Functional Mimicry of a Discontinuous Antigenic Site by a Designed Synthetic Peptide**

Judit Villén,[a, b] Eva Borràs,[a, c] Wim M. M. Schaaper,[d] Rob H. Meloen,[d] Mercedes Dávila,[e] Esteban Domingo,[e] Ernest Giralt,[a] and David Andreu*[a, b]

Functional reproduction of the discontinuous antigenic site D of foot-and-mouth disease virus (FMDV) has been achieved by means of synthetic peptide constructions that integrate each of the three protein loops that define the antigenic site into a single molecule. The site D mimics were designed on the basis of the X-ray structure of FMDV type C-S8c1 with the aid of molecular dynamics, so that the five residues assumed to be involved in antigenic recognition are located on the same face of the molecule, exposed to solvent and defining a set of native-like distances and angles. The designed site D mimics are disulfide-linked heterodimers that consist of a larger unit containing VP2(71 – 84), followed by a polyproline module and by VP3(52 – 62), and a smaller unit corresponding to VP1(188 – 194) (VP = viral protein). Guinea pig antisera to the peptides recognized the viral particle and competed with site D-specific monoclonal antibodies, while inoculation with a simple (not covalently joined to one another) admixture of the three VP1 – VP3 sequences yielded no detectable virus-specific serum conversion. Similar results have been reproduced in two bovines. Antisera to the peptides also moderately neutralize FMDV in cell cultures and partially protect guinea pigs against challenge with the virus. These results demonstrate functional mimicry of the discontinuous site D by the peptides, which are therefore obvious candidates for a multicomponent, peptide-based vaccine against FMDV.

KEYWORDS:

discontinuous epitope · molecular dynamics · peptides · synthetic vaccines · viruses

Introduction

Over the last two decades, peptide synthesis has had a successful record of applications in a number of areas of immunological interest, which include, among others, the delineation and replication of continuous epitopes by means of synthetic peptides,[1, 2] the production of antibodies of predetermined specificity,[3] and, ultimately, the development of peptide-based vaccine candidates.[4–7] Despite these important achievements, there is a consensus in the field that true reconstitution of an antigenic site, defined as the reproduction to a significant extent of the detailed architecture of an antigenic determinant by chemical means, is a rather challenging task that remains largely unachieved. At least two obstacles must be recognized in this direction. Firstly, in contrast with the substantial rigidity of folded proteins, peptides display considerable flexibility in solution. While effective conformational restriction has been achieved in a number of cases,[8–12] particularly through intramolecular cyclization, not all attempts to restrain the mobility of peptide chains are likely to meet with success. Secondly, contrary to a somewhat simplistic view of epitopes as easily mimicked by linear peptides, most antigenic sites are found to be discontinuous; that is, they involve residues that may be spatially close due to the folding of the antigen, but which are distant in the sequence, or even belong to different protein subunits, as is often the case with viral antigens. Therefore, attempted chemical reconstruction of such discontinuous sites[13–15] must not only incorporate different sequences into a single molecular entity, but also ensure that the antigenically critical residues are displayed in a native-like arrangement that favors effective immune recognition by the host.

In a preliminary communication, we have developed a rational, structure-based approach to this problem,[16] relying on two essential types of information: 1) the three-dimensional structure of the antigen, and 2) the identification of key residues

[a] *Prof. D. Andreu, J. Villén,[+] Dr. E. Borràs,[+] Prof. E. Giralt*
Department of Organic Chemistry
University of Barcelona (Spain)

[b] *Present address:*
Department of Experimental and Health Sciences
Pompeu Fabra University
Doctor Aiguader 80, 08003 Barcelona (Spain)
Fax: (+ 34) 935-422-934
E-mail: david.andreu@cexs.upf.es

[c] *Present address:*
Torrey Pine Institute for Molecular Studies
La Jolla, CA (USA)

[d] *Dr. W. M. M. Schaaper, Prof. R. H. Meloen*
Institute for Animal Science and Health
Lelystad (The Netherlands)

[e] *Dr. M. Dávila, Prof. E. Domingo*
Severo Ochoa Center for Molecular Biology
CSIC-UAM, Madrid (Spain)

[+] *These authors contributed equally to this work.*

[**] *Previous communication: E. Borràs, E. Giralt, D. Andreu,* J. Am. Chem. Soc. ***1999***, 121, *11 932 – 11 933. A list of abbreviations can be found at the end of the Experimental Section.*

Supporting information for this article is available on the WWW under http://www.chembiochem.com or from the author.

involved in antigenic recognition events. Foot-and-mouth disease virus (FMDV) is a suitable model to test our approach, because the crystal structures of several of its serotypes are known[17–19] and it has one such discontinuous antigenic site—termed site D in serotype C—involving three amino acid residues from viral envelope protein VP2 and one each from VP1 and VP3.[19, 20] In the crystal structure of FMDV isolate C-S8c1, these five amino acids cluster within a discrete region at the interface between VP1, VP2, and VP3, and four of them are located on highly exposed loops on the viral surface (Figure 1). In serotype O, the region homologous to site D is known to be involved in binding to heparan sulfate residues on the surface of the host cell and thus to play a crucial role in the mechanisms of viral internalization and infectivity.[21, 22] We were thus interested in designing peptides that could achieve a certain level of functional mimicry of this discontinuous antigenic site, capable of eliciting a virus-reactive, neutralizing (ideally protective) immune response. The results in this paper show that these goals have been fulfilled to a significant extent by the peptide constructions that we have designed and assembled.

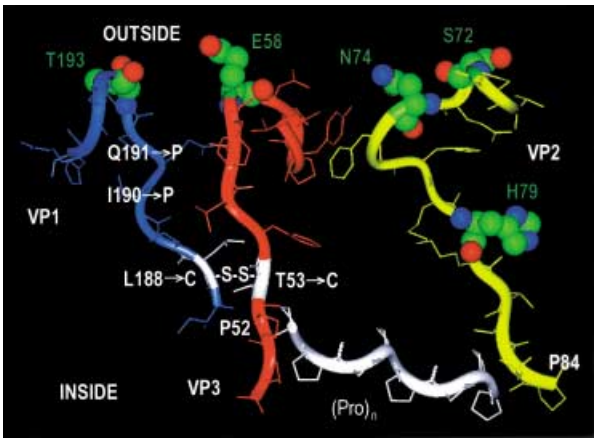


Figure 1. *The discontinuous antigenic site D of FMDV, isolate C-S8c1, involves one loop from each outer capsid protein VP1 (blue), VP2 (yellow), and VP3 (red). Antigenically relevant residues (Thr 193 (VP1), Ser 72, Asn 74, and His 79 (VP2), and Glu 58 (VP3)) on the virus surface are shown as CPK models. In the synthetic replica of this antigenic site, the three loops are covalently joined into a construction that, to a significant extent, reproduces the mutual distances and orientations between these five residues. The VP2 and VP3 segments, antiparallel to each other, are fused into a single sequence by means of a modular PP spacer between the carboxyl of Pro 84 (VP2) and the amino group of Pro 52 (VP3). Ile 190 and Gly 191 (VP1) have been replaced by Pro to reinforce the incipient polyproline conformation of this segment. Thr 53 (VP3) and Leu 188 (VP1) have been replaced by cysteine to allow the connection of VP2/VP3 and VP1 segments through a disulfide bridge.*

Results

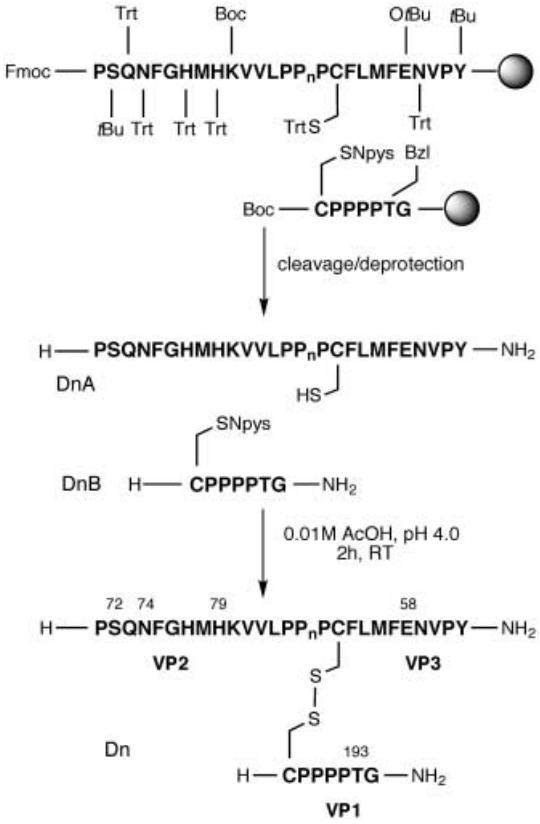
Design of site D mimic D8

Antigenic site D of FMDV, isolate C-S8c1, has been localized on the interface between VP1, VP2, and VP3 coat proteins by a combination of mutational studies and X-ray crystallographic data.[19] Five residues (Thr 193 from VP1, Ser 72, Asn 74, and His 79 from VP2, and Glu 58 from VP3) have been identified as antigenically critical, since FMDV escape mutants selected under immune pressure from monoclonal antibodies (mAbs) consistently display mutations at these five positions. At least four of the five relevant residues of site D (Figure 1) are located on highly exposed loops and define a set of distances compatible with an antibody paratope (approximately 25×25 Å^2). Our goal was to incorporate these five residues and their adjoining regions into a covalent construction that reproduced the distances and relative orientations of these five residues in the viral particle to a significant extent, in particular by allowing all of them to remain on the "outside" of the construction. This requirement precluded constructions in which the connection between the three strands involved residues located too near the surface or the five critical residues. As can be seen in Figure 1, the VP2 and VP3 loops run antiparallel to each other, which suggested the possibility of joining them into a single sequence with the aid of an intervening module of polyproline (PP). The PP motif[23] was chosen because of its relative rigidity, and was positioned between two native Pro residues (Pro 84 of VP2 and Pro 52 of VP3) at the end of each strand. Initially, the length of the PP module was set at eight residues, which should reasonably match the approximately 27 Å distance between the two native prolines, if a PP type II (PPII) conformation is assumed. Unlike the VP2 – VP3 system, VP3 and VP1 run parallel to each other, with the shortest distance that between Thr 53 (VP3) and Leu 188 (VP1). This in turn suggested the possibility of mutating both positions to Cys and linking the strands by means of a disulfide bridge. In addition, the incipient content of the PPII conformation observed in the X-ray structure of the VP1 segment (residues 189 – 192) was reinforced by mutation of both Ile 190 and Gln 191 to Pro. While these mutations may alter productive contacts between VP1 and VP3 strands, close juxtaposition of both strands would still be ensured by the nearby disulfide bridge.

The end result of this design was the disulfide heterodimer D8, synthesized (Scheme 1) from two precursor fragments: 33-residue D8A, with a free Cys, and 7-residue D8B, with its Cys residue protected/activated as a 3-nitro-2-pyridylsulfenyl (Npys) derivative, to facilitate directed disulfide formation under mildly acidic conditions.[24]

Immunological evaluation of D8

The fitness of the D8 construct as a functional mimic of antigenic site D was evaluated in immunization experiments with guinea pigs. Preliminary evidence came from direct enzyme-linked immunosorbent assays (ELISAs) of four anti-D8 sera (Figure 2), which in all cases gave clear recognition of the viral particle, with affinities only one to two orders of magnitude lower than mAbs specific for site D. In addition, a fine epitope mapping (PEPSCAN) analysis of anti D8 serum from guinea pig 1 with 12-residue overlapping peptides covering VP1, VP2, and VP3 showed high responses for peptides VP1(184 – 195) (PRPIL*PIQPTG*D) and VP1(187 – 198) (IL*PIQPTG*DRHK), for a cluster of peptides spanning the VP2(72 – 92) region (*SQNFGHMHKVVLP*HEPKGVYG), and for peptide VP3(50 – 61) (ACPT*FLMFENVP*). The italicized residues correspond to those included in the synthetic construction.



Scheme 1. *Synthesis of Dn peptide constructions reproducing antigenic site D. Precursor peptides DnA (containing the VP2 and VP3 loops separated by a PP segment of variable length) and DnB (VP1 loop) regiospecifically form a disulfide heterodimer through interaction between the free Cys thiol of DnA and the Cys(Npys) group of the DnB fragment.*

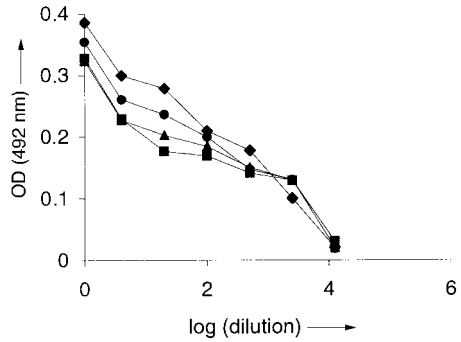


Figure 2. *Direct ELISA result showing recognition of FMDV by antisera (day 63 p.i.) from guinea pigs 1 – 4 (◆, ●, ▲, and ■, respectively) immunized with peptide D8. Data corrected for preimmune sera absorbance.*

Additional evidence of the functional reproduction of site D by the peptide construct came from both neutralization and protection experiments. Thus, the antipeptide sera inhibited the infectivity of FMDV to a moderate extent in a plaque reduction assay (Table 1). In contrast, sera from animals immunized with the mixture of VP1, VP2, and VP3 fragments not covalently joined to one another showed no neutralizing ability. Moreover, one out of three guinea pigs immunized with D8 was protected against a challenge with virus, while a second animal developed only minor lesions at 5 days postchallenge (Table 2).

An obvious extension of the above results was to test the D8 peptide on cattle, the most relevant natural host of FMDV. Preliminary experiments in this direction gave results compara-

While encouraging, these experiments did not constitute sufficiently compelling evidence that antibodies were actually mapping the antigenic site that the peptide had been designed to reproduce. Confirmation of this crucial aspect was provided by a competition ELISA between anti-D8 sera and two canonic site D mAbs, those originally used in mutational studies to define the antigenic site.[19] This experiment (Figure 3, left-hand panels) clearly demonstrated the specificity of our peptide antibodies, which were capable of displacing the binding to FMDV of mAbs specific for site D, but not of mAbs directed to other antigenic sites.

In sharp contrast with the above results, guinea pigs given a mixture (a dose and two boosts) of the three VP1 – VP3 sequences included in the D8 construct but not covalently joined to one another showed no FMDV-specific serum conversion, nor did the sera compete with mAbs specific for site D. All these results clearly argued for a role of the designed covalent structure in the display and orientation of critical site D residues.

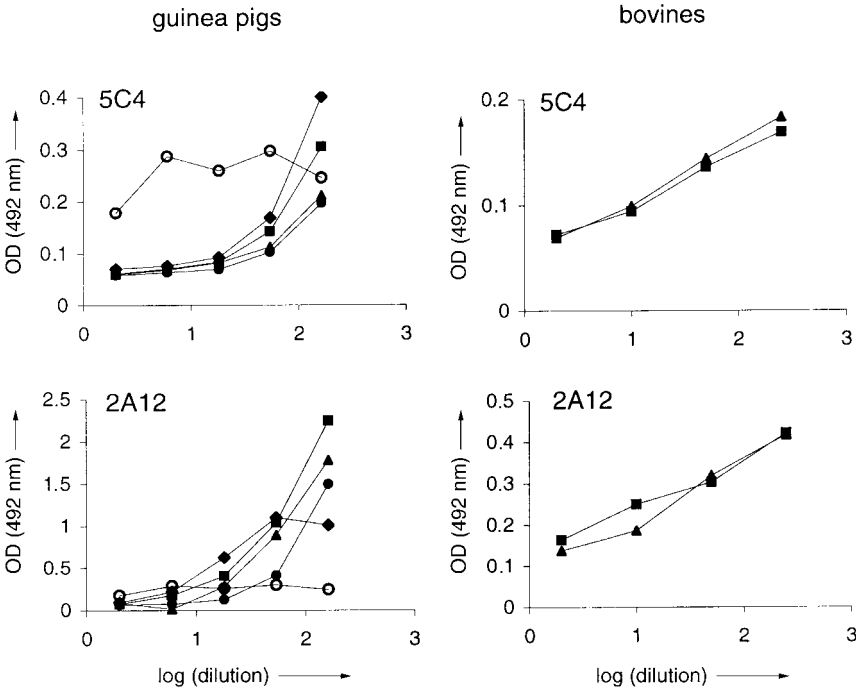


Figure 3. *Competition between guinea pig anti-D8 sera and peroxidase-conjugated mAbs 5C4 and 2A12, specific for site D, for FMDV as a plate antigen. Left-hand panels: Sera are labeled as in Figure 2; ○: negative control with sera from animal 2 and noncompeting mAb SD6, directed to an antigenic site other than site D. Right-hand panels: Competition between bovine anti-D8 sera (day 28 p.i.) and mAbs 5C4 and 2A12 for FMDV as a plate antigen; ▲: animal 1 and ■: animal 2.*

Table 1. *Neutralization titers of guinea pig antisera[a] to peptide D8.*

Serum dilution	% Reduction of infectivity[b] 2	20	200
animal 1	30 (5)	28 (6)	14 (1)
animal 2	47 (0)	40 (8)	20 (4)
animal 3	63 (1)	50 (4)	23 (0)
animal 4	52 (6)	22 (0)	4 (1)

[a] Antisera correspond to day 63 p.i. Preimmune serum titers are given in parentheses. [b] FMDV infectivity measured on BHK cell culture.

Table 2. *Protection of guinea pigs against FMDV by immunization with site D mimic peptide D8.*

Animal	Inoculum	Protection score[a] (days post challenge) 3	4	5
7	D8	2 0 0 0 0	3 2 0 0 0	2 2 0 0 0
8	D8	3 0 0 1 0	3 2 1 1 0	2 1 1 1 1
9	D8	2 0 0 0 0	3 0 0 0 0	2 0 0 0 0
46	vaccine	4 0 0 0 0	4 0 0 0 0	3 0 0 0 0
47	vaccine	3 0 0 0 0	4 0 0 0 0	3 0 0 0 0
48	vaccine	3 0 0 0 0	4 0 0 0 0	3 0 0 0 0
49	none	4 3 0 1 0	4 3 2 1 2	3 3 2 0 2
50	none	4 1 0 0 0	4 3 2 0 0	2 2 2 0 0

[a] See the Experimental Section for scoring procedure.

Table 3. *Neutralization titers of bovine antisera to peptide D8.*

Serum dilution		% Reduction of infectivity[a] 2	10	50	250
animal 1	2 weeks	59	44	43	23
	4 weeks	65	61	43	33
animal 2	2 weeks	31	17	16	18
	4 weeks	22	26	31	16

[a] Measured on BHK cells, as described in the Experimental Section.

ble to those obtained with guinea pigs. For instance, in a neutralization assay, bovine antipeptide sera afforded up to 60% reduction of viral infectivity, still detectable (approximately 20%) at 1:250 dilution (Table 3).

Analogues of D8

The results obtained with the D8 construct, with eight Pro residues between the VP2 and VP3 segments, suggested it would be appropriate to explore different lengths of the connecting module. We used a threefold approach: 1) computational evaluation of analogues with two to twelve intervening Pro residues, 2) in vitro assay of four synthetic structures selected in the previous phase, and 3) immunization of guinea pigs with the same four candidate peptides.

The D8 construct and ten analogues with different lengths ($n = 2-12$) of the Pro module component were evaluated for their ability to emulate the native structure over several cycles of unrestricted molecular dynamics. Analogues for which a significant number of conformations with low relative energies and root mean square deviations (RMSDs) could be found were considered potential candidates for synthesis and immunological evaluation. Figure 4 (top) shows relative energy and RMSD profiles of the 100 conformations sampled for the D4 construct. Three regions (I, II, and III) corresponding to different levels of structural mimicry and energetic accessibility can be arbitrarily defined in the graph. Representative conformers from each region are shown at the bottom of Figure 4, superimposed on the native epitope. Within region III, a considerable number of

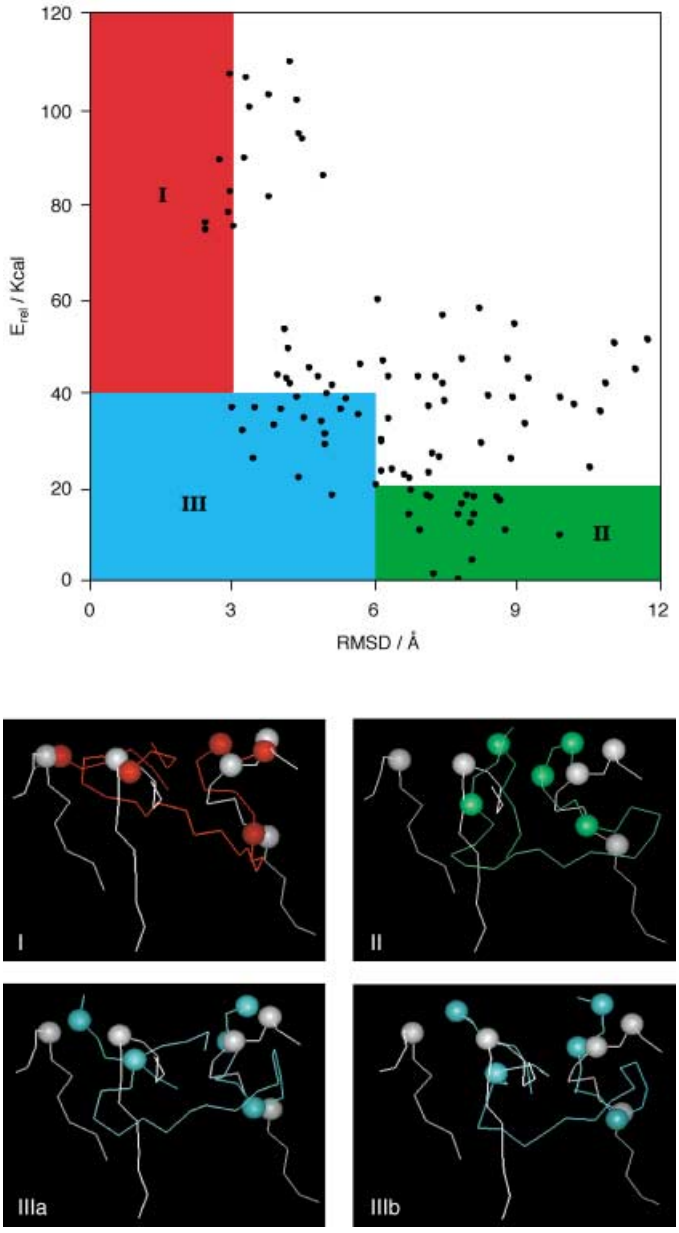


Figure 4. *Evaluation of peptide D4 as a mimic of antigenic site D by unrestricted molecular dynamics at 750 K. Top panel: relative energies (referred to most stable conformer) and RMSD profiles for 100-sampled frames. Three regions can be defined: I) $E_{rel} > 40$ Kcal, RMSD < 3 Å, good epitope mimicry, but energetically inaccessible, II) $E_{rel} < 10$ Kcal, RMSD > 6 Å, populated conformations, but adopting undesirable structures, and III) $E_{rel} < 40$ Kcal, RMSD < 6 Å, conformations with a certain degree of epitope mimicry and energetically possible. Bottom panels: Representative conformations from regions I (one conformer, red), II (one conformer, green), and III (two conformers, blue) superimposed on the native epitope (white). The five antigenically critical residues are shown (Cα) as spheres.*

low energy conformations, relatively close to the native structure, can be found. In these conformations, distances between Glu 58 (VP3) and Thr 193 (VP1) or the three VP2 critical residues (Ser 72, Asn 74, His 79) were quite close to native values, with deviations of the order of 0.2 – 2 Å. Interresidue distances between the VP1 and VP2 segments showed more pronounced deviations.

In the light of these considerations, construct D4, as well as D2, D7, and D10, was selected and further evaluated for accessibility by using Connolly surfaces. The five lowest energy conformations of each construct were analyzed. For each analogue selected it was found that at least one conformation allowed access to the five critical residues similar to or better than that determined on the viral surface.

The selected D2, D4, D7, and D10 analogues were synthesized by the same approach as used for D8 and tested for reactivity with antibodies specific for site D. These antibodies had been obtained by sequential fractionation of anti-FMDV sera through affinity columns corresponding to sites A and C. This assay allows fast and simple screening of potential mimics of site D, in contrast with the rather slow evaluation of D8 by immunization. In this in vitro assay, all four analogues showed immunoreactivities comparable to D8, and considerably higher than those of the controls (mixture of the VP1, VP2, and VP3 peptides not covalently joined to one another, or peptides reproducing antigenic sites A and C).

Finally, the four analogues were used for inoculation in guinea pigs (three or four animals per peptide) and the corresponding antisera were evaluated for FMDV recognition, competition with mAbs specific for site D, and neutralization, as described above for D8. All four peptides elicited a specific response towards site D of FMDV and displayed neutralization levels (Figure 5) that, although modest, allowed a ranking (D8 > D4 > D7 > D10 > D2) that coincided with the results from the in vitro assay of the peptides versus antibodies specific for site D.

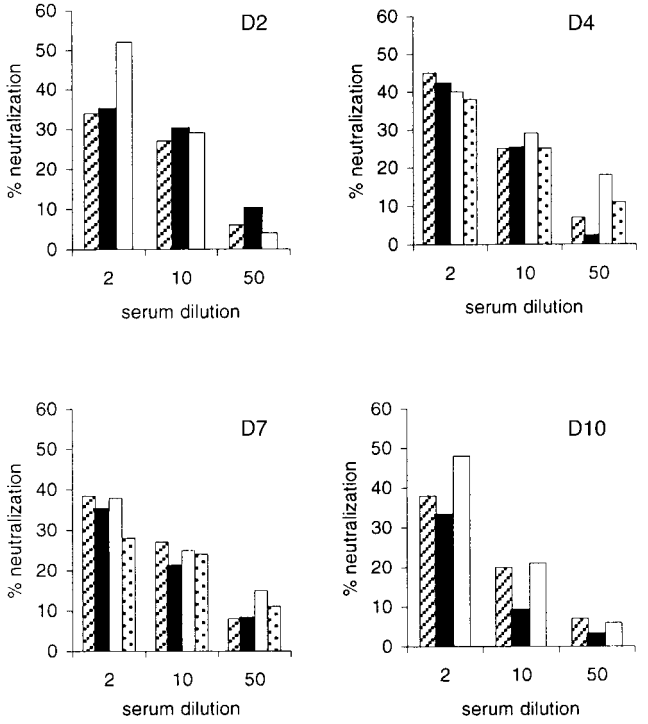


Figure 5. *Neutralization titers of antisera (day 63 p.i.) from guinea pigs immunized with peptides D2, D4, D7, and D10. The corresponding readings of preimmune sera, which did not exceed 5% plaque reduction in the first dilution, were subtracted.*

Discussion

The potential advantages of fully synthetic peptide-based vaccines as alternatives to conventional vaccine preparations have long been recognized[25, 26] and actively pursued,[4, 7] but practical applicability has been demonstrated only in a few cases.[5, 6, 27] Among several factors that hamper this development, the genetic and antigenic heterogeneity of RNA viruses is probably the most serious. The first well-documented example of a synthetic peptide used as vaccine candidate was the linear VP1(140 – 160) sequence of FMDV.[28] This peptide elicited virus neutralizing antibodies but could not fully protect cattle. A combination of this peptide with a second epitope[4] from the C-terminal section of VP1 was again only partially successful in achieving protection of host animals in extensive field trials.[7] These unsatisfactory protection levels can be related to the fast mutation rates of FMDV, typical of RNA viruses, that account for the efficient selection of escape mutants in animals immunized with a single peptide sequence.[7] One obvious way to address this problem and hence to increase the success rate of peptide vaccines is to use multivalent vaccines incorporating several antigenic sites.

In FMDV, site A is often referred to as immunodominant, and it has indeed been shown to attract a substantial proportion (approximately 50%), but not the entirety, of the immune response in natural hosts,[29] a fact that clearly suggests a contribution by other sites, in particular discontinuous site D, to FMDV immunoreactivity. Therefore, any peptide-based vaccine targeted at reproduction of the FMDV antigenic repertory should ideally include representative replicas of site D, in addition to the well-defined, already available mimics of sites A[12] or C.

The results reported here for site D mimics are a first step towards the admittedly challenging goal of synthetic reproduction of discontinuous antigenic sites. We have shown that structure-guided design, aided by molecular dynamics simulations, can help in the selection of candidate molecules displaying the three protein loops of site D in a native-like fashion capable of eliciting, in both guinea pigs and cattle, antipeptide sera that specifically recognize site D. In all these experiments, virus-specific serum conversion has been observed, accompanied by modest but unequivocal levels of neutralization and, in guinea pigs, by partial protection against a challenge with live virus.

Admittedly, peptide D8 and its D*n* analogues can be envisaged as initial stages of a design process open to further improvement. For instance, the molecular diversity implicit in the fairly broad range of intervening Pro residues ($n =$ 2, 4, 7, 8, 10) resulted in D*n* analogues with slighter differences in immune response than could in principle be expected; this

suggests that structural manipulation at other points in the construct may be required in a next generation of constructs. Nevertheless, the basic merits of our approach can easily be appreciated if the immune responses of the D*n* peptides are compared to those of a mixture of VP1 – VP3 sequences not covalently joined to one another; clearly, functional reproduction of site D is only achieved by the integration of the three VP1 – VP3 segments into a single molecular entity.

Our choice of polyproline as spacer was an attempt to find a balance between rigidity and flexibility. The all-*trans* PPII structure expected in aqueous solution (and confirmed by circular dichroism (CD) measurements, see the Supporting Information) provides a certain degree of conformational rigidity in the construction and thus exerts a template-like role. An admitted drawback of this PPII module is that it draws the VP2 – VP3 strands further apart from each other as its length increases. On other hand, a longer PP spacer increases the probability that one of the Pro–Pro bonds is in the *cis* conformation, which would in turn result in the two loops being brought closer. This relative ambivalence has motivated our exploration of different PP module lengths.

In conclusion, peptide D8 and its analogues provide for structural preorganization of the protein segments making up site D, and this amounts to reasonable functional mimicry of this discontinuous antigenic site. From this it can safely be inferred that these peptides, in their current or further refined versions, are obvious candidates for a future multicomponent, peptide-based vaccine against FMDV.

Experimental Section

Materials: Protected (Boc and Fmoc) amino acids and reagents for peptide synthesis were purchased from Bachem (Bubendorf, Switzerland), Neosystem (Strasbourg, France), or Novabiochem (Läufelfingen, Switzerland). *p*-Methylbenzhydrylamine (0.70 mmol g^{-1}) resin and PEG-PS (0.31 mmol g^{-1}) resin were from Novabiochem and Perseptive Biosystems (Framingham, MA), respectively.

Foot-and-mouth disease virus (FMDV) type C-S8c1 is a plaque-purified derivative of isolate C-S8 (C1-Santapau-Sp/70).[30] The previously described neutralizing antibodies[19] used to define site D were elicited against this same isolate and were labeled with peroxidase for competition experiments. Both labeled and unlabeled purified mAbs were tested in a sandwich ELISA to estimate their reactivity with FMDV.

Peptides: The heterodimeric D8 peptide model of antigenic site D (Figure 1 and Scheme 1) was prepared by directed disulfide formation[24] from precursor peptides D8A and D8B.

Peptide D8A (Scheme 1, $n = 8$) was synthesized by Fmoc/TBTU procedures[31] on a PEG-PS resin functionalized with 4-(α-amino-2′,4′-dimethoxybenzyl)phenoxyacetic acid handles.[32] The following side-chain protection was used: *tert*-butyl (Glu, Ser, Tyr), *tert*-butoxycarbonyl (Lys), and trityl (Asn, Cys, Gln, His). Acidolysis with trifluoroacetic acid/thioanisole/ethanedithiol/anisole (90:5:3:2, 25 °C, 2 h) provided a crude product of sufficient quality (approximately 90 % by HPLC; MALDI-TOF MS: found: 3708.2 $[M+H]^+$; calcd: 3708.9) to proceed to the next step. Comparable results were obtained for the analogous DnA peptides (Scheme 1, $n = 2$, 4, 7, 10).

Peptide D8B (Scheme 1) was assembled on *p*-methylbenzhydrylamine resin by using Boc-based synthetic procedures, which are better suited to the use of the 3-nitro-2-pyridylsulfenyl group[33] for Cys protection and directed heterodisulfide formation. The side chain of Thr was protected as a benzyl ether. Couplings were mediated by *N*,*N*′-dicyclohexylcarbodiimide. Acidolysis in HF/anisole (9:1, 0 °C, 1 h) provided the target peptide in > 90 % purity by HPLC, with correct amino acid analysis and MALDI-TOF mass spectra (found: 820.9 $[M+H]^+$; calcd: 821.3).

Peptides D8A (18.2 mg, 5 μmol) and D8B (6.2 mg, 7.5 μmol) were jointly dissolved in 0.01 M AcOH (5 mL, pH 4.5), and left for 1 h at 25 °C, as described;[34] purified heterodimer D8 (> 95 % by HPLC; 16.2 mg, 3.7 μmol, 74 %) was obtained after preparative reverse-phase chromatography (Vydac C18 column, 2.5 × 30 cm) with a 10 – 40 % gradient of acetonitrile in water (with 0.05 % trifluoroacetic acid). Peptide D8 gave satisfactory amino acid analysis and MALDI-TOF mass spectra (found: 4373.0 $[M+H]^+$; calcd: 4373.2). D*n* analogues were prepared similarly (see the Supporting Information for analytical data).

Control sequences corresponding to the VP1 (PIQPTG), VP2 (PSQNFGHMHKVVLP), and VP3 (PTFLMFENVPY) regions of antigenic site D,[19] and peptides A24 (TTTYTASARGDLAHLTTTHARHLP*C*) and C15 (*C*DRHKQPLVAPAKQLL), corresponding respectively to antigenic sites A[35] and C[19] of FMDV, isolate C-S8c1, with an additional Cys residue (in italics) at either the C or the N terminal, were all prepared by conventional Boc solid-phase methods[36] and characterized as above.

Immunizations: Guinea pigs (Dunkin Hartley Hsd Poc: DH) were inoculated subcutaneously with peptide (0.5 mg) in PBS (100 μL) and CFA (100 μL) and boosted intradermally with the same amount of immunogen in PBS and IFA (100 μL each) on days 21 and 42 p.i. Control animals were inoculated with PBS – CFA as above, without peptide. Blood samples were taken at days 0 (preimmune), 21, 42, and 63 p.i. and processed as described.[37] For the protection experiment, three guinea pigs (nos. 7 – 9, Table 2; ID-Lelystad, inbred) were given peptide (0.5 mg) in PBS (100 μL) and CFA (100 μL) and boosted intradermally with the same amount of immunogen in PBS and IFA (100 μL each) on days 22 and 43 p.i. Two controls (nos. 49 and 50) were inoculated once at day 0 with PBS-CFA as above, without peptide. Three controls (nos. 46 – 48) were vaccinated at day 43 with inactivated virus, type C-S8c1 (1 μg), in aluminium hydroxide – saponin adjuvant. Blood samples were taken at days 0 (preimmune), 21, 42, and 56 p.i.

Two bovines were each inoculated subcutaneously with peptide construct (1 mg), emulsified in PBS (1 ml) and IFA (1 ml). Blood samples were taken at days 0 (preimmune), 28, 56, and 70 p.i.

Immunochemical evaluation of antipeptide sera: Direct ELISAs of anti-D*n* sera were performed on microtiter plates coated with FMDV (clone C-S8c1; 1 pmol in PBS (100 μL)) at 4 °C overnight, then blocked with 5 % BSA in PBS for 2 h. Serial dilutions of 63-day sera in 1 % BSA in PBS were incubated for 1 h at 25 °C; plates were washed with 0.1 % Tween in 0.1 % BSA in PBS. Goat peroxidase labeled anti-guinea pig IgG was then added and incubation was carried out for 1 h, followed by treatment with H_2O_2/*o*-phenylenediamine. Absorbance was measured at 492 nm and corrected for background noise (preimmune sera).

For competitive ELISAs, 63-day serum dilutions were preincubated with nonsaturating amounts of peroxidase-labeled mAbs specific for site D (2A12, 2E5, and 5C4) for 90 min at 25 °C, and then added to the plates and quantified as above, with mAb SD6 (specific for site A)[38] as the control.

Neutralization assays: A plaque reduction assay[39] was carried out on BHK cell monolayers. Serial dilutions of sera were preincubated in duplicate with approximately 150 pfu of FMDV for 90 min at 25 °C. Aliquots (200 μL) of each mixture 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 antisera) and corrected for background signals (plaque reduction of preimmune sera).

Molecular dynamics: Molecular modeling was performed with the Insight II/Discover software package, with the cvff force field and $\varepsilon = 4r_{ij}$.[40] Different constructs based on the original D8 design were evaluated, with variable ($n = 2-12$) number of Pro residues connecting Pro 84 (VP2) and Pro 52 (VP3). For each construct, the starting structure (all residues in extended conformation except the PP module) was first minimized by restricting values of dihedral angles and $C\alpha - C\alpha$ distances among the five relevant residues (Thr 193 (VP1), Ser 72, Asn 74, and His 79 (VP2), and Glu 58 (VP3)) to those in the native structure (with a margin of ± 0.5 Å and $\pm 10^\circ$). Standard dihedral angles ($\Phi = -78^\circ$, $\psi = +146^\circ$, $\omega = +180^\circ$) were used for the PPII connecting module.[23] This minimized structure was submitted to 10 cycles of unrestricted molecular dynamics, consisting of a 100 fs equilibration step followed by 3 ps at 750 K. Conformations were sampled every 300 fs and minimized without restrictions (100 different structures). For each sampled conformation, an RMSD parameter was calculated by superimposition of the $C\alpha$ centers of the five relevant residues on the viral structure. Accessibility parameters were derived from Connolly surfaces[41] generated for $r = 1.4$ Å. Solvent-accessible areas were estimated for the five antigenically critical residues in the five most stable conformations of each construct and normalized to the homologous residue on the viral surface.

In vitro screening of candidate peptides: Analogues of D8 peptides were evaluated for recognition by antisera directed against site D in a direct ELISA. Plates were coated with peptide (0.5 μg) in PBS (100 μL) overnight at 4 °C and treated with serial dilutions of antisera. Bound antibody was quantified as described above.

Antibodies directed against site D were obtained by fractionation of sera,[29] from guinea pigs vaccinated with inactivated FMDV, through two consecutive affinity columns, the ligands of which were peptides A24 and C15, respectively (see above), bound to aminohexyl sepharose by means of the heterobifunctional linker MBS.

Challenge experiments: Guinea pigs were challenged at day 64 p.i. with FMDV type C-S8c1 adapted for guinea pigs. A virus suspension (20 μL) containing 10^3 pfu μL^{-1} was injected intradermally into the left hind footpad and the animals were examined at days 3, 4, and 5. The score was based on the lesions and expressed on a scale from 0–4 (0 = no reaction, 4 = severe reaction) for the feet and 0–1 for the tongue in the order: behind left foot, behind right foot, front right foot, tongue, front left foot. Guinea pigs with no lesions or only lesions at the injection site were considered protected; those with more extensive lesions were considered unprotected.

Abbreviations

ALSA	aluminium hydroxide – saponin
BHK	baby hamster kidney
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
CD	circular dichroism
CFA	complete Freund's adjuvant
DMEM	Dulbecco's modified Eagle medium
ELISA	enzyme-linked immunosorbent assay
FMDV	foot-and-mouth disease virus
Fmoc	9-fluorenylmethyloxycarbonyl
HPLC	high-performance liquid chromatography
IFA	incomplete Freund's adjuvant
mAbs	monoclonal antibodies
MALDI-TOF MS	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MBS	3-maleimidobenzoic acid *N*-hydroxysuccinimide ester
Npys	3-nitro-2-pyridylsulfenyl
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PEPSCAN	fine epitope mapping
pfu	plaque-forming units
p.i.	postimmunization
PP	polyproline
PPII	polyproline type II structure
PS	polystyrene
RMSD	root mean square deviation
TBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Trt	triphenylmethyl
VP	viral protein

[1] M. H. V. Van Regenmortel, S. Muller, *Synthetic Polypeptides as Antigens*, 2nd ed., Elsevier, Amsterdam, **1999**..
[2] T. Ben-Yedidia, R. Arnon, *Curr. Opin. Biotechnol.* **1997**, *8*, 442 – 448.
[3] H. J. Dyson, R. A. Lerner, P. E. Wright, *FASEB J.* **1995**, *9*, 37 – 42.
[4] R. DiMarchi, G. Brooke, C. Gale, V. Cracknell, T. Doel, N. Mowat, *Science* **1986**, *232*, 639 – 641.
[5] M. E. Patarroyo, R. Amador, P. Clavijo, A. Moreno, F. Guzman, P. Romero, R. Tascon, A. Franco, L. A. Murillo, G. Ponton, G. Trujillo, *Nature* **1988**, *332*, 158 – 161.
[6] J. I. Casal, J. P. Langeveld, E. Cortés, W. M. M. Schaaper, E. van Dijk, C. Vela, S. Kamstrup, R. H. Meloen, *J. Virol.* **1995**, *69*, 7274 – 7277.
[7] O. Taboga, C. Tami, E. Carrillo, J. I. Núñez, A. Rodríguez, J. C. Sáiz, E. Blanco, M. L. Valero, X. Roig, J. A. Camarero, D. Andreu, M. G. Mateu, E. Giralt, E. Domingo, F. Sobrino, E. L. Palma, *J. Virol.* **1997**, *71*, 2606 – 2614.
[8] V. J. Hruby, F. Al-Obeidi, W. Kazmierski, *Biochem. J.* **1990**, *268*, 249 – 262.
[9] J. Rizo, L. M. Gierasch, *Annu. Rev. Biochem.* **1992**, *61*, 387 – 418.
[10] W. V. Williams, T. Kieber-Emmons, J. Von Feldt, M. I. Green, D. B. Weiner, *J. Biol. Chem.* **1991**, *266*, 5182 – 5190.
[11] P. M. Richalet-Secordel, A. Deslandres, S. Plaué, B. You, F. Barré-Sinoussi, M. H. V. Van Regenmortel, *FEMS Immunol. Med. Microbiol.* **1994**, *9*, 77 – 88.
[12] M. L. Valero, J. A. Camarero, T. Haack, M. G. Mateu, E. Domingo, E. Giralt, D. Andreu, *J. Mol. Recognit.* **2000**, *13*, 5 – 13.
[13] P. Grigera, W. Keil, R. Wagner, *J. Virol.* **1992**, *66*, 3749 – 3757.
[14] S. E. Howie, G. J. Cotton, I. Heslop, J. A. Ross, J. Harrison, R. Ramage, *FASEB J.* **1998**, *12*, 991 – 998.
[15] J. Ottl, L. Moroder, *J. Am. Chem. Soc.* **1999**, *121*, 653 – 661.
[16] E. Borràs, E. Giralt, D. Andreu, *J. Am. Chem. Soc.* **1999**, *121*, 11 932 – 11 933.
[17] R. Acharya, E. Fry, D. Stuart, G. Fox, D. Rowlands, F. Brown, *Nature* **1989**, *337*, 709 – 716.
[18] S. Curry, R. Abu-Ghazaleh, W. Blakemore, E. Fry, T. Jackson, A. King, S. Lea, D. Logan, J. Newman, D. Stuart, *J. Mol. Biol.* **1992**, *228*, 1263 – 1268.
[19] S. Lea, J. Hernández, W. Blakemore, E. Brocchi, E. Curry, E. Domingo, E. Fry, R. Abu-Ghazaleh, A. King, J. Newman, D. Stuart, M. G. Mateu, *Structure* **1994**, *2*, 123 – 139.
[20] M. G. Mateu, C. Escarmís, E. Domingo, *Virus Res.* **1998**, *53*, 27 – 37.
[21] E. Fry, S. Lea, T. Jackson, J. Newman, F. Ellard, W. Blakemore, R. Abu-Ghazaleh, A. Samuel, A. King, D. Stuart, *EMBO J.* **1999**, *18*, 543 – 554.
[22] E. Baranowski, C. M. Ruiz-Jarabo, N. Sevilla, D. Andreu, E. Beck, E. Domingo, *J. Virol.* **2000**, *74*, 1641 – 1647.
[23] F. Rabanal, M. D. Ludevid, M. Pons, E. Giralt, *Biopolymers* **1993**, *33*, 1019 – 1028.

[24] D. Andreu, F. Albericio, N. A. Solé, M. C. Munson, M. Ferrer, G. Barany, *Methods Mol. Biol.* **1994**, *35*, 91 – 169.
[25] M. H. V. Van Regenmortel, G. Guichard, N. Benkirane, J. P. Briand, S. Muller, F. Brown, *Dev. Biol. Stand.* **1998**, *92*, 139 – 143.
[26] F. Brown, *Philos. Trans. R. Soc. London B* **1994**, *344*, 213 – 219.
[27] J. P. M. Langeveld, S. Kamstrup, A. Uttenthal, B. Strandbygaard, C. Vela, K. Dalsgaard, N. C. J. M. Beekman, R. H. Meloen, J. I. Casal, *Vaccine* **1995**, *13*, 1033 – 1037.
[28] J. L. Bittle, R. A. Houghten, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, D. J. Rowlands, F. Brown, *Nature* **1982**, *298*, 30 – 33.
[29] M. G. Mateu, J. A. Camarero, E. Giralt, D. Andreu, E. Domingo, *Virology* **1995**, *206*, 298 – 306.
[30] M. G. Mateu, E. Rocha, O. Vicente, F. Vayreda, C. Navalpotro, D. Andreu, E. Pedroso, E. Giralt, L. Enjuanes, E. Domingo, *Virus Res.* **1987**, *8*, 261 – 274.
[31] G. B. Fields, Z. Tian, G. Barany in *Synthetic Peptides: A User's Guide* (Ed.: G. A. Grant), Freeman, New York, **1982**, pp. 77 – 183.
[32] M. S. Bernatowicz, S. B. Daniels, H. Köster, *Tetrahedron Lett.* **1989**, *30*, 4645 – 4648.
[33] R. Matsueda, T. Kimura, E. T. Kaiser, G. R. Matsueda, *Chem. Lett.* **1981**, 737 – 740.
[34] F. Albericio, D. Andreu, E. Giralt, C. Navalpotro, E. Pedroso, B. Ponsati, M. Ruiz-Gayo, *Int. J. Pept. Protein Res.* **1989**, *34*, 124 – 128.
[35] M. G. Mateu, D. Andreu, E. Domingo, *Virology* **1995**, *210*, 120 – 127.
[36] G. Barany, R. B. Merrifield in *The Peptides. Analysis, Synthesis, Biology, Vol. 2* (Eds.: E. Gross, J. Meienhofer), Academic Press, New York, **1979**, pp. 1 – 284.
[37] E. Harlow, D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, **1988**.
[38] M. G. Mateu, M. A. Martínez, L. Capucci, D. Andreu, E. Giralt, F. Sobrino, E. Brocchi, E. Domingo, *J. Gen. Virol.* **1990**, *71*, 629 – 637.
[39] K. Habel in *Fundamental Techniques in Virology* (Eds.: K. Habel, N. P. Salzman), Academic Press, New York, **1969**, pp. 288 – 296.
[40] M. C. Vega, C. Aleman, C. Alhambra, J. J. Pérez, *J. Biomol. Struct. Dyn.* **1993**, *11*, 429 – 441.
[41] M. L. Connolly, *Science* **1983**, *221*, 709 – 713.

Received: June 25, 2001 [F 259]