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Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus

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A major antibody combining site on foot and mouth disease virus (FMDV) serotype O_1K has been identified in a predicted surface helix of viral protein 1 (VP1) between amino acid residues 144 and 159. A hexadecapeptide covering this sequence elicits high titers of antibodies that specifically recognize and neutralize FMDV. The high quality of the immune response is attributed to a particularly stable conformation of the antigenic amino acid sequence, which is most likely an α-helix.

Key words: FMD virus/synthetic peptide vaccine/peptide antigens

Introduction

Antigenic determinants in a protein can be mimicked by short carrier-linked peptides, and antibodies against such peptides can recognize the corresponding sequential run of amino acids within the native or denatured protein (Sela *et al.*, 1967). More recently this approach has been successfully applied to correlate nucleic acid sequences with conjectured amino acid sequences of known or hypothetical gene products (Walter *et al.*, 1980; Lerner *et al.*, 1981a).

Structural proteins from pathogenic animal viruses provide interesting model systems for such investigations because their immunogenicity, and the structural variations in their antibody combining sites (epitopes), have been studied in great detail (Webster *et al.*, 1982). In addition, peptides with an antiviral immunogenicity provide the possibility of developing synthetic vaccines.

Foot and mouth disease virus (FMDV) is an interesting example for which a detailed epitope analysis at the peptide level is possible: (i) seven distinct serotypes and many more subtypes of FMDV have been well characterized by serological analysis; (ii) the major epitopes have been assigned to the specific viral protein VP1 (viral protein 1); (iii) the primary structure of this protein has been determined for three serotypes and several subtypes (Kurz *et al.*, 1981; Kleid *et al.*, 1981; Boothroyd *et al.*, 1982; Beck *et al.*, in preparation); and (iv) two potentially immunogenic regions have been identified in the carboxy-terminal third of the VP1 polypeptide chain (Strohmaier *et al.*, 1982). Here we describe immunogenic and antigenic properties of peptides from three selected segments of VP1.

Parts of these results have been presented at the Spring Meeting of the Deutsche Gesellschaft für Hygiene und Mikrobiologie, Section Virology, May 25-27, 1982, Heidelberg, FRG.

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Results

Selection of the synthetic peptides

The amino acid sequence of VP1 from FMDV serotype O_1K as deduced from the genomic sequence (Kurz *et al.*, 1981) was used to select peptides for chemical synthesis and immunological analysis. Potentially immunogenic sequences had been identified in the carboxy-terminal third of this sequence by immunological analysis of large VP1 fragments (Strohmaier *et al.*, 1982) and also by a serological analysis of VP1 deletion mutants (H.Küpper, unpublished results).

Independent of these experimental results, we attempted to predict, merely from the known amino acid sequence, antigenic determinants whose structures could be mimicked by peptides. To elicit a strong and specific antipeptide and antiviral immune response, the native conformation of such a segment of the polypeptide chain should be as stable as possible in the protein to ensure that this native conformation is also assumed by the corresponding carrier-coupled peptide. Furthermore, this segment should be located at the protein surface.

A large contribution to protein stability arises from hydrogen bonds (Schulz and Schirmer, 1979). In a short peptide a large number of local hydrogen bonds are formed if this peptide assumes an α-helical conformation. Therefore, amino acid runs that are helical in the native protein are good candidates for assuming a rigid conformation on their own. If such a helix is located at the molecular surface, it often contains well separated hydrophobic and hydrophilic sides, pointing to the protein interior and to the solvent, respectively.

To identify helices in VP1 we applied several methods (Ptitsyn and Finkel'shtein, 1970; Chou and Fasman, 1974; Lim, 1974; Nagano, 1977; Garnier *et al.*, 1978; Rose, 1978) currently used to predict secondary structures from amino acid sequences. As shown by a joint evaluation of five such predictions (Oefner, 1982) (Figure 1a) there are seven regions assigned as being helical by a majority of these methods. For selecting those helices at the protein surface, we used the helix wheel representation, which is a projection of all side chains along the helix axis. An ideal surface helix should split into a purely hydrophobic and a purely hydrophilic half of this wheel (Schiffer and Edmundson, 1967).

From the seven predicted helices of Figure 1, only helices 5 and 6 showed a good separation between polar and non-polar side chains. This separation was particularly striking in helix 5 (Figure 2). Being most clearly predicted with no interference from β-sheet or turn prediction (Figure 1a–c), and being most likely at the surface, helix 5 is the best prediction for an immunogenic surface structure in VP1 that could be mimicked by a peptide.

This prediction is at variance with results of an 'antigenicity plot' according to Hopp and Woods (1981), which essentially searches for regions with a high local hydrophilicity. For VP1 such a plot (Figure 1d) shows highest 'antigenicity' in regions overlapping with helices 1, 4, 6, and 7, and also in non-helical regions, whereas helix 5 has only intermediate antigenicity.

Based on these theoretical predictions and in conjunction

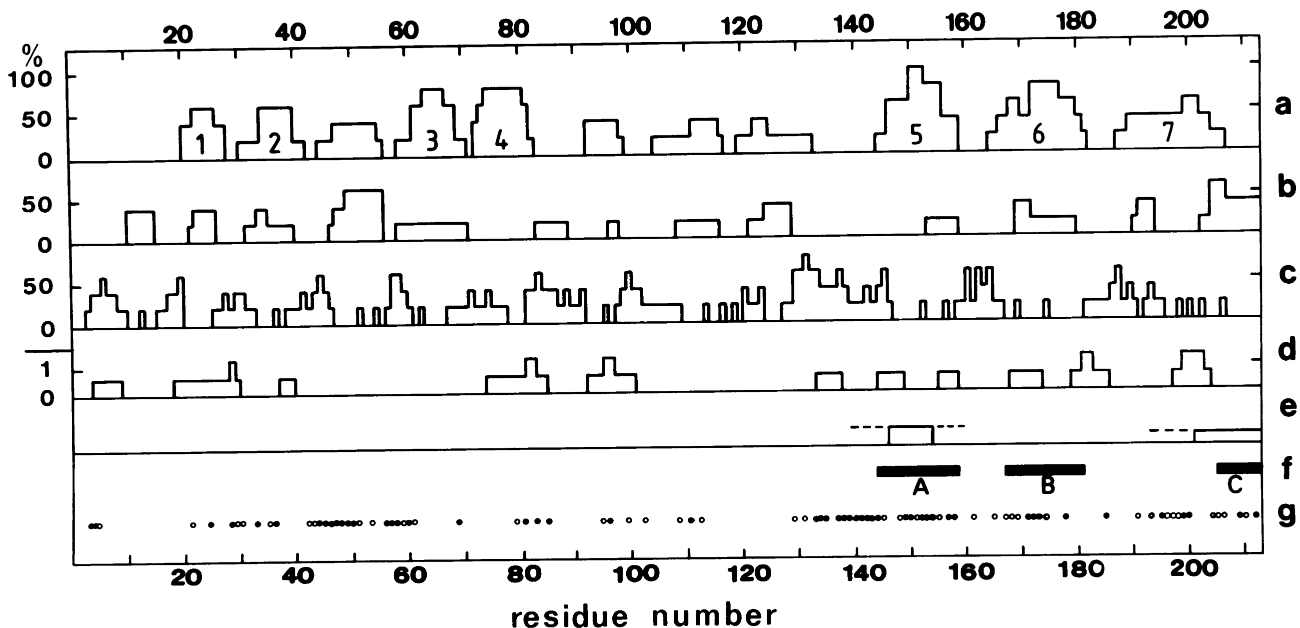

Fig. 1. Selection of segments with predicted high antigenicity of coat protein VP1 of FMDV type O_1K. (**a–c**) Joint prediction histograms (Oefner, 1982) for α-helices (Ptitsyn and Finkel'shtein, 1970; Chou and Fasman, 1974; Lim, 1974; Nagano, 1977; Garnier *et al.*, 1978), β-pleated sheets (Ptitsyn and Finkel'shtein, 1970; Chou and Fasman, 1974; Lim, 1974; Nagano, 1977; Garnier *et al.*, 1978), and reverse turns (Chou and Fasman, 1974; Nagano, 1977; Garnier *et al.*, 1978; Rose, 1978). At each residue position the predictions are added and expressed as the average probability for a particular secondary structure. (**d**) Antigenicity according to Hopp and Woods (1981) given at three levels: ≤ 0, $0-1$, ≥ 1. (**e**) Minimal antigenic regions as predicted from the serological analysis of large VP1 fragments (Strohmaier *et al.*, 1982). (**f**) The selected peptides VP1-A, VP1-B, and VP1-C. (**g**) Sequence variability in coat protein VP1 (Beck *et al.*, in preparation). Open circles denote a change in either serotype C_1O or A_5 as compared with serotype O_1K. Filled circles indicate that both C_1O and A_5 deviate from O_1K.

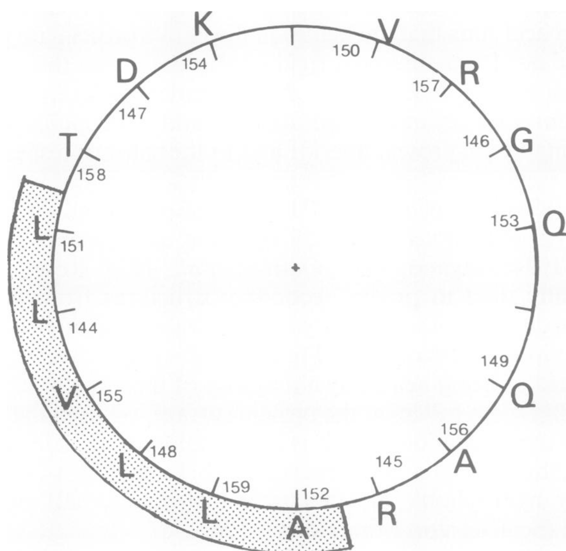

Fig. 2. Helix wheel of segment VP1-A containing residues 144–159. All side chains are considered to be in a regular α-helix at their appropriate azimuthal position. This helix is then projected along its axis (dot) onto the paper plane, revealing that the lower left side of the presumed helix is hydrophobic (marked) whereas the upper right side is hydrophilic.

with the experimental data mentioned above, amino acid sequences from three distinct segments of VP1 (Figure 1f) were selected for chemical synthesis and serological analysis: VP1-A (144 LRGDLQVLAQKVARTL 159) spans the predicted helix 5 and also overlaps a segment of VP1 indicated as immunogenic by experimental data (Strohmaier *et al.*, 1982) (Figure 1e); VP1-B (167 AIKATRVTELLYRLK 181) is the predicted helix 6. This segment is adjacent to the maximum of the antigenicity plot, which should safely predict an antigenic determinant (Hopp and Woods, 1981). For technical reasons the methionine residue in positions 180 was replaced by a leucine residue. We do not expect this change to have much influence on peptide conformation and immune response. VP1-C (205 IVAPVKQTL 213) is a C-terminal peptide. It was selected because: (i) short N- and C-terminal peptides had been already used successfully in the serological identification of other proteins (Walter *et al.*, 1980, 1981); and (ii) experiments suggest that the C-terminal VP1 sequence is also immunogenic (Strohmaier *et al.*, 1982).

Our choice was further supported by sequence determinations of VP1 from different FMDV serotypes (Beck *et al.*, in preparation) which revealed a significant degree of sequence variation in the range of these peptides, in particular within and adjacent to VP1-A (see Figure 1g and Discussion).

Peptide synthesis and antibodies to the synthetic peptides

The peptides chosen for immunological analysis were obtained by custom synthesis (Bachem, Bubendorf, Switzerland). Block condensation in solution was used to minimize the number of side products to be removed. End-products were homogeneous on h.p.l.c. and showed the expected amino acid compositions. The two octapeptides VP1-A_1 (LRGDLQVL) and VP1-A_2 (AQKVARTL), which were used as blocks to build peptide VP1-A, were also obtained in a pure form and tested for their antigenicity together with the three other peptides described before.

To increase their immunogenicity, all peptides were covalently linked to protein carriers using either glutaraldehyde or a water-soluble carbodiimide as coupling reagents. Three different carriers, bovine serum albumin (BSA), bovine thyroglobulin, and keyhole limpet hemocyanin (KLH), were used (Rittenberg and Amkraut, 1966; Erlanger, 1980). The

Table I. Reactivity of antipeptide antisera with the antigens, with coat protein VP1, and with FMDV serotypes O_1K, C_1O, and A_5

Serum or IgG	Antiserum specificity	Titer in the ELISA[a]					
	Antigen:	Peptide		VP1	FMDV serotype		
		Free	Conjugate	O_1K FMDV	O_1K	C_1O	A_5
1	VP1-A/KLH[b]	3.2	3.5	3.3	3.8	1.8	NEG
2	VP1-B/KLH[b]	2.8	3.1	1.0	1.0	NEG	NEG
3	VP1-A/KLH[b] VP1-B/KLH[b]	3.6 3.3	4.4 4.0	4.4	5.1	2.3	NEG
3A IgG[d]	VP1-A/KLH [VP1-B/KLH]	3.5 [1.7]		3.9			
3B IgG[e]	[VP1-A/KLH] VP1-B/KLH	[1.5] 3.1		1.2			
4	VP1-A/KLH[c] VP1-B/KLH[c]	2.8 2.3		4.2	4.9	2.2	NEG
5	VP1-A_1/KLH[b] VP1-A_2/KLH[b] VP1-C/KLH[b]	2.0 2.3 2.7	2.2 2.7 3.0	1.7	2.2	NEG	NEG
6	VP1-A_1/KLH[c]	1.0	2.5	NEG			
7	VP1-A_2/KLH[c]	1.1	2.2	NEG			
8	VP1-C/KLH[c]	1.3	2.7	1.9			

[a] $-\log_{10}$ values; NEG: values identical to those of pre-immune sera (extinction value $\epsilon < 0.05$ at a 1:10 dilution). The values shown in this table were obtained after the third injection.
[b] Coupling of the peptides to the carrier protein using glutaraldehyde.
[c] Coupling of the peptides to the carrier protein using N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide.
[d] IgG purified by an affinity chromatography on a Sepharose-VP1-B column.
[e] IgG purified by an affinity chromatography on a Sepharose-VP1-A column.

different conjugates obtained by the various combinations of carrier, peptide, and reagent were tested in rabbits for their capacity to induce antibodies against the peptide moiety of the conjugates. In some cases (e.g., antisera 3, 4, and 5, see Table I) animals were immunized with mixtures of several conjugates. The immune response of the animals was regularly evaluated in an indirect, enzyme-linked immunoassay (ELISA) against the uncoupled peptides.

The results of this extensive study (to be published in detail elsewhere) showed that all peptides were immunogenic and that the immune response observed was highly specific for the peptides used for immunization. Cross-reactivity was only detected between the related peptides VP1-A and VP1-A_2. KLH proved to be the best carrier, and therefore we report here only on KLH conjugates (Table I). Titers determined in assays with the free peptides as antigens usually ranged from 10^{-2} to 10^{-4}. The best results were obtained with the longer peptides VP1-A and VP1-B. Somewhat higher values were observed if peptide conjugates (to unrelated second carrier proteins) were used as antigens in the ELISA (Table I). This difference can be explained as a technical difficulty of the ELISA because free peptides and their high mol. wt. conjugates may have different affinities to the plastic surface of the microtiter plates used in the assay. Another explanation would be that the peptide molecules undergo conformational changes on direct absorption to the support.

Antibodies to the synthetic peptide react with isolated VP1 and with FMDV

The presence of FMDV-specific antibodies in the rabbit antisera was assessed by reaction with the purified antigenic protein VP1 from FMDV type O_1K, as well as with inactivated virus particles of various strains. The results obtained with the ELISA are presented in detail in Figures 3a and b for antiserum 3. Further data are summarized in Table I. After three immunizations, titers up to 10^{-5} were observed for anti-VP1-A immunoglobulins (e.g., in antisera 3 and 4), whereas antisera against all other peptides reacted only very poorly with either VP1 or virus. The VP1 preparation used in the ELISA had been subjected to denaturing conditions during its isolation (Strohmaier *et al.*, 1982), which may have changed the structure of antigenic determinants. Apparently, however, no such changes persisted in the three VP1 segments analyzed, because their antigenic determinants were recognized equally well in both the virus and the isolated protein.

Further analysis revealed that most amino acid sequences differed in antigenicity if presented either as a short peptide or as part of the VP1 polypeptide chain. Thus, there was a loss in specificity by two orders of magnitude with antisera against VP1-A_1, VP1-A_2, and VP1-B peptides, whereas no such loss, or rather an increase, was observed with anti-VP1-C and anti-VP1-A immunoglobulins. These results indicate that, of the peptides tested, only VP1-A and VP1-C are in similar conformations in either the peptide (and the conjugate) or in VP1 (and the virus). Similarly, these data argue strongly against VP1-B being a stable helix.

The positive reaction observed with anti-VP1-A antiserum was specific for FMDV type O_1K, whose genomic sequence had been used to select the amino acid sequences of the peptides. As shown in Table I, cross-reactivity to FMDV type A_5 was not detected, indicating that it was at least four orders of

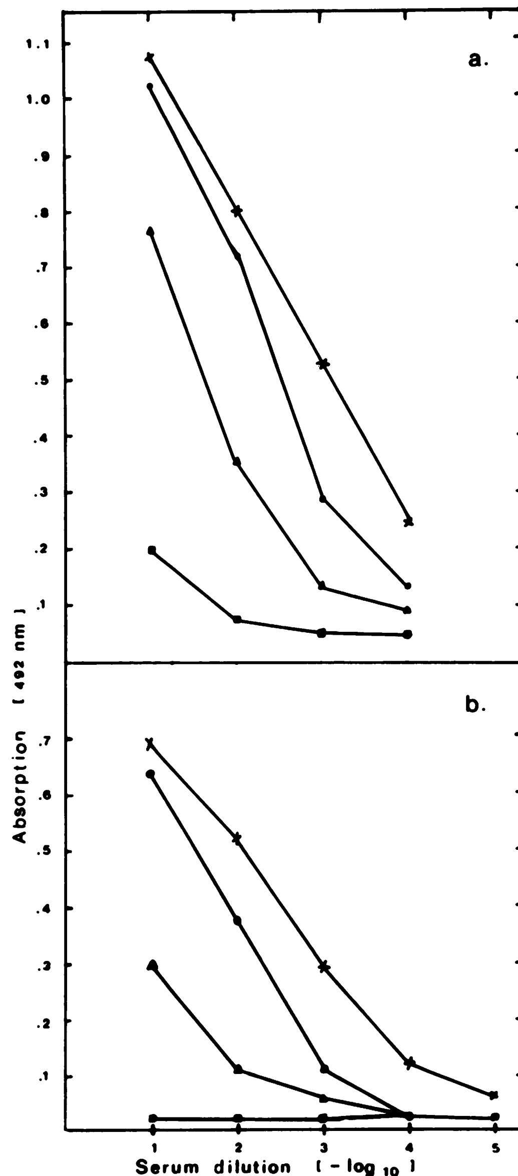

Fig. 3. Antibody response of rabbits to a mixture of a VP1-A/KLH and VP1-B/KLH conjugates. The last sample corresponds to serum 3 in Table I. ELISAs were carried out as described in Table I. (a) With FMDV particles, serotype O_1K. (b) With coat protein VP1, serotype O_1K. (■) Preimmune serum. (▲) Antiserum after first injection of a mixture of VP1-A/KLH and VPl-B/KLH. (●) Antiserum after second injection (same mixture). (x) Antiserum after third injection (same mixture).

magnitude below the reaction with serotype O_1K. However, a significant degree of cross-reactivity was detected with the third European serotype, FMDV C, which was recognized in the ELISA to ~1% of the values determined for serotype O_1K (Table I). This is surprising in view of the drastic variations between the three serotypes in their amino acid sequence in the segment corresponding to peptide VP1-A. Compared with O_1K there are nine and 11 amino acid changes in this region in C_1O and A_5, respectively (Figure 1g, and Beck *et al.*, in preparation).

Neutralization of FMDV by an anti-peptide antiserum

Neutralization of the virus was tested by the capacity of the rabbit antipeptide antibodies to abolish infectivity for suckling mice (Skinner, 1951). Only sera containing anti VP1-A antibodies were highly efficient, with titers well beyond 10^{-3}; antibodies against all other peptides were completely negative (Table II). This was also true if immunoglobulins of mixed specificity had been induced in the same rabbit by a mixture of peptides. An example given in Table I are the specific immunoglobulin fractions 3A and 3B that had been separated to 99% purity by affinity chromatography on peptide Sepharose columns from an antiserum with composite specificity.

Virus neutralization was serotype-specific when tested with serotype O_1K and serotype A_5. However, as observed in the ELISA, a low level of cross-reactivity was detected with FMDV type C_1O. This indicates that our anti-peptide immunoglobulins recognize common antigenic determinants in FMDV serotypes that, by definition, do not cross-react in conventional serological assays.

FMDV-specific antibodies are predominantly directed against segment VP1-A of coat protein VP1

The data presented so far show that anti-VP1-A immunoglobulins can recognize and efficiently neutralize a specific antigenic determinant from VP1 in the isolated protein as well as in the intact virion. We also performed the reverse experiment, in which anti-FMDV antisera from an immunized goat or from guinea pigs were tested for reactivity with the VP1-A hexadecapeptide. In initial ELISAs with free VP1-A, or with VP1-A/KLH conjugate, positive results were obtained at antiserum dilutions beyond 10^{-5}, and the strength of the signal was indistinguishable from those obtained with virus or with isolated VP1 from serotype O_1K as antigens. No antigenic reaction was detected with any other peptides (data not shown).

To determine the contribution of the VP1-A segment to the immunogenicity of coat protein VP1 in the virus, an anti-FMDV antiserum was exhausted for its anti-VP1-A specific immunoglobulins by passage through a column of VP1-A Sepharose. The results (not shown) demonstrated that essentially all (99%) of the anti-VP1 activity in the antiserum was adsorbed to the peptide resin and was, therefore, directed against the VP1-A epitope. Taken together with the fact that VP1 carries the major epitope(s) of FMDV (Bachrach *et al.*, 1975), these results are consistent with the notion that the VP1-A-specified area is the dominant antigenic determinant on the viral surface.

Discussion

Early studies with viral coat proteins of known primary structure, i.e., tobacco mosaic virus (Anderer, 1963) and bacteriophage MS2 (Langbeheim *et al.*, 1976), proved that anti-peptide antibodies can be used to identify amino acid sequences located at the virus surface. Recent advances in recombinant DNA technology have extended this approach to peptide sequences of animal viruses and to the study of the feasibility of synthetic peptide vaccines.

Peptides covering a large fraction of a surface protein of hepatitis B virus and influenza virus have been tested for their immunogenicity (Lerner *et al.*, 1981b; Green *et al.*, 1982). Other groups have selected peptides from the same proteins according to predicted hydrophilicity (Prince *et al.*, 1982; Dreesmann *et al.*, 1982), predicted position at the protein surface (Müller *et al.*, 1982), or high sequence variability between serological subtypes (Dreesmann *et al.*, 1982). In general, antisera were obtained which reacted with target pro-

tein, but which did not or did only poorly neutralize the infectivity of the intact virus.

Our approach with FMDV was to test potentially rigid chain segments on the surface of the viral immunogen VP1. When starting merely from the amino acid sequence without knowledge of the three-dimensional protein structure, the most readily recognizable rigid surface segments are α-helices. Based on this assumption, hexadecapeptide VP1-A was the best choice, and this peptide proved to induce an exceptionally strong immune response as determined in both the ELISA and the neutralization assay. A much lower serological cross-reactivity to coat protein VP1 and to FMDV was observed with several shorter peptides and also with a second long peptide (pentadecapeptide VP1-B), also predicted to be a surface helix. These results are in accordance with the results of Strohmaier *et al.* (1982) in that the stronger of their two minimal 'antigenic portions' of VP1 is contained within peptide VP1-A (see Figure 1), and with the position of exposed trypsin-sensitive sites in VP1 in the virion (Strohmaier *et al.*, 1982; Wild *et al.*, 1969).

With respect to the required length of the peptide, we have a good test in the comparison of the hexadecapeptide VP1-A and its two constituting octapeptides VP1-A_1 and VP1-A_2. The long peptide worked well, whereas both octapeptides were useless in spite of the direct serological cross-reaction between peptides VP1-A and VP1-A_2. Here, one should keep in mind that, because of its length, an α-helical VP1-A would have 50% more hydrogen bonds per residue than an α-helical VP1-A_1 or VP1-A_2, which could well mark the difference between rigid and flexible peptide. A flexible antigenic determinant cannot be bound tightly because a large part of the available free energy of binding has to be spent on solidifying the particular antigen conformation, which is complementary to the elicited antibody. Therefore, we suggest that one should not limit the peptide length to the number of expected antigenic residues, but rather aim at the increase in stability of longer helical peptides.

In addition, one may consider increasing the helix propensity of a given peptide to keep it a helix on the carrier. For this purpose, one may add a proline at the N-terminal end, because Pro is an efficient helix starter. In addition, the hydrophobic side of a helix can be manipulated: valine (like Val-155 in VP1-A) and isoleucine can be replaced by better helix formers as for instance leucine or even an unnatural amino acid such as benzoyl-glutamate. Finally, carriers with hydrophobic surfaces would also stabilize the helix conformation of such a peptide.

These considerations about the requirements in size and conformational stability for an efficient peptide immunogen do not necessarily hold for peptides that correspond to the termini of a polypeptide chain. Usually these chain ends form rather loose structures in the protein (Watenpaugh *et al.*, 1980). As a consequence most of the antibodies that have been produced against the numerous conformations of the carrier-coupled peptide can presumaby force the corresponding segments in the protein into the complementary conformation. However, the required structural change swallows part of the available binding energy and therefore prohibits high binding affinities. On the other hand, chain end flexibility allows the use of peptides as short as six amino acid residues to raise antibodies sufficiently specific for protein recognition (Walter *et al.*, 1981).

In this work, antibodies against the carboxy-terminal nonapeptide VP1-C were shown to interact specifically with the corresponding VP1 as well as with virions of a homologous serotype (Table I). However, as expected, titers were low when compared with anti-VP1-A antisera and no neutralization of FMDV infectivity was observed (Table II). In similar experiments, a much longer C-terminal VP1 fragment (35 amino acid residues) had yielded a very low, but significant titer of virus neutralizing activity (Strohmaier *et al.*, 1982). Together these findings support the theoretical arguments outlined above with respect to the limited applicability of anti-peptide antibodies against terminal amino acid sequences.

Table II. Neutralization of FMDV by anti-peptide antisera and cross-reactivity between different serotypes

Serum	Antiserum specificity	Serum neutralizing assay[a] FMDV serotype			ELISA[b] FMDV
		O_1K	C_1O	A_5	O_1K
1	VP1-A/KLH	3.5	2.4	NEG	3.8
2	VP1-B/KLH	NEG	NEG	NEG	1.0
3	VP1-A/KLH, VP1-B/KLH	>3.6	2.0	NEG	5.1
4	VP1-A/KLH, VP1-B/KLH	>3.6	2.1	NEG	4.9
5	VP1-A_1/KLH, VP1-A_2/KLH, VP1-C/KLH	NEG	NEG	NEG	2.2
	Pre-immune sera	NEG	NEG	NEG	NEG

[a] $-\log_{10}$ values of neutralizing antibody titer (SNT); NEG: SNT <0.3.
[b] See Table I.

Protein antigenic determinants are usually divided into two categories termed 'conformational' and 'sequential' (Sela *et al.*, 1967). Our data indicate that peptide VP1-C relates to a sequential determinant whereas the VP1-A epitope on FMDV combines features of both categories in that a short sequential run of amino acid residues attains a unique local conformation. The data suggest that this particular amino acid sequence comprises the dominant epitope in FMDV. However, there may be additional, purely conformational determinants in VP1 that lose their antigenic conformation after disintegration of the virus (Bachrach *et al.*, 1975) or during isolation of the subunit protein, and which therefore may not be detected with the techniques we used. The presence of epitopes that are only presented to the immune system in the intact virus is indicated by both the very low immunogenicity of isolated VP1 (Kleid *et al.*, 1981; Bachrach *et al.*, 1975) and our finding that essentially all VP1-specific anti-FMDV antibodies can be absorbed on a VP1-A column. In addition, it should be noted that VP1-A is not the region of highest variability in VP1 (Figure 1f) as would be expected for an epitope that underlies constant selection pressure for changes, and it may well be that the highly variable amino acid sequence preceding the VP1-A segment (amino acids 133 – 145, Figure 1, Beck *et al.*, in preparation) is part of a conformational determinant as defined above. This hypervariable region does not need to be included in peptide VP1-A, which elicits high titers of FMDV-neutralizing antibodies in rabbits. These titers are at least 10-fold above the levels needed to protect test animals against virus infection. Thus, the FMDV system seems to be a

most promising first candidate for the development of a synthetic peptide vaccine against an animal virus.

Materials and methods

Synthesis of the peptides

The peptides were synthesized by Bachem, Bubendorf, Switzerland, using the solution method. Peptide A was synthesized by combining the octapeptides A_1 and A_2. The purity of each peptide was assayed by h.p.l.c. and the amino acid composition was determined.

Coupling of the peptides to carrier protein

The synthetic peptides were coupled to carrier protein KLH using two different methods.

Coupling with glutaraldehyde (Glu). We dissolved 2 mg peptide in 10 μl H_2O and added 15 mg carrier protein in 2 ml sodium phosphate buffer (0.1 M, pH 7.0). We then added 1 ml of a water solution of Glu (21 mM) drop-wise over 1 h at room temperature. We allowed the mixture to stand overnight and then dialyzed it against phosphate buffered saline (PBS).

Coupling with water-soluble carbodiimide. We dissolved 2 mg peptide in 10 μl H_2O and 15 mg carrier protein in 1 ml sodium phosphate buffer (0.1 M, pH 6.0). We then added a solution of 120 mg N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide in 150 μl H_2O. After shaking for 1 h at room temperature, we allowed the reaction mixture to stand overnight and dialyzed it against PBS.

Inoculation of the rabbits

For inoculation, the peptide conjugates were dissolved in PBS with 0.1% SDS and mixed with complete Freund's adjuvant at a 1:1 ratio. Doses of 400 μg peptide and 1 mg carrier protein in a total volume of 1.0 ml were injected s.c. into rabbits. Blood samples were taken and injection of antigen with incomplete Freund's adjuvant was repeated after day 28, 38, and 48.

ELISA

The anti-peptide antisera were checked for their ability to react with the peptides, VP1 and virus (146S FMDV particles) by an indirect ELISA (Voller *et al.*, 1976). Microtiter plates (Nunc) were coated with peptide (5 μg/well), VP1 (0.25 μg/well), or whole virus (5 μg/well) and incubated overnight at 37°C. After washing twice with PBS, the wells were saturated with 1% BSA or 1% ovalbumin in PBS (30 min at 37°C). 50 μl anti-peptide antisera dilutions from 1/10 and up to $1:10^5$ were placed in the wells and incubated for 2 h at 37°C. After washing five times with PBS/Triton X-100 the samples were incubated for a further 2 h with peroxidase-labelled goat anti-rabbit IgG at a ratio of 1:500 in 1% BSA/PBS. After repeated washings with PBS/Triton X-100 each well received 50 μl of a solution of 0.4% *o*-phenylendiamine and 0.012% H_2O_2 in 0.1 M phosphate-citrate buffer, pH 5.0. The enzyme reaction was stopped after 30 min and read with a Titertek multiscan photometer at 492 nm. Sera titers at an extinction of 0.1 were expressed as $\log_{10}$ values.

In vivo neutralization assay

The anti-peptide antisera were tested by an *in vivo* neutralization assay. Sera samples were pretreated for 30 min at 56°C and then serially 4-fold diluted with isotonic phosphate buffer, pH 7.5. The sera dilutions were mixed with equal volumes of FMDV suspension containing ~100 LD_{50} units of serotype O_1K, C_1O, and A_5. After incubation at 37°C for 30 min, each mixture was injected intra-abdominally in doses of 0.1 ml into five suckling mice. The serum dilution that protects 50% of suckling mice (SNT) was calculated by the method of Kärber (1931).

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Note added in proof

After this paper had been submitted for publication similar results on the immunogenicity of the VP1-A segment of our FMDV sequence were reported by Bittle *et al.* (1982) in *Nature*, **298**, 30.