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Towards a multi-site synthetic vaccine to foot-and-mouth disease: addition of discontinuous site peptide mimic increases the neutralization response in immunized animals

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Abstract

Synthetic replicas of both antigenic sites A and D of foot-and-mouth disease virus have been tested as a first step towards a multicomponent peptide vaccine candidate. A first evaluation has been performed by neutralization assays on cells with serum mixtures from guinea pigs immunized independently with site A (A24) and site D (D8) peptides. The addition of site D antibodies to site A antibodies has a synergistic effect on neutralization. In a second group of experiments, guinea pigs have been immunized with a dendrimeric tetravalent (MAP) presentation of site A peptide, alone or in combination with D8, using the same total peptide dose. While the first inoculation gives a preferential response to site A-only antigen, specific response to site D and global neutralization levels significantly increase after reimmunization, reflecting a synergistic effect of site D.
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Keywords: Multi-epitope peptide vaccines; Discontinuous epitopes; Dendrimeric presentation

1. Introduction

The demonstrated effectiveness of conventional vaccines [1–3] can be rationalized as the result of diverse and highly immunogenic epitopes being simultaneously presented on a single platform, usually the inactivated or attenuated form of an intact infectious agent. Despite some inherent drawbacks (poor molecular definition, difficult growth of some pathogens in cell culture, cool storage requirements, safety concerns) these vaccines have proven invaluable in the control and eradication of many human and animal diseases. In recent decades, increasing knowledge about the antigenicity, immune response, and structure of infectious agents has fostered the emergence of novel, pathogen-free approaches to vaccination, aiming to reproduce the immunogenicity of the pathogen by means of synthetic constructions that replicate to a certain extent its antigenic sites. Rebuilding the anti-

Abbreviations: AAA, amino acid analysis; Ada, 2-aminodecanoic acid; Boc, *tert*-butyloxycarbonyl; BrAc, bromoacetyl; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CPE, cytopathic effect; DCM, dichloromethane; DIC, *N,N′*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMEM, Dulbecco-modified Eagle medium; DMF, *N,N′*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FMDV, foot-and-mouth disease virus; HOAc, acetic acid; HPLC, high-pressure liquid chromatography; IFA, incomplete Freund's adjuvant; MALDI-TOF, matrix-assisted laser desorption; time-of-flight; MAP, multiple antigenic peptide; MBHA, *p*-methylbenzhydrylamine (resin); MeCN, acetonitrile; MS, mass spectrometry; PBS, phosphate buffered saline; PEG-PS, polyeythyleneglycol-polystyrene (resin); pfu, plaque-forming unit; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; VP, viral protein

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genic features of a complex viral or microbial structure is a challenging task. This explains why all synthetic vaccines tested so far are less immunogenic (and thus less effective) than their pathogen-based counterparts. Plasmid DNA vaccines [4,5], for example, cannot reproduce the discontinuous epitopes that constitute the majority of the antigenic repertory of most pathogens [6,7]. Recombinant protein subunits [8], on the other hand, may adopt folding patterns substantially different from the native protein. Empty capsids, a particularly promising strategy, are notoriously difficult to obtain [9,10]. Finally, synthetic peptide-based approaches have largely tended to focus on a single antigenic site and thus provided limited immunogenicity and protection [11–14].

Foot-and-mouth disease, a highly contagious viral infection of cattle, has devastating economic consequences (commerce restrictions and massive slaughter of infected or suspect animals) every time an outbreak takes place in countries where vaccination is not allowed [15,16]. The disease is caused by foot-and-mouth disease virus (FMDV), a picornavirus composed of a single RNA strand and a protein capsid assembled from 60 units of four structural proteins [2,17]. The fact that FMDV exists in multiple, non cross-neutralizing serotypes and subtypes further complicates its control. For serotype C, three independent antigenic sites have been described: two continuous sites, A and C, located on the surface of VP1, and site D, topologically discontinuous and involving residues from VP1, VP2 and VP3 [18].

Peptide vaccines against FMDV were one of the first examples of synthetic vaccines to be reported and were largely based on immunodominant antigenic site A [12,19–21], occasionally in combination with T epitopes [22–24]. Although these peptide vaccines can elicit neutralizing antibodies, protection has not been convincingly described [23,25,26]. It is also known that roughly one half of the neutralizing antibodies in diseased or vaccinated animals target other antigenic sites [27] and have an important role in the protective response. This highlights the need for replicas of discontinuous site D that, in combination with efficient presentations of site A epitopes, could give rise to peptide-based, multiple site FMDV vaccine prototypes.

The rebuilding of discontinuous epitopes by chemical means, however, implies incorporating into a single molecular entity several segments from various parts of the immunogen which contain the residues known to be implicated in epitope recognition. This must be done in such a fashion that the construct provides a reasonable mimicry of the native state, in terms of inter-residue distances and orientations. Obviously, this is a difficult task that has only been partially fulfilled in a small number of cases. For FMDV in particular, we have shown that a substantial degree of functional mimicry can be achieved by means of a heterotrimer (Fig. 1) that combines one segment each from structural proteins VP1–VP3. This construct, which we have named D8 (after the 8 Pro residues linking the VP2 and VP3 regions), elicits antisera that recognize the FMDV particle and compete with site D-specific monoclonal antibodies [28], and provides moderate but unequivocal neutralizing and protective responses in both guinea pig and cattle [29]. These findings support the need for both sites A and D mimics to improve the neutralizing response of peptide-based FMDV vaccines. Site D replicas such as peptide D8 may thus become relevant components of future multi-component FMDV vaccines. This paper describes our preliminary results in this direction.

2. Materials and methods

2.1. Reagents and viruses

Protected amino acids, resins and reagents for peptide synthesis were from Novabiochem (Läufelfingen, Switzerland), Bachem (Bubendorf, Switzerland), Albatross Chem (Montreal, Canada) or Applied Biosystems (Framingham, MA). 2-Aminodecanoic acid (Ada) was a gift from Prof. W.A. Gibbons (School of Pharmacy, University of London).

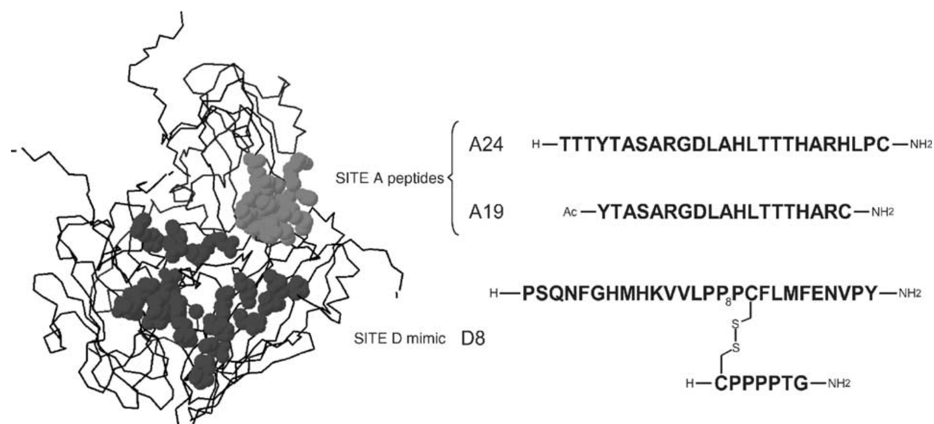

Fig. 1. Antigenic regions included in the multi-site synthetic vaccine are displayed as CPK models on the biological protomer of the capsid. Major antigenic site A is represented by linear peptide A24 or A19, while discontinuous antigenic site D is reproduced by heterotrimeric construction D8. Sequences for all these peptides used in the study are shown.

Other chemicals were from Sigma-Aldrich-Fluka (Madrid, Spain). Peptide synthesis-grade dichloromethane (DCM) and *N*,*N*′-dimethylformamide (DMF), and HPLC-grade acetonitrile (MeCN) were from Scharlau (Barcelona, Spain).

Analytical HPLC was performed on Nucleosil reverse-phase columns (4.6 mm × 250 mm, 5 μm particle size): C_{18} for peptides and C_4 for MAPs. Preparative HPLC runs were done on Vydac C_8 reverse-phase columns. Peptide hydrolysates (6N HCl, 150 °C, 2 h) were analysed in a Beckman 6300 autoanalyser. MALDI-TOF mass spectra were recorded in a Voyager model DE-STR instrument (Applied Biosystems, Foster City, CA). Foot-and-mouth disease virus type C-S8c1 was a plaque-purified derivative of isolate C-S8 (C1-Santapau-Sp/70) [30].

2.2. Peptide immunogens

Peptide sequences for D8, A24 and A19 are shown in Fig. 1. The synthesis of the heterotrimeric D8 replica of site D has been described [29]. Peptides A24 and A19 were prepared by standard Boc-based solid phase peptide synthesis [31,32]. All three peptides were characterized by AAA and MALDI-TOF MS, with results in agreement with the expected compositions.

A lipidated tetravalent lysine core (lipoMAP) with bromoacetyl (BrAc) functionalization, $BrAc_4$-Lys2-(Lys)-Ada_2-NH_2, was manually assembled by Boc synthesis protocols on 0.2 mmol (286 mg) of MBHA resin. The initial two Ada residues (0.4 mmol per coupling, 2 eq.) were followed by one, then (after deprotection of both α and ε amino groups) two residues of Lys(Boc) (0.6 mmol per amino group). Couplings were mediated by TBTU and DIEA (0.6 and 1.2 mmol per amino group, respectively; 1.5 h). The BrAc units were next incorporated (1.2 mmol each of bromoacetic acid and DIC, 2 h plus overnight recoupling) to give the tetravalent core. After HF/anisole treatment (9:1, v/v, 0 °C, 1 h), the $BrAc_4$-lipoMAP was solubilized in aqueous HOAc and purified by HPLC.

The A19-lipoMAP conjugate was prepared by thioether ligation as follows. Twenty mg of A19 in free thiol form (9 μmol, 1.5 eq. per branch) was added to 1.8 mg (1.5 μmol) of $BrAc_4$-lipoMAP in 1 mL of DMF at pH 8, over N_2 for 20 h. The reaction mixture was purified by HPLC. Fractions shown by MALDI-TOF MS to contain four copies of the A19 peptide coupled to the dendrimer core were pooled to give 3.7 mg of immunogen (25%).

2.3. Immunizations

For single peptide immunizations, guinea pigs (male Dunkin Hartley Hsd Poc: DH) were inoculated subcutaneously with peptide D8 (0.5 mg, 125 nmol) or A24 (0.5 mg, 185 nmol) in phosphate buffered saline (PBS) (100 μL) and complete Freund's adjuvant (CFA) (100 μL) and boosted intradermally with the same amount of immunogen in PBS and incomplete Freund's adjuvant (IFA) (100 μL each) on days 21 and 42 p.i. Blood samples were taken at 0 (preimmune), 21, 42 and 63 dpi, stored overnight at 4 °C and centrifuged at 10,000 rpm and 4 °C to obtain clarified sera.

To evaluate the multi-site immunogen, two groups of five guinea pigs each were used. Group 1 was inoculated with 0.20 mg of A19-lipoMAP (80 nmol of A19) in 200 μL PBS/CFA (1:1) and boosted on day 21 with the same amount in PBS/IFA. The same regimen was used to inoculate and boost group 2 animals with a mixture of 0.10 mg of A19-lipoMAP (40 nmol of A19) and 0.15 mg (40 nmol) of D8. Blood samples were taken at 21 and 42 dpi, and processed as above. Two animals injected with PBS only were used as negative controls.

2.4. ELISA

Antisera of animals immunized with the A19-lipoMAP/D8 mixture were tested by direct ELISA. Microtiter plates (Costar polystyrene, high binding, Corning, NY) were coated overnight with either A19 or D8 (250 ng) in 50 μL (PBS) at 4 °C, then blocked with 100 μL 2% bovine serum albumin (BSA) in PBS for 2 h. Serum serial dilutions (preimmune, 21 and 42 dpi), 50 μL in 1% BSA in PBS, were incubated for 1 h at 25 °C; plates were washed three times with 100 μL 0.1% Tween, 0.1% BSA in PBS, then goat peroxidase-labeled anti-guinea pig IgG (Sigma) was added at 1/5000 dilution in 0.1% BSA in PBS and incubation was carried out for 1 h, followed by washes and treatment with H_2O_2/*o*-phenylenediamine. Absorbance was measured at 490 nm and corrected for background noise (preimmune sera).

2.5. Neutralization assays

A plaque reduction assay [33] was carried out on BHK-21 cell monolayers. Serum serial dilutions were preincubated in duplicate with 150 plaque-forming units (pfu) of FMDV C-S8 for 90 min at 25 °C. Aliquots (200 μL) of each sample were added to p60 Petri plates and incubated for 60 min at 37 °C. Cell monolayers were washed with DMEM and agar medium was added. After 24 h, cells were fixed with 10% formaldehyde and stained with crystal violet. Plaque reduction levels were determined relative to a positive control (150 pfu of FMDV, no sera) and corrected for background signals (plaque reduction of preimmune sera).

Microneutralization assays were performed on 96-well plates containing IB-RS cell monolayers. Serial dilutions of serum samples (1/20, 1/40, 1/80, 1/160, 1/320 and 1/640) in 50 μL of DMEM were incubated in the presence of virus at 37 °C for 1 h. At that time, 50 μL cells were added to all wells and plate was further incubated for 48 h. Wells containing cells alone and dilutions of FMDV C-S8 with 100, 10, 1, 0.1, 0.01 and 0.001 infective doses were included in the assay. The neutralization titer was calculated as the lowest serum dilution preventing cytopathic effect (CPE) (i.e., rendering an intact cell monolayer).

3. Results

3.1. Dual site-based FMDV peptide immunogens

Two well-defined antigenic sites on FMDV, continuous site A and discontinuous site D [18], spanning a substantial area of the FMDV capsid, were obtained in the form of synthetic peptides, using the approach illustrated by Fig. 1. Site A replicas included two peptides: A24, used to obtain site A-directed antibodies, and a shorter version, A19, incorporated on a tetravalent dendrimeric (lipoMAP) scaffold. This presentation of site A was combined with the replica of site D [29], with the aim of producing a more comprehensive immune response, resembling that obtained with the native virus.

3.2. Viral neutralization with serum mixtures

Several mixtures containing different ratios of guinea pig antibodies to sites A and D were used to obtain evidence of in vitro cooperation between the two antigenic sites towards FMDV neutralization. One antiserum against A24 and one against D8 were used. Viral neutralization levels were first determined separately for each antiserum; dilutions providing 30–60% neutralization (1/10,000 for anti-A24; 1/20 for anti-D8) were considered optimal for mixing experiments. Five samples covering the entire 0–100% mixture range for both A24 and D8 antisera were then prepared. For each mixture, antisera were appropriately diluted to achieve the above optimal neutralization levels. Five additional mixtures were obtained by fivefold dilution of the initial ones. All serum mixtures were incubated with virus and tested for viral infectivity in a plaque-reduction assay in BHK-21 cells.

As shown in Fig. 2, neutralization ability mainly depended on the presence of site A antibodies in the mixture. However, mixtures containing up to 50% of D8 antisera afforded equal or higher neutralization titers than the 100% anti-A24 serum, implying a synergistic effect of antibodies to site D on neutralization.

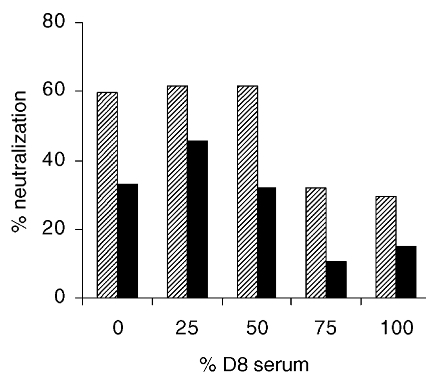

Fig. 2. Neutralization abilities of guinea pig anti-A24 and anti-D8 serum mixtures. Five mixtures at different ratios of A24 and D8 antisera were evaluated on cultured BHK-21 cells at two different dilutions (black bars correspond to fivefold dilutions of mixtures designed by dashed bars). See Section 3 section for details on mixture composition and determination of neutralization percentages.

3.3. Immunization with peptide mixtures

The observed cooperation between site A and site D antibodies in FMDV neutralization prompted us to explore the possibility of synergistic effects upon co-immunization with replicas of both antigenic sites.

The tetravalent A19-lipoMAP dendrimer (Fig. 1) chosen as site A replica included two residues of a lipophilic amino acid conferring adjuvant properties [34,35]. A similar scaffold, containing linear [36] or cyclic [37] peptide epitopes of serotype O, has shown an excellent protective response in guinea pigs (De Oliveira et al., in preparation). For antigenic site D, the heterotrimeric peptide D8 (Fig. 1) was selected, based on earlier evidence that it provides significant functional mimicry of this discontinuous site [29]. Since the goal of this experiment was to explore cooperation between both A and D sites, we chose the site A peptide (A19-lipoMAP) as control and tested the immunogenicity of a 1:1 equimolar combination of A19-lipoMAP + D8. In this way, any improvement relative to the single site A immunogen could be associated with the ability of discontinuous D site peptide to stimulate the immune response.

Thus, five guinea pigs (group 1) were immunized with peptide A19-lipoMAP alone (80 nmol) and the antibody response observed was compared to that elicited in five additional animals (group 2) immunized with a non-covalent mixture of peptides A19-lipoMAP and D8 (40 nmol each).

3.4. Antibody specificity to site A and site D

Sera from animals of group 2, immunized with the A19-lipoMAP + D8 peptide mixture, were collected at 0 (preimmune), 21 and 42 dpi and evaluated for their content in site A and site D antibodies using a direct ELISA against peptides A19 and D8, respectively, as shown in Fig. 3. Antibodies to peptide A19 were clearly detected at 21 dpi and no boost effect was observed upon re-immunization of animals with the peptide mixture. Conversely, induction of antibodies to peptide D8 was not observed until a second dose of peptide mixture was injected to the guinea pigs. Significant ELISA titers were detected after the boost (42 dpi), with values lower than those exhibited by animals immunized with peptide A19-lipoMAP.

3.5. Neutralization assays

Serum neutralization titers of animals of groups 1 and 2, at 21 and 42 dpi, were determined by microneutralization assays on IB-RS-2 cells, and are shown in Table 1 (see Section 2 for details). In both groups of animals, a single immunization resulted in the induction of neutralizing antibodies, with higher titers observed in animals immunized with peptide A19-lipoMAP alone (group 1). Consistent with the ELISA

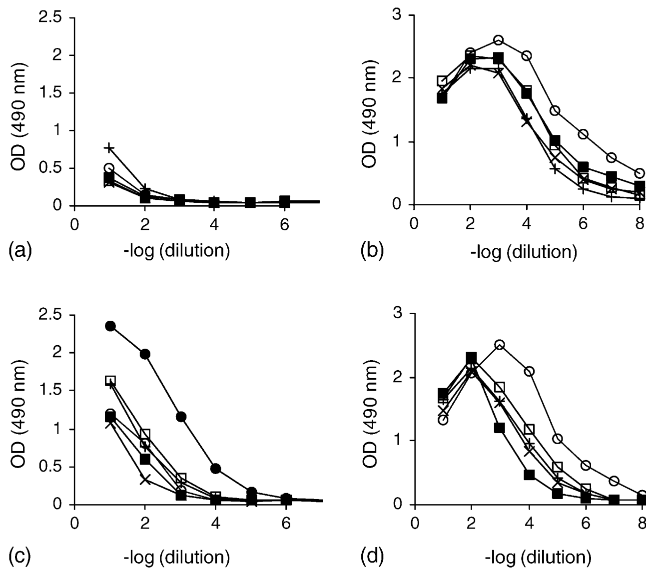

Fig. 3. Antibody response to peptides D8 (a, c) and A19 (b, d) of five guinea pig immunized with a mixture of A19-MAP and D8 (+: animal 6, ×: animal 7, □: animal 8, ■: animal 9, ○: animal 10). Sera obtained three weeks after the first (a, b) and the second (c, d) inoculation of antigens are analysed by direct ELISA. A control sera from a guinea pig immunized with three doses D8 is also shown on panel c (●). Apparently response to D8 is delayed but appears after a second peptide dose. The control after three doses D8 show much higher antibody content.

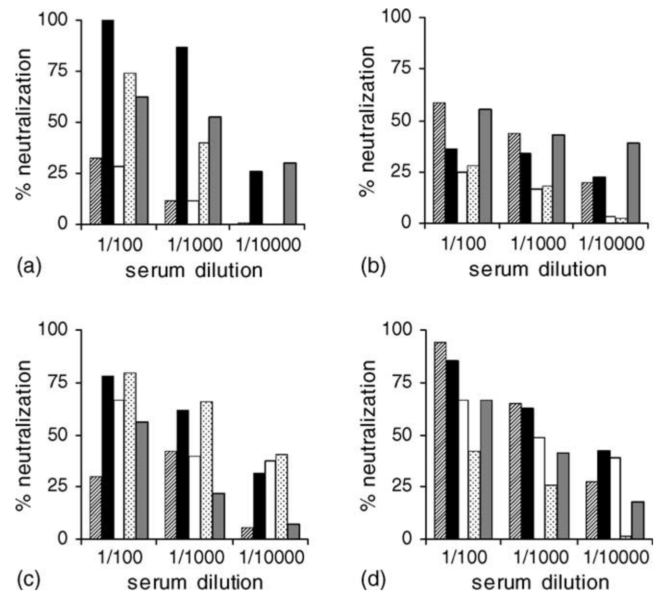

Fig. 4. Neutralization values of guinea pig sera obtained 21 days after the first antigen inoculation (a, b) and 21 days after the boost (c, d), determined in BHK-21 cells at three different serum dilutions. Different panels represent guinea pig group 1 sera (a, c) immunized with 80 nmol A19-MAP and group 2 immunized with a mixture of 40 nmol A19 MAP and 40 nmol D8 (b, d). After a single dose despite its intraspecific variability, group 1 sera contain higher concentration of neutralizing antibodies. This situation changes after a second dose of immunogen, concluding that response against D8 peptide requires a boost to appear but is highly effective.

results, a significant increase in neutralization titers was noticed upon boosting in most sera of group 2 relative to group 1. Thus, at 42 dpi (21 days after boosting), animals immunized (and boosted) with A19-lipoMAP + D8 showed neutralization titers (1.96 on average) higher than those immunized (and boosted) with 19-lipoMAP peptide alone (1.6 on average) (Table 1).

Similar results were observed in a plaque-reduction assay in which the presence of neutralizing antibodies was assessed for different serum dilutions (Fig. 4). Following the first dose, titers at dilutions 1/100 and 1/1000 were higher in group 1, which had received twice the A19-lipoMAP dose of group 2. However, serum titers from animals immunized with the A19-lipoMAP + D8 mixture showed lower animal-to-animal variation. Also, a significant increase in neutralization titers in most of group 2 sera was observed after boosting with 19-lipoMAP + D8, resulting in neutralization titers that were, on average, slightly higher than those observed in animals boosted with 19-lipoMAP peptide.

Table 1
Microneutralization titers of guinea pig sera on IB-RS cells

Immunogen	Animal	Serum neutralization titer		
		Preimmune	21 dpi	42 dpi
A19-lipoMAP	1	<0.6	1.6	1.0
	2	<0.6	2.5	1.6
	3	<0.6	1.6	1.9
	4	<0.6	1.9	2.2
	5	<0.6	1.3	1.3
A19-lipoMAP + D8	6	<0.6	1.3	2.8
	7	<0.6	1.3	1.9
	8	<0.6	1.0	1.6
	9	<0.6	<1.0	1.0
	10	<0.6	1.3	2.5
PBS	11	<0.6	<1.0	<1.0
	12	<0.6	<1.0	<1.0

In addition to groups 1 (animals 1–5) and 2 (animals 6–10), immunized with A19-MAP and a mixture of A19-MAP and D8, respectively, a control group inoculated with PBS (11, 12) is shown. Titer is defined as −log of the minimum dilution able to keep unaltered cell monolayer. Limits of detection were 0.6 for preimmune and 1.0 for immune (21 and 42 dpi) sera.

4. Discussion

In all reported vaccination trials against FMDV using synthetic peptides [19,20,23], only partial protection has been achieved, in contrast with conventional vaccines based on the whole inactivated virus. These results suggest that the peptides employed in those attempts, based on continuous antigenic sites, lack some of the relevant antigenic information present in viral particles which is able to elicit a full-fledged immune response.

The complexity of peptide vaccine approaches using B cell epitope mimics is due to the fact that most of these sites are topologically discontinuous and are difficult to reproduce synthetically in the correct conformation by means of inherently flexible peptides.

All current approaches to FMDV synthetic peptide vaccines are based on the continuous antigenic site A [18,38], either alone [12,19,21] or in combination with an additional site at the C-terminus of capsid protein VP1 [20,23]. Attempts to reinforce site A immunogenicity by including FMDV specific [23,39] or heterologous T cell peptide epitopes [40] have not improved protection in natural hosts.

In this work, we have analyzed in a guinea pig model the potential of peptide D8, a novel construct mimicking discontinuous antigenic site D, to complement the anti-FMDV response elicited by peptides from site A.

The ability of D8 to induce FMDV-specific, neutralizing antibodies was confirmed [29] by guinea pig immunization experiments where significant neutralization titers were found for anti-D8 sera, though of lower magnitude (1/20 versus 1/10,000) than those observed in an animal immunized with A24, a linear peptide reproducing site A. An indication of the potential of anti-D8 antibodies to cooperate with those induced by A24 was obtained when appropriate dilutions of each serum were mixed in different proportions and assayed for neutralization (Fig. 2). Although neutralization titers were directly related to the proportion of anti-A24 serum in the mixture, samples containing 25 and 50% of antiserum to D8 showed similar or superior titers.

Further evidence of the immunogenic potential of peptide D8 came from the analysis of sera from guinea pigs immunized with a combination of peptides D8 and A19-lipoMAP, the latter carrying four copies of peptide A19 on a tetravalent dendrimeric scaffold. ELISA revealed the presence of significant levels of anti-D8 antibodies upon re-immunization with the A19-lipoMAP + D8 mixture. These titers were only slightly lower than those determined for anti-A19 antibodies. Interestingly, no similar boost effect towards site A was observed in the same group of animals (Fig. 3).

This lack of boost effect was also observed in the microneutralization titers of sera from animals immunized with A19-lipoMAP alone, while animals receiving the peptide combination evidenced a clear increase in those titers (Table 1), on average well above those obtained using a dose of A19-lipoMAP double of that in the mixture.

These results are consistent with the plaque reduction titers determined for these sera (Fig. 4), which again reflect a clear boost effect only in animals immunized with the D8 + A19-lipoMAP combination, along with reduced animal-to-animal titer variation relative to guinea-pigs immunized with A19-lipoMAP alone.

We have thus obtained evidence that the inclusion of peptide D8 in the vaccine formulation leads to an improved FMDV neutralizing response. This effect results in an enhancement of serum microneutralization and plaque reduction titers upon re-immunization. While improved neutralization need not directly translate into better protection; the observation of a certain correlation between both parameters is not completely unusual in peptide-based FMDV vaccines [12,19,20,23]. This fact, plus the safety concerns associated with FMDV handling, made it expedient to exclude, for now, challenge with live virus from our experimental design.

Further work is required to clarify why no boost effect is observed in the neutralization titers of animals immunized with A19-lipoMAP. A tentative explanation might be related to the described ability of peptide immunogens in multimeric presentation to elicit B cell responses less dependent on T cell activation than those induced by the monomeric forms [41]. Thus, the enhancer effect observed in animals immunized with peptide D8 could be due, not only to neutralizing antibodies that are elicited, but also to specific T cells induced by this peptide which could cooperate in the production of antibodies against site A.

In summary, our results indicate the potential of a novel peptide that mimics an important discontinuous B cell site of FMDV to enhance the neutralizing response elicited by the main discontinuous B cell site on FMDV. This finding has obvious implications for the design of improved, multi-site FMDV synthetic peptide vaccines.

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