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Neutralizing antibodies to all seven serotypes of foot-and-mouth disease virus elicited by synthetic peptides

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SUMMARY

Uncoupled peptides from all seven serotypes of foot-and-mouth disease virus (FMDV) protein VP1 have been used to elicit neutralizing antibody responses in guinea-pigs. The responses were largely serotype specific, although some significant cross-neutralization was observed. Dimeric tandem peptides have also been used to simultaneously elicit neutralizing antibodies to two different FMDV serotypes. The possible existence of structural features common to the B-cell neutralization sites or the guinea-pig helper T-cell sites within all seven peptides are analysed and discussed.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the only member of the Aphthovirus genus in the family Picornaviridae. Cattle, swine, sheep and goats are the species most frequently affected by the virus, although all ruminants and cloven-footed animals are susceptible. It is considered to be an extremely contagious disease and morbidity in fully susceptible contact stock is often close to 100%. Not only was FMDV the first animal virus to be described (Loeffler & Frosch, 1897) but it was also the first virus in which antigenic differences between strains were recognized (Vallée & Carré, 1922). There are seven distinct serotypes (O, A, C, SAT1, SAT2, SAT3 and ASIA1).

In recent years studies on the antigenic structure of the virus have led to the identification of an immunogenic region within one of the four virus coat proteins, VP1, which could be mimicked using synthetic peptides (Bittle *et al.*, 1982; Pfaff *et al.*, 1982). This region is now known to form a disordered loop on the virus surface (Acharya *et al.*, 1989). Studies using carrier-linked (Bittle *et al.*, 1982, 1984; Pfaff *et al.*, 1982; Rowlands *et al.*, 1983; Francis *et al.*, 1985; Murdin & Doel, 1987; Meloen *et al.*, 1987) and uncoupled (Bittle *et al.*, 1984; Francis *et al.*, 1985, 1987b; DiMarchi *et al.*, 1986; Bolwell *et al.*, 1989) synthetic peptides have concentrated on the three most common serotypes O, A and C, although there has been one report of neutralizing antibody elicited using a peptide from a Southern African serotype, SAT2 (Francis *et al.*, 1987a). For the concept of a synthetic peptide vaccine against FMD to gain universal acceptance, it is essential to demonstrate that similar antigenic regions in all seven serotypes are immunogenic. Furthermore, since a helper T-cell (Th cell) site for guinea-pigs and mice has been identified within this major antigenic region (Francis *et al.*, 1987b), it is of interest to determine whether this is also common to all serotypes.

In this report we have used data provided by the VP1 sequences from all seven serotypes (B. E. Clarke *et al.*, manuscript in preparation) to synthesize peptides from the major antigenic loop region. These peptides were then used without carriers to inoculate laboratory animals and to study the neutralizing activity and serotype specificity of the antibodies elicited. We have also explored the possibility of producing multivalent peptide vaccines by the continuous synthesis of two peptides from different serotypes in tandem. Finally we comment on data provided by predictive models regarding conserved structural features of B-cell and Th cell sites within the peptide sequences.

MATERIALS AND METHODS

Synthetic peptides

Peptides were synthesized using an adaptation of the Merrifield (1963) technique described by Houghten (1985). Seven peptides were produced according to the VP1 sequences of seven FMDV serotypes (B. E. Clarke *et al.*, manuscript in preparation). Peptide lengths ranged from 28 to 35 residues, based on alignment with the 136–165 region of O_1BFS VP1 (Fig. 1). Single- and double-copy tandem peptides of O_1 Kaufbeuren (Kurz *et al.*, 1981) and A_{12} 119B (Rowlands *et al.*, 1983) 137–162 were also synthesized in the following combinations: O_1, O_1/O_1, O_1/A_{12}, A_{12}/O_1, A_{12}/A_{12} and A_{12}. Each peptide had an additional non-natural cysteine residue at its carboxy-terminus to facilitate coupling, should it be required, and to enhance its immunogenicity (Francis *et al.*, 1987b). The SAT2 peptide was coupled to keyhole limpet haemocyanin (KLH; Calbiochem, CA) through its C-terminal cysteine residue using m-maleimidobenzoyl-N-hydroxysuccinimide ester as a coupling agent.

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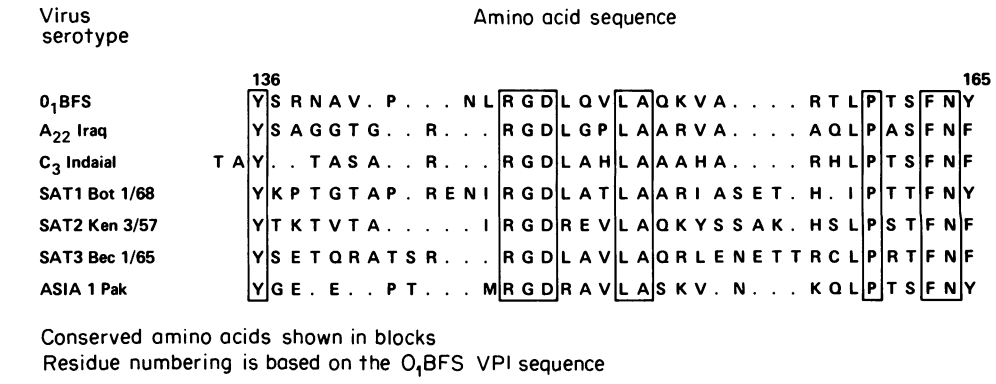

Figure 1. Foot-and-mouth disease virus VP1 peptide sequences from seven different serotypes.

Animals

Female Dunkin–Hartley guinea-pigs, approximately 12 weeks old and weighing between 450 g and 500 g, were housed at the Institute for Animal Health, Pirbright Laboratory, Surrey.

Neutralization assay

The neutralizing activity of serum samples against 100 $TCID_{50}$ of virus was determined using a microneutralization test in IBRS2 cells (Francis & Black, 1983). Each test was performed in duplicate and the results were recorded as the mean $\log_{10}$ reciprocal of the serum dilution that gave confluent cell sheets in 50% of the microplate wells (SN_{50}).

The degree of serological relatedness between pairs of virus serotypes was determined by cross-neutralization tests. The results are expressed as a ratio (Rweyemamu *et al.*, 1978):

$$r = \frac{\text{heterologous neutralization titre}}{\text{homologous neutralization titre}}.$$

Enzyme-linked immunosorbent assay (ELISA)

A modification of the indirect ELISA technique described by Voller & Bidwell (1976) was used to assay anti-peptide IgG responses. Briefly, microplates were coated overnight at room temperature with uncoupled synthetic peptide at a concentration of 2 μg/ml. The plates were washed, and test serum samples at a range of doubling dilutions from 1:10 were added. After incubation for 1 hr at 37°, plates were washed and anti-guinea-pig IgG–peroxidase conjugate was added. After a further hour at 37°, the plates were washed and an enzyme substrate (0·04% o-phenylenediamine + 0·004% hydrogen peroxide in phosphate/citrate buffer) added. The resulting colour development was stopped with 12·5% sulphuric acid after 5–7 min and the absorbance at 492 nm measured in a Titertek Multiskan (Flow Laboratories, Irvine, Ayrshire).

The A_{492} values obtained from doubling dilutions of post-inoculation samples were plotted against the $\log_{10}$ reciprocal antiserum dilution and the antibody titres calculated by reference to a negative standard (a 1:10 dilution of pre-inoculation serum). The results reported are the means of two tests, using duplicate wells for each serum dilution in each test.

Structural analysis

Secondary structural analysis was carried out on all peptides using a suite of 10 programs assembled at the Department of Biophysics, University of Leeds (Eliopoulos *et al.*, 1982). The peptides were also analysed for T-cell epitopes using two published algorithms. The first, proposed by DeLisi & Berzofsky (1985), suggests that T-cell sites tend to be amphipathic structures, possessing opposed hydrophobic and hydrophilic domains, which frequently form an α-helix. The originators of this hypothesis have published a computer program to assist in the identification of amphipathic helices within a primary amino acid sequence (Margalit *et al.*, 1987). The second method, proposed by Rothbard (1986), suggests that each T-cell epitope has within it a sequence composed of a charged residue or glycine followed by two hydrophobic residues, with in many cases the next residue being charged or polar.

RESULTS

Anti-peptide and neutralizing antibody responses to seven serotype peptides

Seven groups of four guinea-pigs were inoculated intramuscularly with 200 μg of uncoupled peptide in a water-in-oil emulsion, currently used for commercial FMD vaccines. All animals were re-inoculated with the same material at 42 days. Serum samples collected at frequent (1–2 week) time intervals were analysed for anti-peptide and neutralizing activity.

The results of this analysis (Fig. 2) showed that all seven peptides elicited anti-peptide antibodies in the absence of a carrier protein. In general these anti-peptide responses were paralleled by virus neutralizing activity. In this respect it appears that the three SAT serotypes and the ASIA1 serotype peptide were as immunogenic as the O and A serotypes, which have received most attention to date. The two apparent exceptions were the results with the C peptide, where anti-peptide antibodies, which were poor after the primary inoculation, appeared to be significantly boosted by re-inoculation while the neutralizing antibodies were not, and those with the SAT2 peptide, where re-inoculation appeared to boost neutralizing antibodies without significantly affecting the anti-peptide response. Indeed, it is interesting that immunization of a group of four guinea-pigs with a similar SAT2 peptide coupled to KLH via an added C-terminal cysteine residue resulted in the production of significant levels of anti-peptide antibodies ($>3{\cdot}0$ $\log_{10}$) with no detectable neutralizing activity ($<0{\cdot}6$ $\log_{10}$ SN_{50}).

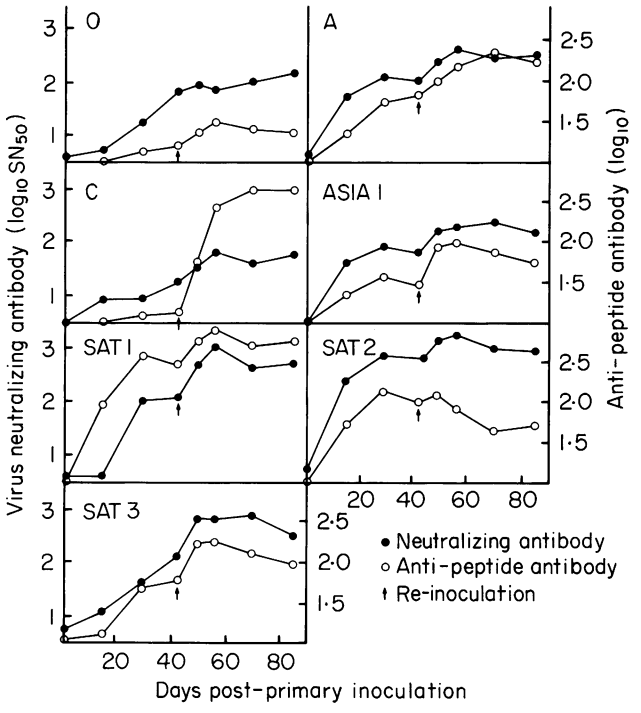

Figure 2. Neutralizing and anti-peptide antibody response to synthetic peptides corresponding to all seven serotypes of FMDV.

Serotype specificity

Serum samples collected at 84 days post-primary inoculation (42 days post-re-inoculation) were analysed for their neutralizing activity against all seven viruses from which the peptide sequences were derived. In general, the results showed that the anti-peptide responses were largely serotype specific (Table 1), with the neutralizing activity of each serum sample with its homologous virus being the greatest value observed. Nevertheless, there were some significant levels of cross-serotype neutralization observed, notably the O_1 peptide serum with viruses belonging to serotypes SAT2 ($r=0{\cdot}32$), SAT3 ($r=0{\cdot}50$) and ASIA1 ($r=0{\cdot}40$), the C3 peptide serum with SAT3 virus ($r=0{\cdot}32$), the SAT1 peptide serum with SAT3 virus ($r=0{\cdot}40$) and the ASIA1 peptide serum with SAT2 virus ($r=0{\cdot}20$). All other r values were $<0{\cdot}14$.

Immunization with multivalent tandem peptides

Six groups of four guinea-pigs were inoculated intramuscularly with 100 μg of uncoupled peptide in the water-in-oil emulsion. Serum samples were collected at regular (14-day) intervals for 70 days, at which time all sera were analysed for neutralizing activity against virus serotypes O_1 and A_{12}. Bleeds at 0–56 days were treated as pools, while 70-day bleeds were kept separate.

The results showed that neutralizing activity against type O virus was similar in the groups inoculated with single or tandem O peptides and the tandem divalent O/A or A/O peptides (Table 2a). In contrast it appears that the tandem A_{12} peptide produced significantly more neutralizing antibody than the single copy peptide (Table 2b). This difference was particularly noticeable in the individual 70-day bleeds. Furthermore the O/A or A/O divalent tandem peptides were as immunogenic as the tandem A_{12} dipeptide in terms of eliciting neutralizing antibodies against the serotype A virus.

Analysis of peptide sequences

The ability of peptides from all seven serotypes of FMDV to elicit neutralizing antibodies in the absence of carrier molecules shows that each contains appropriate B- and Th cell epitopes, which may be represented by common structural features within the sequences.

Regarding the common B-cell sites capable of eliciting a neutralizing antibody response, sequence alignment (Fig. 1) reveals a conserved block of amino acids consisting of RGD---LA within the hypervariable region 137–159 based on the O_1BFS sequence. Indeed, secondary structural analysis predicts the presence of a conserved beta-turn within all seven peptides incorporating the Arg-Gly residues of this conserved block.

Analysis of the primary sequences for possible Th cell sites (Table 3) reveals that each peptide is predicted to have between 22% and 45% alpha-helical content between residues 141 and 157 of the O_1BFS sequence, with the Ala residue at position 152 being common to all predicted helices. Using two published algorithms for T-cell epitopes we also identified amphipathic helical segments (Margalit *et al.*, 1987) in six of the seven

Table 1. Cross-serotype neutralizing activity of anti-peptide antisera

Virus	Peptide antiserum							Normal guinea-pig serum
	O_1BFS	A_{22} Iraq	C_3 Indaial	SAT1 Bot 1/68	SAT2 Ken 3/57	SAT3 Bec 1/65	ASIA 1 Pak	
O_1BFS	1·9*	<0·6	0·9	1·3	<0·6	1·0	0·7	<0·6
A_{22} Iraq	<0·6	2·7	<0·6	<0·6	<0·6	0·6	<0·6	<0·6
C_3 Indaial	0·7	<0·6	1·9	1·0	<0·6	1·3	<0·6	<0·6
SAT1 Bot 1/68	<0·9	<0·9	<0·9	2·3	<0·9	<0·9	<0·9	<0·9
SAT2 Ken 3/57	1·4	<0·8	<0·8	0·9	3·4	<0·8	1·7	<0·8
SAT3 Bec 1/65	1·6	0·9	1·4	1·9	<0·7	2·7	<0·7	<0·7
ASIA 1 Pak	1·5	1·1	1·0	1·0	0·9	1·0	2·4	<0·6

*$\log_{10}$ SN_{50}.

Table 2. Neutralizing antibody response of guinea-pigs to tandem peptides of serotypes O and A

Peptide antigen	Days post-inoculation					
	0	14	28	42	56	70 (individual bleeds)
(a) O_1 neutralizing antibody						
O_1	<0·6*	1·5	1·9	1·8	1·9	1·4, 1·5†
O_1–O_1	<0·6	1·7	2·3	1·9	2·0	2·1, 1·7, 1·4, 2·0
O_1–A_{12}	<0·6	1·1	1·8	1·5	1·4	1·8, 1·2, 1·0, 1·5
A_{12}–O_1	<0·6	1·7	2·1	1·8	1·9	2·1, 1·8, 0·7, 1·8
A_{12}–A_{12}	<0·6	<0·6	<0·6	<0·6	<0·6	<0·6, <0·6, <0·6, <0·6
A_{12}	<0·6	<0·6	<0·6	<0·6	<0·6	<0·6, <0·6, <0·6, <0·6
(b) A_{12} neutralizing antibody						
O_1	<0·6*	<0·6	<0·6	<0·6	<0·6	<0·6, <0·6†
O_1–O_1	<0·6	0·7	<0·6	<0·6	<0·6	<0·6, <0·6, <0·6, <0·6
O_1–A_{12}	<0·6	1·9	2·0	1·7	1·8	2·0, 1·7, 1·7, 2·0
A_{12}–O_1	<0·6	1·7	1·9	2·1	1·8	2·2, 2·3, 1·1, 1·7
A_{12}–A_{12}	<0·6	2·0	1·7	1·7	1·7	1·3, 2·0, 0·9, 1·3
A_{12}	<0·6	1·0	1·2	1·1	1·1	1, 1, <0·6, <0·6, <0·6

*$\log_{10}$ SN_{50}.
†Two animals in this group died.

Table 3. Analysis of peptide sequences for possible T-cell epitopes

Peptide	No. residues	Alpha-helical content		Amphipathic helix model (Margalit *et al.*, 1987)			Linear pattern model (Rothbard, 1986)	
		Residue numbers	Percentage	Mid-points of blocks	Range of angles	Amphipathic score	Residue numbers	Sequence
O_1BFS	30	12–23	40	P18–25	85–100	20·3	19–22	KVAR
A_{22} Iraq	29	16–23	28	P18–23	85–100	12·5	18–22	RVAAQ
C_3 Indaial	29	11–23	45	P18–20	85–90	4·8	11–14	DLAH
				P22–23	90–95	4·1	22–25	HLPT
SAT1 Bot 1/68	35	15–26	34	21–23	80–90	5·1	15–18	DLAT
							22–25	RIAS
							28–31	HIPT
SAT2 Ken 3/57	32	12–18	22	—	—	—	13–17	EVLAQ
SAT3 Bec 1/65	35	17–24	23	6–8	80–95	6·0	—	—
				P24–30	90–105	14·3	—	—
ASIA 1 Pak	28	11–19	32	P16–23	80–95	15·0	—	—

The presence of proline within the first 10 residues at the C-terminus of predicted amphipathic segments is indicated by a P to the left of the fifth column.

peptides between O_1BFS residues 153 and 160, and linear patterns (Rothbard, 1986) between O_1BFS residues 147 and 161 in five of the seven peptides. The SAT2 Ken 3/57 peptide, that had no predicted amphipathic region using a block length analysis of 11 residues, contained a predicted linear pattern (EVLAQ). In addition to these published algorithms we also looked for sequence and charge similarities within the peptides. This revealed that there was a region of two conserved hydrophobic residues (Leu and Ala) at positions 151 and 152 of the O_1BFS sequence followed by a single hydrophobic or uncharged polar residue and then a positively charged Lys or Arg residue in six of the seven peptides. The only exception was the C_3 peptide where two hydrophobic residues fell between Leu-Ala and a positively charged His residue.

DISCUSSION

We have shown that it is possible to elicit significant levels of neutralizing antibodies to all seven serotypes of FMDV using synthetic peptides. Moreover, this has been achieved using a

commercially acceptable adjuvant and no carrier molecule. Indeed, in one case (serotype SAT2) carrier linkage was detrimental to the immunizing activity of the peptide. Therefore, it appears that within the major antigenic loop on VP1 of FMDV there is a common B-cell site capable of eliciting neutralizing antibodies when presented as a linear peptide as well as a common Th cell site appropriate for the immunization of outbred guinea-pigs. These results confirm previous observations with FMDV that peptides covering the 141–160 region of VP1 contain B- and Th cell epitopes (Francis *et al.*, 1985, 1987b) and support the increasing literature on short peptides with dual T- and B-cell reactivity (Milich, 1989). Furthermore, the fact that the helper function of these peptides is maintained in such a hypervariable region suggests that conserved structural features necessary for MHC class II–Th cell interaction may exist. This observation could have important implications for other viral and non-viral pathogens displaying serotype diversity.

The conserved Arg-Gly-Asp (RGD) sequence identified by sequence alignment has previously been shown to contribute to the binding site of several FMDV monoclonal antibodies (Parry *et al.*, 1985; 1989a; Thomas *et al.*, 1988; Pfaff *et al.*, 1988; Bolwell *et al.*, 1989). This sequence has also been shown to play an important role in virus–cell interaction and to form part of the cell attachment site on the virus (Fox *et al.*, 1989). Despite the conserved nature of this triplet, the cross-neutralization results reveal that the neutralizing antibody response is largely serotype specific, suggesting that the RGD sequence forms part of a loop that is seen by the immune system only in the context of the highly variable flanking regions. Nevertheless, the antibody response to this region when presented as a linear peptide has been shown to be quite distinct from that against the virus (Francis *et al.*, 1988) and some significant levels of cross-serotype neutralizing activity are elicited, particularly with the O_1 peptide, which supports previous work on the cross-reactivity of FMDV peptide antibodies (Parry *et al.*, 1989b). Furthermore, it has been shown that following a single amino acid substitution within an O_1 peptide at position 148 adjacent to the RGD region, the anti-peptide antibody produced will equally neutralize and protect against challenge O_1 or A_{12} virus (Parry *et al.*, 1989b) suggesting that synthetic peptides provide the opportunity for producing highly cross-protective vaccines against FMDV. This feature of peptide immunogens introduces the posssibility of producing cross-protective responses against pathogens where antigenic variation presents problems for conventional vaccine approaches.

In addition we have shown that it is possible to produce dimeric vaccines against FMDV by synthesizing peptides of different serotypes in tandem without encountering problems of antigenic competition. This approach may also be suitable for the production of peptide vaccines against a number of different diseases and supports the concept of using peptide cocktails for multivalent vaccination purposes. Indeed it has been shown that both mycobacterial and viral B-cell epitopes can be immunogenic within the same hybrid peptide (Cox *et al.*, 1988). There is also an indication from our results that a tandem peptide of the same serotype is more immunogenic than a single copy peptide, which would support our results obtained with peptide/β-galactosidase fusion proteins (Broekhuijsen *et al.*, 1987).

Despite the fact that neither predictive algorithm identified a common T-cell site on all seven serotypes, those we used selected residues within the 150–160 region of O_1BFS sequence as being strong candidates. This result supports the *in vitro* identification of an active T-cell site on the O_1 peptide within this region (Francis *et al.*, 1987b). Indeed the α-helical content of the peptides and the location of hydrophobic residues Leu 151 and Ala 152 followed by a positively charged Lys, Arg or His at 154 (or 155) appear to be important features. It should be stressed that this helper T-cell site may not be appropriate for all target species and thus the addition of appropriate Th cell epitopes (Francis *et al.*, 1987c) or the use of a strongly immunogenic carrier (Clarke *et al.*, 1987) will probably be required for effective vaccination of target species.

In conclusion we have demonstrated that it is possible to immunize animals against all seven serotypes of FMDV using linear peptides. Furthermore, a common T-cell site for guinea-pigs allows this to be achieved in the absence of carrier protein. It has also been shown that dimeric peptide vaccines can be produced by co-linear synthesis of appropriate B-cell epitopes.

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