. Other MAbs were directed to conformational epitopes on the virion surface. It is suggested that the major neutralizing site of $O_1$ Campos may be more conformation-dependent than those observed for $A_{24}$ Cruzeiro and $C_3$ Indaial viruses (*6,13,24*).

In a previous study (*15*), the epitopes recognized by the $C_3$ Indaial MAbs 7EE6 and 7JA1 on the $VP_1$ region corresponded, respectively, to the amino acids 138 to 156 (site A) and 192 to 209 (site C). In the present study, these MAbs were found to cross react with $O_1$ Campos and $A_{24}$ Cruzeiro viruses (figure 1). Neutralizing activity in MPT was observed for the homologous virus with the MAb 7EE6, and was recorded also for the heterologous $O_1$ Campos and $A_{24}$ Cruzeiro viruses. Although the MAb cross-reaction titers were considered low, these findings are suggestive of similarities amongst the neutralizable epitopes defined by 7EE6 MAb both in the homologous and the other two heterologous virus types. Studies are in progress to further clarify these findings.

The VNT *in vitro* assay is selective for a particular type of antibody, which is capable of neutralizing viral infectivity mainly at high concentrations (*17*). This is in contrast to what really happens in the natural host or in *in vivo* MPT, where other immunological mediators are involved, such as antibody opsonization and antibody plus complement, which enhance phagocytosis of viral particles (*18*). In addition, the antibody class and subclass play an important role in relation to complement activation and to the interaction of antibody with Fc receptors in macrophages or in neutrophils (*21*). In fact many MAbs with very low VNT titers had high MP index. The reverse situation was not seen. This has important implications for experiments and assays in which titer is related to protective capacity of an antigen. While the MPT is generally regarded to be the most relevant to protection in cattle it has to be said that a high titer in the mouse of a given MAb may reflect the peculiarities of a particular murine isotype and its interactions with murine phagocytes rather than the situation in cattle.

Relationships among FMDV strains can be established clearly on the basis of the nucleotide sequence (*5*). Although this technique may serve as a useful epidemiological tool, it has limitations for predicting viral antigenicity, for example in relation to determining vaccine strategy (*3*). A single amino acid substitution within the 138-156 region of $VP_1$ has been shown to modify the antigenic specificity of the virus (*15*) and variation in domains other than antigenic site A, may be also relevant to the overall antigenic diversity of FMDV in the field (*9*).

In general, these considerations stress the potential significance of the MAbs described in this report for use in the antigenic characterization of FMDV obtained from field outbreaks as well as vaccine strains. Information along these lines seems

particularly of value to identify the four or five antigenic sites of FMDV described (*6,7,9,12,13,23*).

Furthermore, evidence was found (*14*) using MAbs described in this paper that a very limited sequence variation in the independent antigenic sites of FMDV, which contain several epitopes, may constitute the basis for FMDV antigenic diversity in the field. Finally, some of these MAbs have proven useful during the process of FMD vaccine production (*1,2*), quantifying antigenic mass and for determining the fit between inactivated antigens and challenge viruses.

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