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Vaccine 23 (2005) 2647–2657
Vaccine
www.elsevier.com/locate/vaccine

Analysis of the immune response against mixotope peptide libraries from a main antigenic site of foot-and-mouth disease virus

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Received 19 August 2004; accepted 22 October 2004
Available online 7 December 2004

Abstract

The design of vaccines for RNA viral diseases is complicated by the high genetic variability of the viruses, which favors the selection of escape mutants. A case in point is foot-and-mouth disease virus (FMDV), for which only limited protection has been observed in vaccination with single peptides. We have explored the potential of immunogens of higher sequence diversity, covering a broad range of field or culture-induced mutations at the immunodominant site A of FMDV, serotype C. Four mixotope-type peptide libraries, containing ca. 3×10^3 or ca. 3×10^5 peptides each, in either linear or cyclic form, and combining most significant mutations found or induced at site A have been synthesized and used to immunize guinea-pigs. Substantial levels of serum conversion have been observed for all four mixotope libraries, as well as for single peptides, linear or cyclic, corresponding to the consensus site A sequence. The specificity and neutralizing ability of the anti-mixotope and -peptide antibodies have been evaluated by direct ELISA and by plaque reduction and micro-neutralization assays, respectively. Challenge experiments with an infectious, guinea-pig-adapted FMDV strain, have shown higher protection rates in animals immunized with the cyclic versions, either in single sequence or in combinatorial mixotope form.

Keywords: Foot-and-mouth disease virus; Peptide library; Mixotope; Synthetic vaccine

1. Introduction

Foot-and-mouth disease virus (FMDV) is a paradigm of the challenges posed by RNA viruses to vaccine development. At the genetic level, the high variability of FMDV results in a *quasi-species* [1,2] structure, in which each genome population is conformed by one or several master sequences and an spectrum of mutant sequences differing in one or more amino acid residues. This genetic heterogeneity and its derived high mutation rates underlie the substantial diversity of FMDV serotypes (A, O, C, Asia 1, SAT1, SAT2 and SAT3; each with a large number of subtypes and variants) [3–5], and are a recognized obstacle in vaccine development and implementation. Thus, conventional vaccination with classical inactivated virus of one particular FMDV serotype does not confer protection against other serotypes, and the level of protection may vary among viruses of a single serotype [1]. With regard to synthetic vaccines, the situation is even less favorable. Despite some initial reports of success in the vaccination of cattle with a single FMDV peptide [6], later experiments using adequate statistics [7] have revealed limited (39% at most) protection rates and concomitant selection of escape mutants. A recent paper claiming almost

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doi:10.1016/j.vaccine.2004.10.041

full protection of swine with a peptide-based FMDV vaccine [8] has been followed by another report where the same immunogen is found to be totally ineffective in cattle [9].

A particular type of combinatorial library named mixotope [10] was proposed as a candidate immunogen in rabbits by the group of Tartar some time ago, in an attempt to address the problem of the considerable antigenic diversity of HIV-1 [11]. The library contained ca. 10^5 peptide sequences combining the mutations found in 21 isolates of the hypervariable V3 loop region of gp120. The prospect was that an immunogen of such high variability could improve protection levels by eliciting a broad enough antibody response to target not only reported mutations but, given its combinatorial nature, any potential new ones as well, which would in practice significantly reduce the escape rate. A similar approach was also used by the same group [12] in rhesus macaques immunized with a mixotope based on the hypervariable region of the envelope glycoprotein (gp130) of simian immunodeficiency virus (SIVmac142). In both experiments, antibodies specifically binding the native proteins and related peptides were observed. However, to our knowledge, no reports on the capacity of these complex mixtures of related immunogens to elicit neutralizing antibodies and protection in animal models have been reported.

This fact prompted us to explore the applicability of the mixotope approach to FMDV. We report here the results of an immunization experiment using four types of mixotope libraries, with two different diversities (3×10^3 and 3×10^5, termed MixLo and MixHi, respectively) and in either linear or cyclic form. The mixotopes are based on a 22-residue peptide corresponding to the main antigenic region (site A) of FMDV, serotype C, located on the exposed hypervariable G–H loop of capsid protein VP1 [13,14], and combine amino acid variations found in either field isolates or in cell culture that affected FMDV antigenicity [7,15–17]. Prior to their preparation and use as immunogens, a low-diversity mixotope has been synthesized and exhaustively analyzed by chemical means to validate the quality of the synthetic methodology. The specificity and neutralizing ability of the antibodies elicited in guinea-pigs by the mixotopes has been evaluated, respectively, by ELISA and by plaque reduction and micro-neutralization assays, respectively. Challenge with a serotype C, guinea-pig-adapted infectious FMDV isolate, 2.10b [18], has been performed 62 d.p.i.

2. Experimental

2.1. Chemicals and biologicals

Protected amino acids, *p*-methylbenzhydrylamine resin (MBHAR) (0.70 mmol/g) and reagents for peptide synthesis were from Neosystem (Strasbourg, France), Bachem (Bubendorf, Switzerland), or Novabiochem (Läufelfingen, Switzerland). Solvents were from Scharlau (Barcelona, Spain); trifluoroacetic acid (TFA) from Solvay (Bad Wimpfen, Germany) and all other reagents from Sigma-Aldrich (Madrid, Spain).

Amino acid analysis (AAA) of peptide hydrolyzates [6 M HCl/phenol (99:1, v/v), 130 °C, 4 h] were done in a Beckman System 6300 analyzer. Analytical and preparative HPLC were performed on Nucleosil C_{18} reverse phase columns (0.46 cm × 25 cm, 5 μm and 2 cm × 25 cm, 10 μm, respectively) eluted with water–acetonitrile (both containing ca. 0.05% TFA, v/v) gradients. MALDI-TOF and ES mass spectra were recorded in Voyager DE-RP (Applied Biosystems, USA) and VG-Quattro (Micromass, UK) instruments, respectively.

2.2. Single peptide synthesis

The consensus A21 sequence (TTYTASARGDLAHPTTTHARHL) was assembled on MBHAR in an automated synthesizer (Applied Biosystems 430A) using Boc/Bzl chemistry and preformed symmetrical anhydride couplings. Side chain protections were benzyl (Thr, Ser), 2-bromobenzyloxycarbonyl (Tyr), 4-methylbenzyl (Cys), tosyl (Arg), cyclohexyl (Asp) and benzyloxymethyl (His). After chain assembly, the peptide-resin was deprotected at the N-terminus with 40% TFA in DCM, then treated with HF/*p*-cresol (9:1, v/v; 0 °C, 1 h) to release the free peptide, which was dissolved in 10% HOAc, lyophilized and purified by preparative reverse phase (C_{18}) HPLC using a linear gradient of 5–25% MeCN into H_2O (both containing 0.05%, v/v, TFA). Fractions corresponding to the main peak were examined by analytical HPLC using a linear 10–40% gradient of MeCN (+0.036% TFA) into H_2O (+0.045%) over 30 min at 1 mL/min. HPLC-homogeneous fractions were pooled, lyophilized and further characterized by AAA and MALDI-TOF MS (Table 1). Other related C-serotype sequences and the randomized version (TLRTRHTLHATGHTSAPAYTDA, rA22) of the peptide were obtained likewise. For the cyclic version, TTCTASARGDLAHPTTTHACHL, Tyr^{136} and Arg^{153} of the consensus sequence were replaced by Cys and the bis-thiol precursor obtained upon HF treatment was HPLC-purified and characterized as above, then dissolved to 50 μM in 100 mM NH_4HCO_3, pH 8.5, and air-oxidized for 26 h. Progress of the oxidation was monitored by HPLC of neutralized 100 μL-aliquots of the solution.

The 64 individual sequences included in the test mixotope (see Section 3.1.2) were prepared by simultaneous parallel Fmoc synthesis in an AMS422 multiple peptide synthesizer (Abimed, Langenfeld, FRG), using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate as coupling agent. Side chain protecting groups were *t*-butyl (Thr, Ser, Tyr, Asp), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg), and trityl (Cys, His). After TFA cleavage and deprotection, crude products containing >85% (by HPLC) of the target peptide sequence (determined by MALDI-TOF mass spectrometry) were used without purification; those

Table 1
Chemical characterization of consensus A22(147P) peptide (linear, cyclic and random versions)

Peptide	Purity (%, HPLC)	Molecular weight theoretical (found[a])	AAA[b]
A22(147P)	98	2377.21 (2378.70)	Asp, 1.09 (1); Thr, 5.37 (6); Ser, 1.21 (1); Gly, 1.00 (1); Ala, 4.16 (4); Leu, 2.01 (2); Tyr, 0.98 (1); His, 2.75 (3); Arg, 1.99 (2)
cycA22(147P)	88	2262.07 (2262.49)	Asp, 1.05 (1); Thr, 5.01 (6); Ser, 1.33 (1); Gly, 1.09 (1); Ala, 4.28 (4); Leu, 1.97 (2); His, 2.63 (3); Arg, 0.97 (1)
A22ran	99	2377.21 (2377.54)	Asp, 1.05 (1); Thr, 5.40 (6); Ser, 1.21 (1); Gly, 1.00 (1); Ala, 4.05 (4); Leu, 1.90 (2); Tyr, 1.10 (1); His, 3.05 (3); Arg, 1.95 (2)

[a] Determined by MALDI-TOF mass spectrometry.
[b] Experimental followed by theory values in parenthesis.

of lower content were purified by HPLC to meet the desired specifications.

2.3. Mixotope synthesis

All mixotopes were synthesized by manual solid phase methods, as described in [10], starting from 0.1 mmol (144 mg) of MBHAR and using a Boc protection scheme. Couplings at non-degenerate positions were done with 3 equivalents of the corresponding Boc-amino acid in the presence of dicyclohexylcarbodiimide/1-hydroxybenzotriazole (3 equivalents) in *N*-methylpyrrolidone. At degenerated positions, a first coupling (1.5 h to overnight) was done with 0.1 mmol (1 equivalent) of an equimolar mixture of the Boc derivatives of the amino acid mutations reported for that position. *N*,*N*-diisopropylethylamine (0.1 mmol) was added to the coupling reaction during the last 30 min. If a positive Kaiser ninhydrin test was observed, a second coupling with 0.2 mmol of the same Boc amino acid mixture was performed. The mixotope libraries were cleaved off the resin with HF/*p*-cresol (9:1, 0 °C, 1 h), taken up in 10% HOAc and lyophilized several times. Exhaustive chemical characterization of the preliminary 64-peptide test library was done by HPLC, MALDI-TOF mass spectrometry and amino acid analysis (AAA) as discussed in the Results section.

For the cyclic disulfide mixotopes, an approach similar to that of Section 2.2 was followed, i.e., Tyr^{136} and Arg^{153} of the consensus sequence were replaced by Cys and the bis-thiol precursor obtained upon cleavage (9:1, HF/*p*-cresol; 0 °C, 1 h) was isolated, dissolved to 45 μM in 100 mM NH_4HCO_3, pH 8.5, and air-oxidized for 26 h. Since HPLC monitoring of the reaction progress was obviously unfeasible, Ellman colorimetric tests were performed, as follows: 500 μL-aliquots of the mixotope solution were neutralized (dilute HOAc), lyophilized and redissolved in 10 μL of 20 mM Tris buffer, pH 8, then mixed with 25 μL of saturated 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution in 20 mM Tris buffer, pH 8. Cysteine and cystine samples were used as positive and negative controls, respectively.

The four libraries to be used as immunogens were characterized exclusively by AAA [10]. Experimental values were matched against expected amino acid molar ratios calculated from the theoretical composition of the mixotope.

2.4. Viruses and cells

Five FMDV, serotype C1, isolates (CS8, CS15, CS20, CS35 and MAR 2.1) were used in the neutralization assays of sera derived from mixotope libraries. The amino acid sequences of their GH loops (positions 137–157 of capsid protein VP1) [19,20] are shown in Fig. 1. For guinea-pig challenge experiments, FMDV isolate 2.10b was used. This virus was derived from isolate C-S8c1 by 10 serial passages in guinea-pigs and was able to consistently produce vesicular lesions in this species [18]. FMDV 2.10b was unable to infect cell cultures; it was propagated in guinea-pig and the virus recovered from lesions was titrated by means of its capacity to kill suckling mice [21].

2.5. Animal immunization and viral challenge

Male guinea-pigs (Dunkin Hartley, 250–350 g) were immunized by subcutaneous injection with 1 mg of peptide immunogen (consensus sequence, MixHi and MixLo, in both linear and cyclic versions) dissolved in 150 μl of PBS and emulsified with an equal volume of FCA. Animals were boosted twice, at 21 and 42 d.p.i., with an equivalent dose in FIA administered intradermally. Sera from immunized animals were collected at 61 d.p.i. for serological analysis. Twenty days after the second vaccine boost (62 d.p.i.), animals were challenged by intradermal injection of 3×10^4 LD_{50} in suckling mouse of FMDV 2.10b, in the metatarsal pad of the left hind foot. The appearance of lesions in any of the feet or in the mouth was monitored by visual inspection for seven days. A clinical score (0, absence; 4, large vesicles) was used to evaluate the severity of the lesions.

2.6. Serological analyses

2.6.1. Detection of anti-peptide/mixotope antibodies

A direct ELISA, based on a previously described method with slight modifications, detected antibodies recognizing either the consensus peptide or the mixotopes [22]. All procedures were performed at room temperature (ca. 25 °C) unless otherwise indicated. Briefly, 96-well plates were coated with 2 μg of peptide or 8 μg of mixotopes in water, by evaporation overnight at 37 °C, then blocked for 1 h with 0.5% BSA

FMDV Serotype C	135			140							145						150					155
C-S8c1	T	T	Y	T	A	S	A	R	G	D	L	A	H	L	T	T	T	H	A	R	H	L
2.10b				T	A	S	A	R	G	D	L	A	H	P	T	T	T	H	A	R	H	L
C-S15				T	T	S	T	R	G	D	L	A	H	I	T	A	T	H	A	R	H	L
C-S20				T	A	S	T	R	G	D	L	A	H	L	T	A	T	H	A	R	H	L
C-S35				T	T	S	T	R	G	D	L	A	H	I	A	A	T	H	A	R	H	L
MAR2.1				T	A	R	A	R	G	D	L	A	H	L	T	T	T	H	A	R	H	L

Fig. 1. Amino acid sequences at VP1 site A of serotype C of FMDV isolates. The conserved RGD region is boxed. Virus 2.10b was recovered upon adaptation from isolate C-S8c1 to guinea-pig (mutation of Leu to Pro at position 147), and was used for challenge experiments.

in PBS (blocking buffer), and incubated with serial dilutions of sera in blocking buffer plus 0.1% Tween 20 for 2 h. After washing with PBS–0.1% Tween-20 (PBST), wells were incubated with protein A-horseradish peroxidase conjugate for 40 min in PBST. After extensive washing, the substrate tetramethyl-benzidine (TMB) was added to the wells, the reaction was allowed to develop for 10 min and then stopped with 2 M H_2SO_4. Titers of antibodies to the peptides or mixotopes were defined as the reciprocal of the dilution giving OD 1.0 at 450 nm.

Antibodies against peptides reproducing site A single or multiple mutations were detected by a slightly different ELISA in which peptide (2 μg in 100 μL PBS) was coated overnight at 4 °C; blocking, washes and serum incubations were as above, and *o*-phenylenediamine (OPD, detected at 490 nm) was used as peroxidase substrate.

2.6.2. Detection of anti-FMDV antibodies

Antibodies recognizing FMDV were assessed by direct or sandwich ELISA. In the direct protocol, concentrated FMDV CS8 supernatants were directly coated into 96-well plates by overnight incubation at 4 °C, the plates were washed and blocked as above, then incubated with serum dilutions in blocking buffer for 90 min. Protein A-peroxidase-OPD detection was performed as above.

In the sandwich format, plates were coated overnight with serotype C-specific rabbit anti-FMDV antiserum, diluted 1/2500 in 0.1 M carbonate–bicarbonate buffer, pH 9.6. After washing with PBS, FMDV C_1 supernatant at 5×10^5 pfu/well was added and incubated for 1 h at 37 °C. After washing with PBS–0.1% Tween-20, serum dilutions in PBS–0.1% Tween-20–2% skimmed milk were incubated for 1 h at 37 °C. Antibody detection was as above.

2.7. Viral neutralization assay

For fast screening, a micro-neutralization assay (VN) [23] was performed as follows: two-fold serial dilutions of serum (starting with 1:25) were incubated in duplicate for 1 h at 37 °C with 50 pfu FMDV in 100 μL DMEM supplemented with antibiotics and glutamine, then transferred to IB-RS2 cell monolayers grown in 96-well microtiter plates containing 50 μL/well of DMEM supplemented with glutamine, antibiotics and 5% FCS (final volume: 150 μL/well). After overnight incubation (18–20 h), cells were fixed and stained with crystal violet in 4% formaldehyde for 1 h at 25 °C, washed with water to remove excess dye, and the cytopathic effect (cpe) measured as a function of dye incorporation, by incubation with methanol (200 μL/well) for 1 h at 25 °C, and recording of the OD at 620 nm. 100% and 0% cpe were determined in wells incubated with virus alone or without virus, respectively. Neutralization titer was defined as the reciprocal of the dilution giving 50% cpe reduction.

3. Results and discussion

3.1. Synthetic antigens

3.1.1. Single sequences

A 22-residue peptide, TTYTASARGDLAHPTTTHARHL, corresponding to residues 134–155 (GH loop) of VP1 of the guinea-pig-adapted FMDV 2.10b isolate (Figs. 1 and 2), was taken as consensus sequence of antigenic site A for the present study. This sequence contains the amino acid residues most frequently found at each position in a guinea-adapted version of FMDV, and differs from the CS8c1 isolate used in earlier work (e.g., [16]) in that Pro instead of Leu is present at position 147 (Fig. 1). It was synthesized as a single peptide in both linear [A22(147P)] and cyclic disulfide [cA22(147P), Tyr^{136} and Arg^{153} mutated to Cys] versions, and used to evaluate the affinity and specificity of the different antisera. Linear sequences corresponding to other related type C isolates (Fig. 7) were also made in order to test the antigenic spectrum recognized by mixotope sera. A random version of A22(147P), rA22, was prepared as negative control.

3.1.2. Test mixotope

To assess the reliability of the synthetic methods to be used in the preparation of mixotope libraries, a preliminary low-diversity mixotope, based on the 136–150 VP1 residues of FMDV2.10b and containing only 64 sequence combinations, was synthesized [24]. In parallel, all 64 individual peptide sequences theoretically present in the mixotope were synthesized and characterized by HPLC and MALDI-TOF mass spectrometry. The peptides were then combined in equimolar amounts and the mixture compared to the mixotope by HPLC–electrospray mass spectrometry. This analysis showed the mixotope library to contain all the peptide masses of the artificial mixture, with generally comparable peak intensities on both samples. In a few cases (less than 5%) the

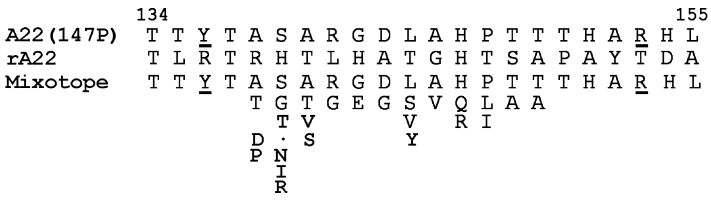

Fig. 2. Composition of the low variability mixotope, MixLo (3.4×10^3 sequences), is shown in normal type. The high variability library, MixHi (3.2×10^5 sequences), includes in addition the residue shown in boldface. Cyclic mixotopes were obtained by intramolecular disulfide formation between positions 136 and 153 (underlined), both mutated to Cys.

intensity of a certain peptide mass in the mixotope was <50% than in the control, suggesting not strictly equimolar composition. Since the AAA of both mixtures were also coincident within a 5% error, it was concluded that the test mixotope provided a reasonably accurate representation of the combined mutations in FMDV2.10b.

3.1.3. Mixotope design and synthesis

Once reliable methods for mixotope synthesis had been worked out, mixotope libraries representing a broad array of FMDV antigenic site A mutations were built (Fig. 2). The mixotopes were designed on the basis of the FMDV 2.10b consensus sequence shown in Fig. 1. Although most mutations altering virus antigenicity, previously observed in either field or cell-cultured mutants [15–17], cluster around a central, 15-residue region (residues 136–150) of VP1, we opted for a slightly longer (22-residue) sequence, as previously used in single peptide vaccination trials [7]. Many of these mutations are known to be critical in terms of both antibody and cellular receptor recognition. Among the latter, some involve the Arg–Gly–Asp (RGD) triplet. This motif is involved in host cell recognition via integrins such as $\alpha_V\beta_3$ and $\alpha_V\beta_6$ [25–27] and in interaction with neutralizing antibodies [28–30] and is highly conserved in all FMDV serotypes. For type C FMDV, escape mutations affecting Arg^{141} have been detected in cattle vaccination trials with site A-based peptide vaccines [7], as well as the other two residues in viral passage experiments in cell culture [15].

Other positions where mutations of interest have been reported are those immediately up or downstream from RGD. For instance, changes at Leu^{144} and Leu^{147} (and to a lesser extent in Ala^{145}) are known to induce critical losses in both antigenicity and neutralizing ability [16,31] and are selected in cattle vaccinated with type C, site A-based peptide vaccines upon viral challenge [7].

The level of chemical complexity of the mixture is an important decision in the design of mixotope libraries [10]. The aim is to find a proper balance between, on the one hand, sufficient coverage of significant mutations and, on the other hand, adequate representation (i.e., concentration) of each combinatorial sequence in the immunogen. Given the relative lack of data on the subject, we decided (i) to discard mutations with reported frequencies below an arbitrary 2% lower limit; (ii) to test two levels of diversity, differing in roughly two orders of magnitude, and (iii) to evaluate the effect of conformational restriction through an internal disulfide on the immune response, an approach reported to enhance both the antigenicity and immunogenicity of site A peptide vaccines [32]. Thus, our low-diversity mixotopes, MixLo and cMixLo, linear and cyclic, respectively (Fig. 2), contained the most frequent mutations observed at Ala^{138} (Thr), Ser^{139} (Gly), Ala^{140} (Thr), Leu^{144} (Ser, Val) Ala^{145} (Val), His^{145} (Gln, Arg), and Pro^{147} (Leu, Ile), all with a frequency above 2%, in addition to those at the RGD motif (Gly, Glu, Gly, respectively). This amounted to a theoretical number of $2^7 \times 3^3 = 3456$ peptide sequences. For the cyclic disulfide version, and on the basis of previous results [33], Tyr^{136} and Arg^{153} were mutated to Cys. In the two high-variability mixotopes, MixHi and cMixHi, mutations with frequencies under 2% at the hypervariable region were also included, as well two Ala→Thr changes at positions 148 and 149 (frequency above 2% in both cases), altogether defining a theoretical number of $2^6 \times 3^2 \times 4^2 \times 5 \times 7 = 322\,560$ combinatorial peptide sequences. Mutations representing reported gaps (e.g., at positions 138 and 139, Fig. 2) were also represented. This was done by removing a residue aliquot corresponding to $1/n$ of the total batch, n being the number of variant residues at this position.

Amino acid analysis is the only possible characterization for mixture libraries of such complexity. Fig. 3 shows a comparison between the experimental and theoretical compositions of all four libraries. In general, satisfactory correlations were obtained for most residues. The larger discrepancies were observed for β-branched residues such as Val or Thr, known to hydrolyze poorly, especially when juxtaposed, as is the case in many of the sequences in the mixotope.

3.2. Antibody response to mixotope immunogens

Direct ELISA were performed to evaluate the immune response elicited in guinea-pig by either single peptides [A22(147P), cA22(147P] or combinatorial libraries (MixLo, cMixLo, MixHi and cMixHi) against each respective parent immunogen peptide, as well as to detect cross-reactivities among them (Fig. 4). For this purpose, groups of four animals were immunized with each of the peptides, as described in Section 2.5. Antibody responses detected by ELISA within each group before viral challenge (at 61 d.p.i.) were consistent and no significant variability among animals was observed. The highest titers were obtained in the group of

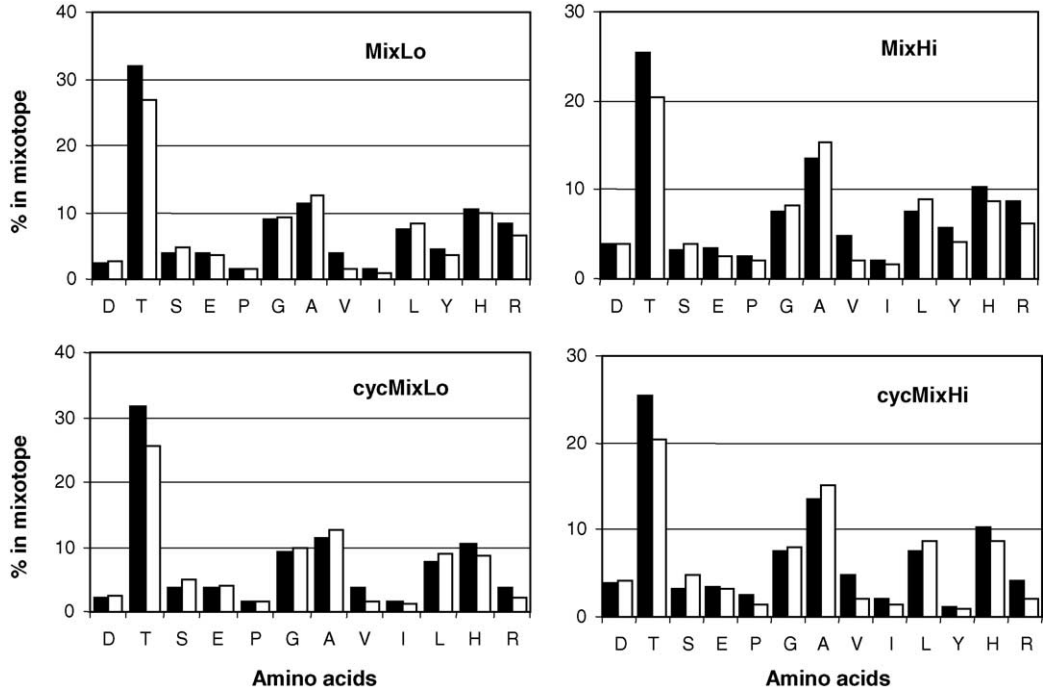

Fig. 3. Amino acid composition of the low variability mixotope, MixLo and cyMixLo (3.4×10^3 sequences), and high variability mixotope library, MixHi and cyMixHi (3.2×10^5 sequences), determined after hydrolysis with 6 M HCl for 1.5 h at 155 °C (white bars). Theoretical composition (dark bars), calculated on the basis of an equimolar amount of each amino acid introduced in the degenerated position. D and E columns represent Asp + Asn and Glu + Gln, respectively.

animals immunized with linear MixHi. Remarkably, the titers of these sera against the consensus A22(147P) sequence were even higher than those of animals immunized with the single peptide, despite the ca. 1:300 000 dilution of this sequence in the MixHi immunogen. In contrast, sera from animals immunized with A22(147P) recognized mainly this consensus sequence and only weakly the mixotopes. The general pattern of antiserum reactivity was MixHi > MixLo > consensus, with lower serum titers observed for animals immunized with cyclic peptides. These results are in fair agreement with the study of Gras-Masse et al. [34], which showed that increased immunogen degeneracy does not entail a commensurate loss in either serum titer or specificity.

3.3. Specificity of anti-mixotope sera

The cross-reactivity of anti-mixotope sera with sequences bearing mutations included in the mixotope is well

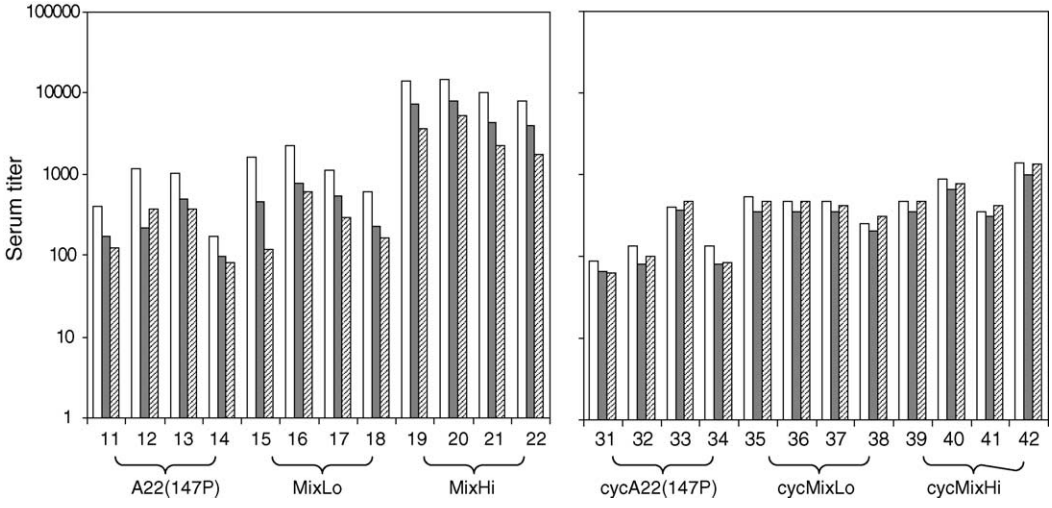

Fig. 4. Direct ELISA of peptide or mixotope antisera. Groups of four animals were vaccinated with consensus peptide, linear (11–14) or cyclic (31–34); MixLo, linear (15–18) or cyclic (35–38); MixHi, linear (19–22) or cyclic (39–42). Plate antigens were the consensus peptide (white bars), the MixLo (black bars) and the MixHi (grey bars), linear or cyclic, respectively. Titers are expressed as the inverse of the dilution giving a OD = 1 at 450 nm.

Table 2
Serum neutralization (plaque reduction) and protection against viral challenge in guinea-pigs immunized with peptides and mixotopes

Animal	Immunogen	Neutralization titer[a]	Protection[b]
11	A22(P147)	<5	+
12		<5	−
13		5	+
14		5	−
15	MixLo	5	−
16		0	−
17		0	−
18		0	−
19	MixHi	0	−
20		0	−
21		<3	−
22		<5	−
31	cycA22(147P)	<5	−
32		20	+
33		20	+
34		5	+
35	cycMixLo	0	[c]
36		0	[c]
37		<5	+
38		0	−
39	cycMixHi	<5	+
40		<5	−
41		<5	±
42		0	−
43	PBS	<5	−
44		0	−

[a] Titer defined as the lowest dilution giving 50% plaque reduction. Each antiserum assayed in duplicate. Other details in Section 2.
[b] Challenge with CS8c1 FMDV; (+) and (−) indicate protected (no lesions on either pads or tongue) and non-protected, respectively, on day 8 post-challenge; (±) minor lesion on left hind pad (inoculation site) observed on day 8 post-challenge.
[c] Animal dead before challenge.

established [10,34]. We attempted to go one step further, by exploring the recognition by mixotope antisera of peptides with residues both included in and absent from the library. Thus, three of the FMDV peptides used as coating agents in the ELISA employed (peptides A15S30, A21S30 and cyc16S30; Table 2), all related to isolate C-S30 [31], had Val instead of Leu at position 147, a change not included in either mixotope, plus other mutations covered by the mixotopes. In addition, cyc16S30 included a non-natural residue (6-aminohexanoic acid) used originally to enhance the flexibility of the cyclic structure [31]. Another of the coating peptides, A15(FPSI), had a far more heterologous sequence, none of its four mutations included in the mixotopes. The rA22 random sequence was used as negative control.

Mixotope antiserum reactivity (at 61 d.p.i.) toward this panel of antigens was, broadly speaking, consistent with expectations. Thus, antisera from linear mixotopes (Fig. 5, A panels) recognized peptide A22(147P) quite well, peptide A15S30 to a lower extent, and peptide A21S30 only faintly. Variability among different sera was not significant (only two representative animals in each group appear in Fig. 5). In terms of specificity, no striking differences were observed between MixLo and MixHi antisera, other than an increased recognition of A21S30 in the latter, consistent with the fact that only the high-variability mixotopes contained the Ala → Thr mutation at position 149. It seems reasonable to assume that the diminished reactivity of all anti-mixotope sera against peptides A15S30 and A21S30 relative to the A22(147P) consensus peptide may be substantially due to the non-inclusion of the Val^{147} mutation in either mixotope. Earlier studies using site A monoclonal antibodies and single-replacement peptides had shown this position to be particularly sensitive, even to seemingly conservative changes such as Val → Leu (absent and present, respectively, from mixotope) [31]. Similar reasoning can explain the total lack of reactivity of the A15(FPSI) peptide against all mixotope sera. In this case, the four replacements (none included in the mixotope) would clearly abolish any advantage related to the Leu^{147} mutation being present in both sequence and libraries.

The cyclic mixotopes (Fig. 5, B panels) behaved again fairly much as expected. Reactivity was highest against the consensus sequence (in cyclic form), with titers comparable to those of anti-linear mixotope sera, and with no appreciable differences between low- and high-variability libraries. The antisera showed clear specificity for cyclic antigens (consensus and cyc16S30) and practically no recognition of linear, multiply mutated sequences.

We next investigated the ability of peptide A22(147P) and the linear mixotope-derived antibodies to recognize type C FMDV particles. The guinea-pig-adapted FMDV 2.10b, whose site A sequence was taken as consensus for the present work, could not be used to assess the antigenicity and neutralizing activity of sera samples, due to its inability to grow in cell culture [18]. Therefore, the type C isolate C-S8c1 (Fig. 1), whose sequence was also considered in the MixLow and MixHigh mixotope design (Fig. 2), was used as coating antigen for the ELISA. An antiserum against A24 (TTYTASARGDLAHLTTTHARHLP), a peptide that strictly reproduces site A of type CS8c1 isolate, was used as positive control. As can be seen in Fig. 6, panel A, sera from guinea-pigs immunized with peptide A22(147P), with the exception of one of the four test animals, were significantly less reactive with the viral particle than anti-A24 serum. This can be plausibly attributed to the Leu → Pro replacement at the sensitive position 147 (see above). Interestingly, the anti-mixotope sera (Fig. 6, panels B and C) reacted to C-S8c1 virions in a similar manner as those from the consensus peptide A22(147P), with slightly better recognition observed for MixLo versus MixHi antisera. To ensure that the effect was not due to a change in the native virus conformation upon binding to the plate, a capture ELISA with FMDV in solution was performed, which showed similar results to those observed with the direct ELISA (data not shown). Thus, a significant binding to viral particles corresponding to one of the sequences included in the mixotopes was detected in both linear MixLow and MixHigh sera.

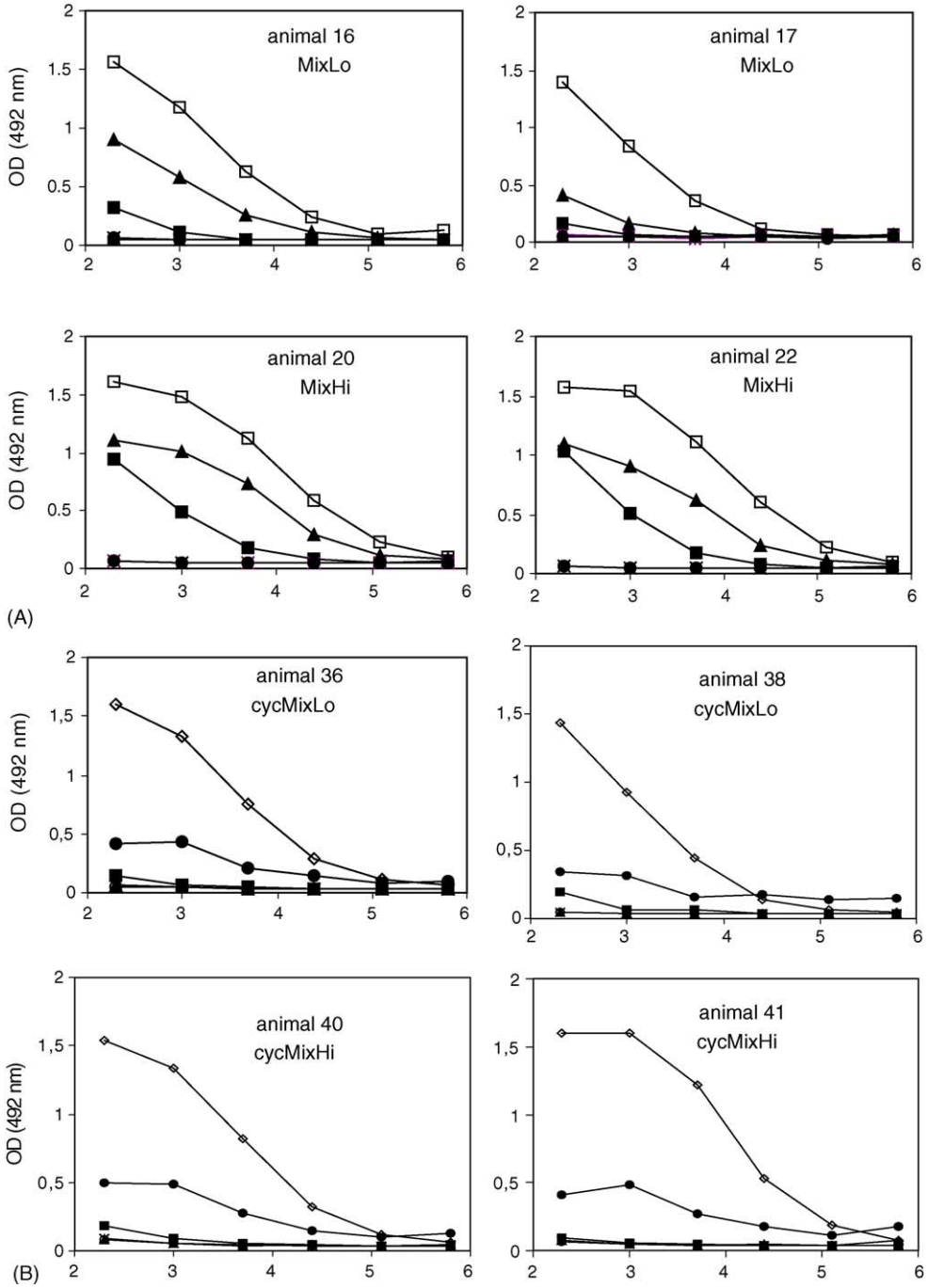

Fig. 5. Direct ELISA of linear and cyclic consensus peptide and mixotope antisera against mutated peptides related to site A of FMDV. Sera from animals immunized with linear MixLo (16 and 17) and MixHi (20 and 22) (panel A), and with cyclic MixLo (36 and 38) and MixHi (40 and 41) (panel B) were tested. Plate antigens were the A22(147P) consensus peptide in either linear (□) or cyclic (◊) form, as well as the A21S30 (■), A15S30 (▲), cyc16S30 (●), and FPSI (*) mutants, plus the random peptide rA22 (○).

3.4. Neutralization

A micro-neutralization assay (see Section 2.7), which allows fast processing of small amounts of many samples, was used to study the ability of the different antisera to neutralize FMDV. Using this assay, the 28 antisera produced [four animals each for A22(P147), MixLo, MixHi, plus cyclic versions, plus four controls inoculated with PBS] were tested against five different FMDV isolates (Fig. 1). All mutations (relative to 2.10b) in antigenic site A of the viruses included in this assay were covered by the mixotope library (Figs. 1 and 2).

The highest titers in this micro-neutralization assay were found for animals immunized with peptide cycA22(P147),

Coating peptide	Sequence
A15S30	Y T **T** S **T** R G D L A H V T **A** T
A15(FPI)	Y T F S P R S D L A H L I T T
cyc16S30*	C T **T** S **T** R G D L A H V T **A** U C
A21S30	Y T **T** S **T** R G D L A H V T **A** T H A R H L P
A22(147P)	T T Y T A S A R G D L A H **P** T T T H A R H L
cycA22(147P)*	T T C T A S A R G D L A H **P** T T T H A C H L
rA22	T L R T R H T L H A T G H T S A P A Y T D A

* cyclic disulfide

Fig. 6. Mutant peptides related to serotype C of FMDV used in ELISA. Residues present and absent from the mixotopes are shown in bold and underlined, respectively. Cyclic peptides were obtained by mutation of Tyr^{136} and Arg^{153} to Cys and internal disulfide formation. U stands for 6-aminohexanoic acid.

followed by those of peptide A22(P147) (Fig. 8). These results are in agreement with previous observations on the higher capacity of cyclic peptides to elicit neutralizing antibodies to FMDV [32,35], which is most likely related to the adoption of native-like conformations upon cyclization. Interestingly, 7 out of 16 mixotope sera showed significant levels of neutralization against some of the type C viruses analyzed. Even in those cases when, on the whole, a considerable individual animal variation was observed, and serum from no single animal neutralized all the five FMDV isolates tested, the results illustrated the capacity of MixHi, cycMixLo and cycMixHi to elicit detectable levels of anti-FMDV neutralizing antibodies. In the linear series (Fig. 8, panel A), animals immunized with MixLo were non-responsive, and of those receiving MixHi two antisera (21 and 22) were able to neutralize the cellular infection by four out of five viruses tested. For cyclic mixotopes, individual animal variation was higher, and neutralizing activity was observed in sera from mixotopes of high and low complexity (Fig. 8, panel B).

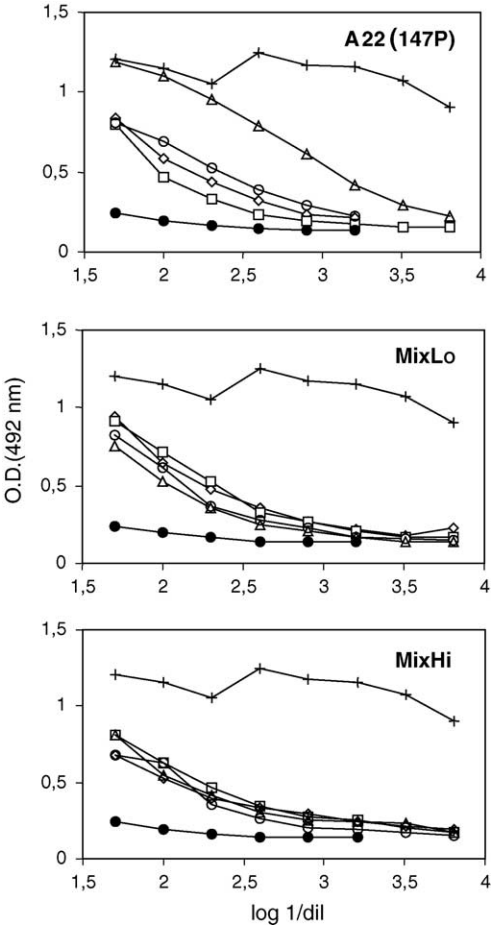

Fig. 7. ELISA of antisera from animals immunized with linear consensus peptide, MixLo and MixHi (panels A–C, respectively) against inactivated CS8 FMDV. Sera from animals immunized with A24-peptide and PBS: (+) and (●) used as positive and negative controls, respectively.

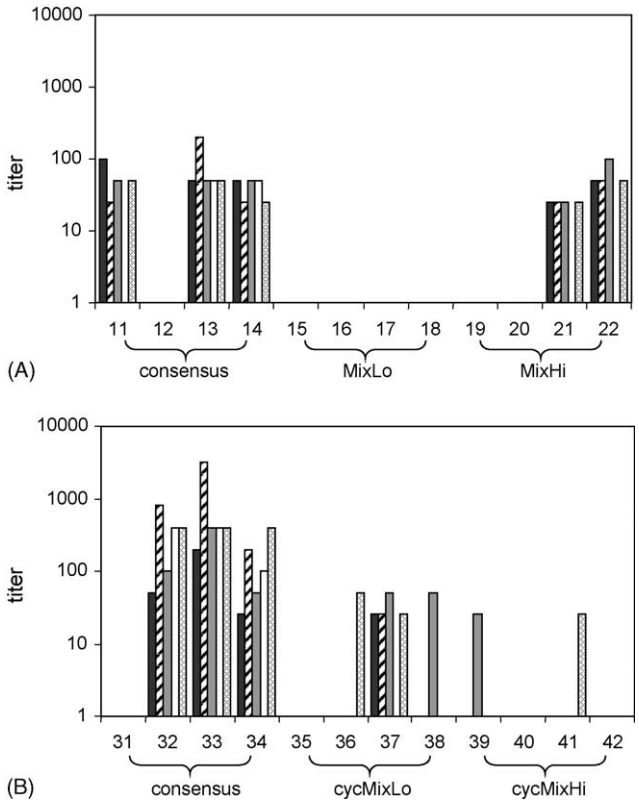

Fig. 8. FMDV neutralization by sera from animals immunized with linear (panel A) or cyclic (panel B) versions of either consensus peptide or the mixotopes. See Fig. 4 for serum assignments. FMDV test isolates were CS8 (■), CS15 (▨), CS20 (▩), CS35 (□) and MAR2.1 (▧). Titer was defined as the reciprocal of the maximum dilution giving 50% of cytopathic effect reduction at 620 nm. Lowest dilution was 1/25.

3.5. Viral challenge and protection

The guinea-pigs immunized with the mixotopes or consensus peptides (linear and cyclic) were challenged with FMDV isolate 2.10b to assess the levels of protection conferred. Two animals were inoculated with PBS to be included in this assay as control.

As discussed above, the estimation of the individual neutralization titers elicited against the challenge virus 2.10b was not possible. Despite this limitation, a correlation was observed in animals vaccinated with consensus peptides between protection against challenge with 2.10b virus and neutralization titers to related type C viruses (Table 2 and Fig. 8). Thus, the three protected animals (32, 33, 34) vaccinated with peptide cycA22(147P), showed a consistent neutralizing response to all the type C isolates analyzed. Likewise, two of the three animals (11, 13) vaccinated with peptide A22(147P) that showed consistent neutralization titers were protected.

This trend was also observed, albeit to a lesser extent, in animals vaccinated with cyclic mixotopes. Protected animals 37 (cycMixLo) and 39 (cycMixHi) elicited neutralizing antibodies against four and one of the type C isolates, respectively. Also, animal 41, which developed a mild lesion upon challenge, exhibited neutralization titers against the Mar 2.1 isolate. However, no protection was found in animals immunized with linear mixotopes, even in guinea-pigs (21 and 22) exhibiting significant neutralizing response (Fig. 8). These results suggest that a qualitative difference exists in the capacity of antibodies elicited by cyclic versus linear mixotopes in conferring protection in an animal model (Fig. 8).

The role in protection of the antibodies induced by peptide vaccines is not fully understood, and it has been reported that the correlation between in vitro serum neutralization and protection is lower than that observed in conventionally vaccinated and convalescent animals [36–38]. On the other hand, the molecular processes underlying the complexity of the antigen recognition processes and the specific activation of B and T cells by complex mixtures of different related immunogens, such as mixotopes, will require considerable research to be elucidated.

However, and despite the statistical limitations of the results here discussed, we believe that the finding that mixotopes of considerable diversity can elicit antibodies recognizing and neutralizing a complex structure such as the FMDV viral particle offers interesting possibilities in the peptide-based vaccine approaches designed to deal with highly variable pathogens. In particular, the results obtained with cyclic mixotopes suggest the possibility of inducing a protective immune response against viral challenge.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Technology (BIO 2002-04091-C03-01 to D.A. and -02 to F.S.), the European Union (QLK2-2001-1304 to F.S.) and Fundación Severo Ochoa (to F.S.).

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