DETAILED CHARACTERIZATION OF SOME MONOCLONAL ANTIBODIES WHICH RECOGNIZE ANTIGENIC SITES ON VP_1 OF FOOT-AND-MOUTH DISEASE VIRUS

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SUMMARY. **Most monoclonal antibodies (MAbs) raised against the O_1 Campos, A_{24} Cruzeiro and C_3 Indaial strains of foot-and-mouth disease virus (FMDV), and which were previously shown to react with VP_1 in Western Blots, recognized synthetic peptides corresponding to the 141-158 sequence of this protein. A significant number of the MAbs which recognized synthetic peptides did so in a heterologous as well as homologous manner. Thus, many of the A_{24} Cruzeiro MAbs recognized an O_1 Kaufbeuren peptide and the cross-reactivity was ascribed to the 141-158 region of the VP_1. One C_3 Indaial MAb recognized peptides corresponding to three serotypes and the cross-reactivity was ascribed to the 200-213 sequence of the VP_1. Usually, the cross-reactivity of a given MAb was observed with either the virus or the peptide but not with both antigens.**

Monoclonal antibodies (MAbs) raised against foot-and-mouth disease virus (FMDV) have been widely used in fundamental and applied investigations. Notable applications include the definition of antigenic sites on the virus (*10*) and strain differentiation with particular respect to the relationship among field strains and to vaccine strains. Usually, panels of MAbs are used in the latter application to support data obtained with polyclonal reagents. While the value of these panels in discriminating between closely related viruses or detecting slight antigenic changes is indisputable, the precise relevance of the information obtained to practical aspects in the diagnosis and control of the disease may be more obscure.

One reason for this is that the MAbs chosen may not be fully representative of the polyclonal antibody response of a target species to vaccination or infection with FMDV. Indeed, a combination of fundamental immunological and technical considerations make it unlikely that any one laboratory would be successful in developing a comprehensive library of MAbs. It is worth reflecting that the evidence for at least five antigenic sites in type O FMDV was obtained with MAbs from four different laboratories (*3,11*). A further complication is the tendency to select MAbs and MAb panels on the basis of their ability to neutralize virus *in vitro*. While it is not intended to devalue neutralization *per se*, both MAbs and polyclonal sera do exist which neutralize strongly *in vitro* but are considerably less effective in *in vivo* protection tests and vice-versa (*15*).

It is clear, therefore, that MAb libraries should be characterized comprehensively to en-

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sure that any interpretations made are meaningful. To this end, there has been a continuous programme within the Pan American Foot-and-Mouth Disease Center (PANAFTOSA) to develop and characterize fully MAbs against strains of major importance to South America. A previous publication (*1*) described a range of properties of MAbs raised against the O_1 Campos, A_{24} Cruzeiro and C_3 Indaial strains of FMDV including their ability to recognize conformation-independent epitopes, as deduced from reactivities with denatured virus proteins as well as whole virus, or conformation-dependent epitopes, as deduced from reactivity with virus alone. In the present study, we have extended the work with these and other MAbs to examine the recognition of some synthetic peptides with particular reference to identifying the binding sequences.

MATERIAL AND METHODS

Synthetic Peptides. These were either a kind gift from Dr. Richard DiMarchi (Eli Lilly Research Laboratories, Indianapolis, U.S.A.) or prepared by one of the authors (T.R. Doel) at the Institute for Animal Health, Pirbright, U.K. and donated by Dr. Mike Parkhouse. Peptides were synthesized by T-boc solid phase methods.

Most of the basic screening was done with three peptides based on the VP_1 sequences of the O_1 Kaufbeuren, A_{24} Cruzeiro and C_3 Indaial strains of FMDV (*12*). Each peptide incorporated two antigenic regions of the VP_1 sequence and, in the case of the O_1 Kaufbeuren and A_{24} Cruzeiro peptides, contained additional amino acids between the two sequences and at each end (*7*). That is:

O_1 peptide was C-C-X_O-P-P-S-Z_O-P-C-G
A_{24} peptide was C-C-X_A-P-P-S-Z_A-P-C-G

where C,G,P and S are the single letter codes for cysteine, glycine, proline and serine and:

X_O is the 200-213 sequence of the O_1 K strain of FMDV and is:

R-H-K-Q-K-I-V-A-P-V-K-Q-T-L

X_A is the equivalent sequence of the A_{24} Cruzeiro strain of FMDV and is:

R-H-K-Q-K-I-I-A-P-A-K-Q-L-L

Z_O is the 141-158 sequence of the O_1 K strain of FMDV and is:

V-P-N-L-R-G-D-L-Q-V-L-A-Q-K-V-A-R-T

Z_A is the equivalent sequence of the A_{24} Cruzeiro strain of FMDV and is:

G-S-G-R-R-G-D-M-G-S-L-A-A-R-V-V-K-Q

The C_3 peptide had the construction Z_C-X_C where

Z_C is the 134-160 sequence of the C_3 Indaial strain of FMDV and is:

T-G-T-T-A-Y-T-A-S-A-R-R-G-D-L-A-H-L-A-A-A-H-A-R-H-L-P

X_C is the 196-213 sequence of the C_3 Indaial strain of FMDV and is:

P-A-G-D-R-H-K-Q-P-L-I-A-P-A-K-Q-L-L

Other peptides used included the individual 141-158 and 200-213 sequences of the O_1 and A_{24} viruses and overlapping peptide sets corresponding to the peptides C-C-X_O-P-P-S-Z_O-P-C-G and C-C-X_A-P-P-S-Z_A-P-C-G. Each set was made by the tea-bag procedure (*9*) and was composed of 29, 12-mer peptides with each peptide overlapping the next peptide in the sequence by 11 amino acids.

Monoclonal Antibodies. Most of the MAbs used in the present study have been described elsewhere (*1,17*). McA OC3 (anti-O_1 Caseros) was kindly provided by Dr. Sandra Farias, Federal University, Porto Alegre, Brazil. MAb B2 (anti-O_1 Switzerland 1965) was originally described by Crowther et al. (*4*).

Immunoassays. ELISA was carried out as described previously (*7*) with a few minor variations. Briefly, plastic plates (Nunc Immunoplate 1*) were coated overnight at 4°C with 3 µg/ml synthetic peptides in 15 mM carbonate/bicarbonate buffer, pH 9.6. The following day, dilutions of MAbs were added to the plate and incubated at 37°C for 30 minutes. Following incubation with goat anti-mouse immunoglobulin conjugated to horseradish peroxidase, positive reactions were detected with O-phenylenediamine/H_2O_2. The carrier protein used in the diluent for the MAbs and enzyme conjugate was 1% ovalbumin in PBS containing 0.05% v/v Tween 20.

RESULTS

Table 1 summarizes some of the properties of the MAbs in the present study, including their ability to recognize synthetic peptides corresponding to two sequences within VP_1. With respect to the latter property, two reactivities are clearly distinguishable from table 1. None of the MAbs, which were previously categorized as recognizing conformation-dependent epitopes (*1*), recognized the homologous VP_1 peptides nor, when tested, any of the heterologous peptides. The other category of MAbs, previously characterized as conformation-independent because of reactivity with VP_1 in Western Blotting experiments, consistently recognized the peptides and, in particular, the 141-158 region as deduced with peptides corresponding to shorter sequences. A number of MAbs, not previously categorized with respect to recognizing conformation-dependent or-independent epitopes were tested blind against the peptides. Those which recognized one of the peptides were also found to bind to the homologous VP_1 in Western Blotting experiments, whereas those which did not recognize any of the peptides did not bind any of the denatured structural proteins of the virus (results not shown). Thus, peptide binding represents a rapid and easy method to make an initial grouping of a large number of MAbs.

Dealing first with the serotype specific recognition, the O_1 MAbs, 11HC10, 36AF8 and 36BE8 reacted with O_1 12-mer tea-bag peptides covering the Arg-Gly-Asp (RGD) motif in the 141-158 region of VP_1. The profile obtained with the 11HC10 MAb is shown in figure 1 and differed slightly from those of 36AF8 and 36BE8 in terms of the number of peptides recognized around the RGD sequence. Thus, 11HC10 reacted strongly with only three peptides whereas 36AF8 and 36BE8 had broader reactivities similar to that of the MAb OC3 shown in figure 1. The profiles for MAbs B2 and OC3 are given because of their historical importance in relation to defining those antigenic sites of O_1 virus which involve VP_1 140-160, namely site 1 and site 5 respectively (*3*). All of the A_{24} MAbs which recognized the large A_{24} peptide indicated in table 1, with the possible exception of 31FE7 which was not tested, also recognized the A_{24} 141-158-Pro-Cys-Gly sequence. In general, the reactivity of the A_{24} MAbs with the A_{24} tea-bag set of peptides was low, although 31DF4 recognized strongly one peptide within the A_{24} 141-158 sequence (table 1). The failure to define more precisely the amino acids recognized by the A_{24} MAbs may be due to the relative sizes/conformations of the tea-bag 12 mers and the 141-158-Pro-Cys-Gly sequence. Many of the C_3 MAbs which were categorized as recognizing a continuous epitope (VP_1 in Western Blotting) bound strongly to the C_3 peptide which contained both the 140-158 and 200-213 regions of the VP_1. With the exception of 7JA1, all of these MAbs also recognized a smaller peptide corresponding to the 140-160 sequence (results not shown, and ref. *14*).

A significant feature of many of the McAs was the ability to recognize one or both of the heterologous peptides described in table 1. For example, the O_1 MAb, 36AF8, recognized both the C_3 Indaial peptide and the homologous peptide. Given the tea-bag data with this MAb, it is suggested that the cross-reactivity occurs within the

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Table 1. Summary of properties of monoclonals

AcMs	Type[a]	Serum[b] Neut	Serum[b] Prot	Try[c]	Reactivity with peptides[d] O_1	C_3	A_{24}	Sequence
O_1 CAMPOS MAbs								
12BB5	CON	<1.2	3.25	N	0	-	-	
36AC6	CON	>3.6	2.5	N	0	-	-	
12FA5	CON	<1.2	0.88	N	0	-	-	
12DB7	CON	1.9	2.83	N	0	-	-	
13EG1	CON	<1.2	0	N	0	-	-	
11HC10	VP_1	<3.5	4.27	Y	++++	0	0	PPSVPNLRGDLQ
11AD6	CON	<1.2	2.35	N	0	-	-	
12GG11	CON	<1.2	0	N	0	-	-	
36AF8	VP_1	-	-	-	++++	++	?	PPSVPNLRGDLQ
36BE8	VP_1	-	-	-	++++	?	?	PPSVPNLRGDLQ
A_{24} CRUZEIRO MAbs								
31EF12	CON	1.8	4.25	Y	-	-	0	
32DH3	CON	<1.2	4.25	Y	-	-	0	
31DF4	VP_1	3.4	4.03	Y	++++	0	++++	LPPSGSGRRGDM
32AA5	VP_1	<1.2	0.85	Y	++++	0	++++	
31FA4	VP_1	<1.2	3.15	Y	++++	?	++++	
16EC11	VP_1	<1.2	3.0	Y	0	0	++++	
31GA6	CON	<1.2	3.85	Y	0	0	0	
31GA3	VP_1	<1.2	3.35	-	0	0	++++	
31DB8	VP_1	<1.2	4.02	-	++++	0	++++	
31DC7	VP_1	<1.2	2.24	-	+++	0	++++	
31DE3	VP_1	<1.2	3.0	-	+++	0	++	
31EH2	CON	1.5	2.75	-	0	0	0	
31FE7	VP_1	<1.2	3.1	-	++++	0	++++	
31CG4	VP_1	<1.2	1.25	-	+++	0	++	
31ED1	CON	1.4	4.5	-	0	0	0	
31EA12	CON	1.3	2.0	-	0	0	0	
C_3 INDAIAL MAbs								
7FC12	VP_1	3.3	3.0	Y	0	++++	0	
7LA5	CON	<1.2	4.25	N	-	0	-	
7CA8	VP_1	>3.6	3.25	Y	0	+++	0	
7CH1	CON	1.9	3.75	Y	-	0	-	
7AB5	VP_1	3.5	> 4.75	Y	0	++	0	
7DF10	CON	1.5	4.32	N	-	0	-	
1BH8	VP_1	1.9	2.22	Y	0	+++	?	
2LC9	VP_1	<1.2	1.7	Y	0	++++	0	
7EE6	VP_1	3.4	3.07	Y/N	0	+++	0	
7AH1	VP_1	<1.2	0.6	-	0	++++	0	
7CA11	VP_1	3.5	3.6	Y/N	0	++++	0	
7JA1	VP_1	<0.61	0.46	-	++++	++++	++++	KIVAPVKQTLPP
7JD1	VP_1	>3.61	3.5	-	0	++++	?	

Adapted from Ref. 1.

[a] Type. Indicates type of antigen with which MAb reacts. All MAbs were initially selected on basis of recognition of virus in ELISA but were discriminated subsequently on their reactivity (VP_1) or non-reactivity (CON) with VP_1 protein in Western Blotting.

[b] Titre of MAb in either tissue culture serum neutralization test (Neut) or protection test in mice (Prot)

[c] Reactivity of MAb with trypsin-treated virus. Y indicates that binding of antibody to trypsin treated virus is abrogated.

[d] O_1 peptide was C-C-X_o-P-P-S-Z_o-P-C-G, A_{24} peptide was C-C-X_A-P-P-S-Z_A-P-C-G and the C_3 peptide had the construction Z_c-X_c. Sequences recognized with tea-bag peptides are given and correspond to the peptide which gave the peak absorbance value. See Materials and Methods for more information. Reactivity was assessed on a scale from 0 (no binding of MAb to peptide) to + + + + (strong binding);

- indicates experiment not done.

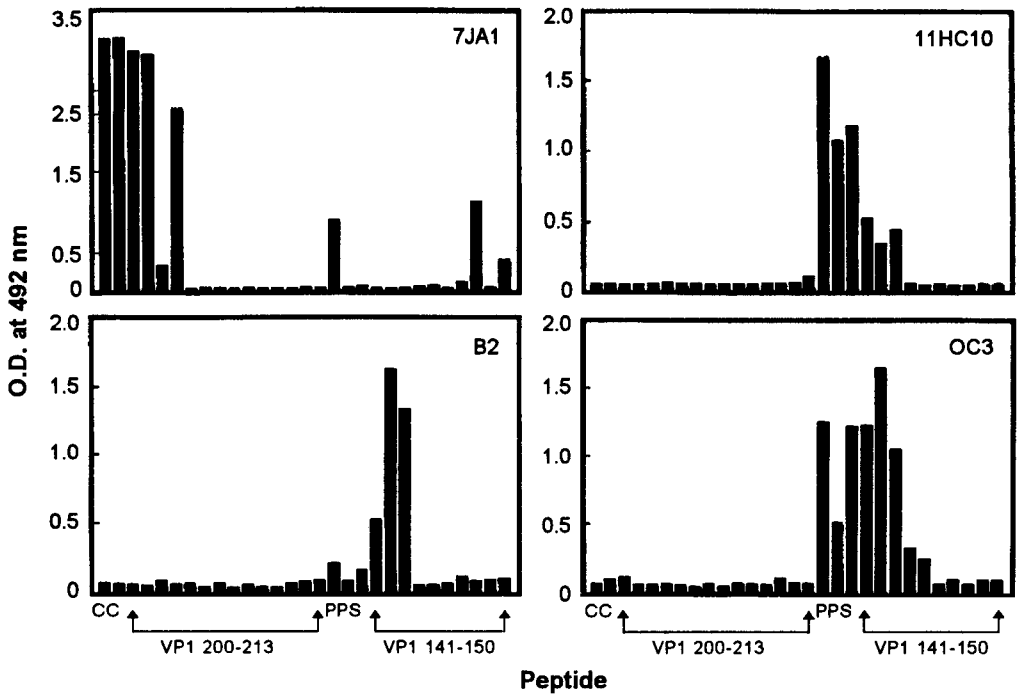

Figure 1. ELISA of four MAbs against tea-bag peptides. Each panel shows 29, 12-mer peptides based on peptides C-C-X_A-P-P-S-Z_A-P-C-G or C-C-X_O-P-P-S-Z_O-P-C-G (See Materials and Methods). The 12-mer on the extreme left of each panel is C-C-(200-209), to the right of which are peptides C-(200-210), (200-211), (201-212), (202-213), (203-214)-P etc. Thus, the peptide on the extreme right is (150-158)-P-C-G. MAbs 11HC10, B2 and OC3 were assayed against the O_1 Kaufbeuren peptide set whereas MAb 7JA1 was assayed against the A_{24} Cruzeiro peptide set.

141-158 region of the VP_1. The same MAb also cross-reacted in ELISA with the heterologous viruses but to a significantly lesser extent than the homologous reaction (results not shown). With the exception of 16EC11 and 31GA3 all of the conformation-independent A_{24} MAbs recognized the O_1 serotype peptide to the same or a similar extent as the homologous sequence. On the basis of the strong reactivities of the A_{24} MAbs with the A_{24} 141-158-Pro-Cys-Gly sequence and the weak but significant reactivities of most A_{24} MAbs with the equivalent O_1 peptide, this cross-reactivity was attributed to a site or sites within this sequence. Interestingly, all of the conformation-independent A_{24} MAbs recognized very strongly an O_1 peptide having the general structure (141-158) (141-158)-Pro-Cys-Gly, i.e., a dimeric form of the O_1 141-158 sequence, suggesting the importance of peptide size or stearic considerations in the ELISA results. For reasons which are not clear at this time, the only reactivity observed when the cross-reactive A_{24} MAbs were assayed against the O_1 tea-bag set of peptides was a low but significant reactivity with 12-mer peptides corresponding to the C terminus of the 141-158-Pro-Cys-Gly sequence, namely QVLAQKVARTPC and MAb 31DC7, i.e., a different reactivity to that seen with 31DF4 and the homologous tea-bag set. The level of cross-reactivity seen with the A_{24} MAbs and the O_1 and A_{24} peptides was not reflected in ELISA assays with the different viruses in which little or no cross-reactivity was observed. Only one of the C_3 MAbs reacted with more than one peptide. Figure 2 shows that 7JA1 recognized each of the three

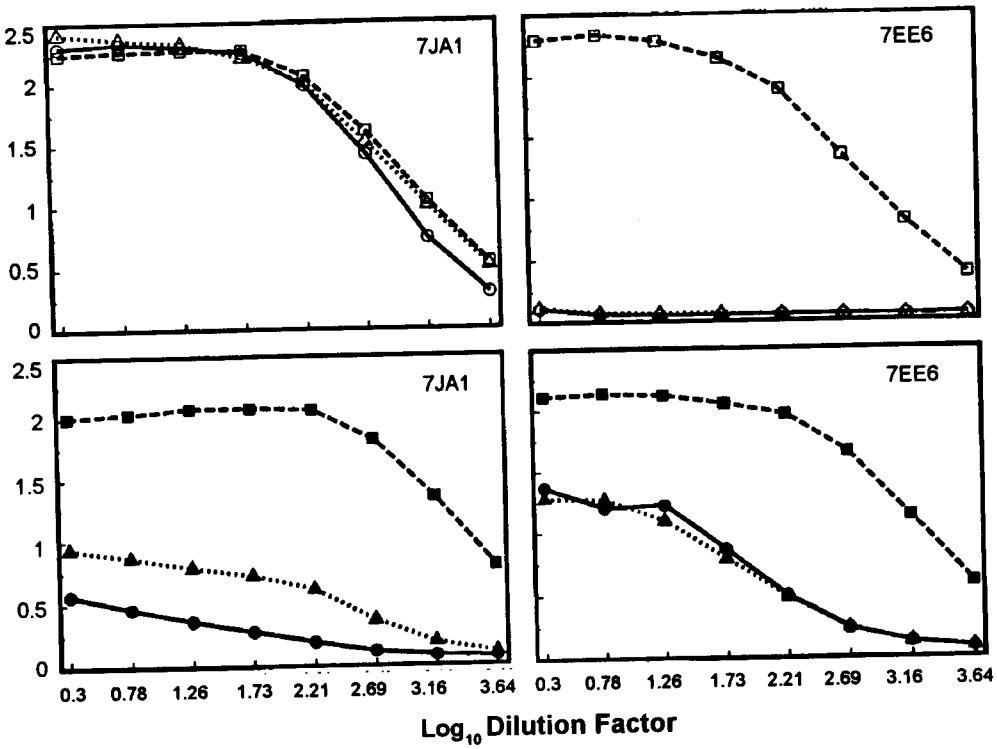

Figure 2. ELISA of two C_3 Indaial MAbs (7JA1 and 7EE6) against three synthetic peptides and three viruses. Open and closed squares are C_3 Indaial peptide Z_C-X_C and virus respectively, open and closed triangles are A_{24} peptide C-C-X_A-P-P-S-Z_A-P-C-G and virus respectively, and open and closed circles are O_1 Kaufbeuren peptide C-C-X_O-P-P-S-Z_O-P-C-G and O_1 Campos virus respectively. See Materials and Methods for full peptide sequences.

peptides to an equal extent but was relatively specific in its reaction with the C_3 virus. In contrast, another C_3 MAb, 7EE6, only recognized the C_3 peptide but was considerably more cross-reactive in ELISA with the three viruses (figure 2). In keeping with its peptide specificity, 7EE6 only neutralized the homologous C_3 virus in serum neutralization tests. MAb 7JA1 also recognized the O_1 and A_{24} tea-bag sets in the 200-213 region of the peptide (figure 1) and is the only clear example in the present work of reactivity at this site. In the ELISA shown in figure 1, 7JA1 also appeared to react with several peptides in the Pro-Ser-141-158-Pro-Cys-Gly sequence although the signal strength for these peptides was considerably weaker than with the 200-213 sequences. The O.D. values for the latter were in excess of 2.

DISCUSSION

The antigenic complexity of FMDV has been demonstrated in a number of studies utilizing MAbs (*2,3,10,11,14,16*). Mateu et al. (*14*) screened 31 South American isolates and 15 European isolates of the C serotype against a large panel of

C virus MAbs, which included many of those indicated in table 1. They suggested the existence of at least 12 epitopes involved in the neutralization of viruses of this serotype. More recently they reported that 23 of 36 MAbs tested recognized VP_1, and distinguished at least 13 virion conformation-independent epitopes involved in neutralization of FMDV type C (*13*). Furthermore, at least 10 distinguishable, overlapping epitopes were attributed to VP_1 residues 138-150. Kitson et al. (*10*) sequenced single and multiple neutralizing MAb resistant viruses of type O FMDV, on the basis of which they proposed four major sites for this serotype. Site 1 was associated with amino acids in the 140-160 sequence of VP_1 and its C terminus (residue 208) and was considered to be an essentially conformation-independent epitope whereas the other three sites were considered to be conformational sites in nature. Site 2 involved residues 70-77 and 131 of VP_2, site 3 involved residues 43-48 of VP_1 and site 4 involved residue 58 of VP_3. Using the MAb OC3 and an OC3 resistant mutant, Crowther et al. (*3*) recently proposed a fifth conformation-dependent site. However, the recognition of the VP_1 141-158 region by the MAb OC3 (present work) and the amino acid changes observed in this sequence in the OC3 resistant mutant (*3*) indicate a very close association between sites 1 and 5 of the O_1 serotype. Kitson et al. (*10*) also reviewed the literature on antigenic sites of the A serotype. Site 1 consisted of residues in VP_1 140-160 as well as involving several amino acids at the C terminus of VP_1. Residues 175 and 178 of VP_3 were also implicated for A_{12} virus. Site 2 involved residues within VP_2 only, site 3 involved amino acids around position 170 of VP_1, and site 4 involved residues 58-61, 69-70, 136-139 and 195 of VP_3. The variability in numbers of defined antigenic sites per serotype is due to a number of factors. For example, some authors have reported multiple sites for a given sequence (*13*) considered by others to be a single site. Also, the number of sites reported for a given virus strain may reflect the intensity of the investigation and the number of MAbs available.

A key question in these studies is the relevance of the sites identified to protection of the host. Certainly, site 1 has been considered important for many years based on observations such as the considerable diminution in immunogenicity of vaccines following treatment of virus with trypsin (*5*) and the ability to protect animals with synthetic peptides representing this site (*7*). With the O serotype there is also persuasive evidence from Crowther et al. (*3*) suggesting the importance of all of the four/five antigenic sites of this serotype. Their work demonstrated that recognition by post-infected or vaccinated cattle sera against the parental strain was only negated with a MAb escape mutant virus in which all four/five antigenic sites had changed. There is evidence from field isolates that sites other than site 1 may be important in protection. Feigelstock et al. (*8*) attributed the failure of the vaccine strain C_3 Resende Br/55 to protect cattle against the field isolate C_3 Argentina/84 to changes in sequence at three antigenic sites other than VP_1 135-160 and 200-213.

In the present work, a considerable number of the MAbs, but particularly those against the A_{24} and C_3 viruses, recognized VP_1 141-158. This has implications for the MAb groups used to analyse viruses submitted to PANAFTOSA (*1*). For example, of the eight A_{24} MAbs and one C_3 MAb normally used to screen A serotype viruses, five recognized VP_1 141-158. Of the nine C_3 MAbs normally used, five recognized VP_1 141-158. This is not to say that each of the five MAbs within a group is recognizing exactly the same epitope. Indeed, the data of Mateu et al. (*13*) demonstrated at least 10 distinguishable epitopes within VP_1 138-150 and the unique patterns observed by Alonso et al. (*1*) when they examined the reactivities of the MAbs against a large number of viruses suggest that the MAbs were recognizing distinguishable epitopes. Rather, the present observations emphasize the need to fully characterize MAb libraries in general and the MAbs held in PANAFTOSA specifically, with the aim of grouping them in the context of important (relevant) antigenic sites. This ought to include MAbs which neutralize weakly or not at all given the fact that *in vitro* neutralization does not necessarily correlate with *in vivo* protection (table 1; ref. *15*). However, Mateu et al. (*14*) made the interesting observation that all of the non-

neutralizing C serotype MAbs in their work reacted equally with all of the South American and European isolates of C virus tested. From this they deduced that the epitopes recognized by these MAbs were highly conserved, and therefore, unlikely to be of significance serologically. Efforts are now directed to producing MAb resistant mutants which will allow grouping of the MAbs and, with selected sequencing, identification of the antigenic sites involved.

Many of the MAbs recognized more than one serotype at the peptide level whereas virus recognition was invariably serotype specific. This was notable with the A_{24} MAbs, eight of which recognized the O_1 and A_{24} peptides, whereas two were serotype-specific in the peptide sense. The cross-reactivity observed with both the A and O serotypes was attributed to the 141-158 region of VP_1 in contrast to the highly cross-reactive C_3 MAb 7JA1 which clearly recognized peptides in the 200-213 region (present work; ref. *13*). There are several aspects in relation to cross-reactivity which merit further comment. Despite the absence of cross-reactivity and/or cross-protection between serotypes following infection or multiple vaccination with whole virus, it is clear that cross-reactive antibodies can be stimulated under some circumstances. For example, the neutralizing MAb 7EE6 recognizes a determinant common to all three serotypes albeit to differing extents. In addition, 7JA1 recognizes the 200-213 region of VP_1 of at least three serotypes which, in the form of the synthetic peptide Cys-Cys-(200-213)-Pro-Pro-Ser-(141-158)-Pro-Cys-Gly stimulates cross-reactive anti-peptide antibodies and cross-protection in a guinea pig challenge model (*6, 7*). It is also interesting to note that cross-reactive sites were identified in the 141-158 region of VP_1 by some of the A_{24} and O_1 MAbs in the present study. The intriguing question is whether it will be possible to design novel vaccines which will allow the presentation of conventional virus particles or, more probably, synthetic or recombinant antigens, to the immune system of the host in such a way as to stimulate preferentially the desired antibody population.

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