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Peptide vaccine candidates against classical swine fever virus: T cell and neutralizing antibody responses of dendrimers displaying E2 and NS2–3 epitopes

Marta Monsó,[a†] Joan Tarradas,[b†] Beatriz G. de la Torre,[a] Francisco Sobrino,[c] Llilianne Ganges[b] and David Andreu[a*]

Three peptide-based systems integrating B and T antigenic sites of CSFV and displaying the B epitopes in fourfold presentation have been designed and produced, and shown to bring about significant enhancements in immunogenicity over the peptides in monomeric form. Of the different strategies tested for producing the dendrimeric constructs, stepwise SPPS using 3,6-dioxaoctanoic acid as flexible, PEG-like spacer units at the branching points is clearly advantageous, in particular over ligation in solution. The constructs have been used for immunization of domestic pigs, in order to evaluate the protective response induced by each peptide constructs, and to characterize the B- and T-cell response against CSFV in the natural host. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: classical swine fever; dendrimeric peptides; peptide vaccines; PEG-like flexibilizing units

Introduction

Multimerization has long been recognized as an effective strategy for enhancing the response of peptides in immunization experiments [1–3]. Among the different approaches such as simple polymerization [4], or conjugation to sequential oligopeptide carriers [2,5], or display on lysine scaffolds [6,7], incorporation into dendrimeric systems such as the MAP platforms pioneered by Tam [1,8,9] has proven particularly successful for immunological and microbiological applications [9–12]. In an extension of the MAP paradigm, we have recently shown that dendrimeric constructs (named B_4T) combining B and T epitopes of FMDV can elicit immune responses far superior to those of the constituent linear epitopes and result in complete protection in challenge experiments [13].

CSF is a highly contagious viral infection affecting domestic and wild pigs. It is considered to be one of the most devastating diseases for the pig industry throughout the world, from both the economic and sanitary points of view [14]. Protection against CSFV is associated with the induction of neutralizing antibodies; however, the role of the cellular response elicited upon viral infection is not fully understood [15]. The induction of specific T lymphocytes in infected animals has been described, and there is increasing evidence on the role of the T-cell responses in protection against CSFV [15–18]. Antibodies against CSFV elicited by infected swine mainly target structural proteins E^{rn} and E2, plus nonstructural protein NS2-3. Glycoprotein E2, which is regarded as the most immunogenic of CSFV proteins, is mainly responsible for the induction of neutralizing antibodies [19]. Live attenuated vaccines against CSFV, introduced some 60 years ago and based on glycoprotein E2, induce high titers of neutralizing antibodies and are the only vaccine candidate that can confer protection when administered alone [20–22]. However, although they guarantee high protection rates, such CSFV vaccines have the serious disadvantage of not being marker or DIVA vaccines, i.e. their antibody patterns are very similar to those observed in naturally infected animals, making it extremely difficult to differentiate vaccinated animals from infected ones. This has prompted the search for new DIVA vaccine strategies against CSFV, including DNA [15,23–29], viral vector [20,22,30–33],

* Correspondence to: David Andreu, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain. E-mail: david.andreu@upf.edu

a Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain

b Centre de Recerca en Sanitat Animal (CReSA), Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

c Centro Biología Molecular 'Severo Ochoa'-CSIC, Universidad Autónoma de Madrid, 28049 Cantoblanco, Spain

† These authors contributed equally to this work.

Abbreviations used: *Ahx, 6-aminohexanoic acid; BSL3, biosafety level 3; ClAc, chloroacetyl; CSF, classical swine fever; CSFV, classical swine fever virus; CTL, cytotoxic T cell; DIEA, N,N-diisopropylethylamine; DIVA, differentiation of infected from vaccinated animals; d.p.c., days postchallenge; d.p.i., days post-immunization; EDT, ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; FMDV, foot-and-mouth disease virus; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high performance liquid chromatography; IFN, interferon; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MAP, multiple antigenic peptide; MS, mass spectrometry; NPLA, neutralizing peroxidase-linked assay; O_2Oc (O), 3,6-dioxaoctanoic acid; PEG, polyethyleneglycol; SPPS, solid phase peptide synthesis; $TCID_{50}$, 50% infective dose in tissue culture; TFA, trifluoroacetic acid; TIS, triisopropylsilane.*

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chimeric pestivirus [34–36], and peptide [14,37–42] vaccines. Because peptide vaccines contain no genetic component, no risk of pathogen replication by any vaccine component exists, thus making them practically, by definition, DIVA vaccine candidates.

In the search for peptide-based CSFV vaccines, several immunogenic peptides corresponding to different regions of the A or B-C domains of E2 have been proposed and used in either mono- or multi-peptide formulations [38–42] which have been evaluated in immunization and challenge experiments. Although some peptide mixtures have been reported to induce CSFV-specific neutralizing antibodies as well as protective activity against CSFV challenge infection, in most cases they have failed to provide complete protection from clinical signs, viraemia, and virus shedding [38,39]. Thus, the need for safer CSFV marker vaccines remains.

As all CSFV candidate vaccines hitherto described are based on linear peptides, in our approach to novel peptide-based CSFV marker vaccines we have chosen a multimerization strategy similar to that of our B_4T vaccine [13] for FMDV. Using a library of overlapping peptides spanning the main antigenic domain of glycoprotein E2, Dong *et al*. [38] identified residues 693–716 as those inducing the best protective response on an individual basis [39]. Other *in vitro* studies have proposed residues 829–837 [43] as B-cell epitopes of E2, and several epitopes inducing CTL/helper responses have been identified in the nonstructural protein NS3 of CSFV [44]. This information provides a basis for a first generation of dendrimeric, B_4T-type CSFV vaccine candidates.

Materials and Methods

Peptide Synthesis

Fmoc-protected amino acids and chemicals were obtained from Iris Biotech (Marktredwitz, Germany). Solvents for synthesis were obtained from SDS (Peypin, France). Automated syntheses were performed in an ABI433 peptide synthesizer (Applied Biosystems, Foster City, CA) running standard Fmoc (FastMoc) protocols [45]. For simple linear peptides, 0.1-mmol resin batches of Fmoc-Rink-amide ChemMatrix (Matrix Innovation, Montreal) were used. For dendrimeric peptides of the general structure shown in Table 1, the linear (T epitope) region was built as above and branching was introduced by means of Fmoc-Lys(Fmoc) residues. The resulting resin was then processed in one of the two possible ways: (i) Fmoc deprotection and coupling of chloroacetic acid to each branch to give a tetra-chloroacetyl (Cl_4Ac) peptide used in the ligation experiments (see Section on Results) or (ii) elongation by standard SPPS. In this case, the resin was divided into three portions (*ca* 130 μmol of amino groups), one for each of the constructs in Table 1, and on each portion a 3,6-dioxaoctanoic acid (O_2Oc) unit was introduced at each of the four branches by means of the corresponding Fmoc derivative (Iris Biotech), followed by the chosen B epitope sequence (one copy at each branch), again built by the FastMoc program in systematic double coupling fashion (reaction scheme, Figure 1). The side-chain functionalities were protected with ^{t}Bu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys, Trp), Pbf (Arg), and Trt (Asn, Gln, His) groups. Couplings were done with an eightfold molar excess of Fmoc-amino acid, HBTU, and HOBt, in the presence of double that amount of DIEA, in DMF as solvent. Peptide resins were deblocked with 20% piperidine/DMF before full deprotection and cleavage by acidolysis with TFA/H_2O/EDT/TIS (94 : 2.5 : 2.5 : 1 v/v, 90 min, RT). Cleaved peptides were precipitated by the addition of chilled diethyl ether, taken up in aqueous AcOH (10% v/v), and lyophilized.

Analysis and Purification

Analytical reversed-phase HPLC was performed on C_{18} columns (4.6 × 50 mm, 3 μm, Phenomenex, Torrance, CA) in a model LC-2010A system (Shimadzu, Kyoto, Japan). Elution was done with linear gradients of solvent B into A over 15 min (Figures 2–4 legends for further details) at a flow rate of 1 ml/min, with UV detection at 220 nm. Preparative HPLC was performed on C_{18} (10 × 250 mm, 10 μm, Phenomenex) in a Shimadzu LC-8A instrument. Solvents A and B were 0.1% TFA (v/v) in water and acetonitrile, respectively, and elution was again done with linear gradients of solvent B into A over 30 min, at a flow rate 5 ml/min, with UV detection at 220 nm. Preparative fractions of satisfactory

Table 1. Dendrimeric peptide vaccine candidates used in this study

No.	Structure[a]	B epitope	T epitope	Mass[b] (calcd.) (Da)	Mass[c] (found) (Da)
1	(KEDFRYAISSTNEIGLLGA-O)$_2$K / (KEDFRYAISSTNEIGLLGA-O)$_2$K > KKKHKVRNEVMVHWFGD	B-C domain of E2 (aa 694–712)	Nonstructural protein NS2-3 (aa 1446–1460)	11 239.56	11 283
2	(AEGLTTTWKDYDHNLQ-O)$_2$K / (AEGLTTTWKDYDHNLQ-O)$_2$K > KKKHKVRNEVMVHWFGD	B-C domain of E2 (aa 712–728)	Nonstructural protein NS2-3 (aa 1446–1460)	10 470.37	10 505
3	(TAVSPTTLRTEVVK-O)$_2$K / (TAVSPTTLRTEVVK-O)$_2$K > KKKHKVRNEVMVHWFGD	A domain of E2 (aa 829–842)	Nonstructural protein NS2-3 (aa 1446–1460)	8 909.32	8 935

[a] O stands for 3,6-dioxaoctanoic acid, O_2Oc.
[b] Calculated for the $[MH]^+$ ion.
[c] Determined by MALDI-TOF MS in the linear mode.

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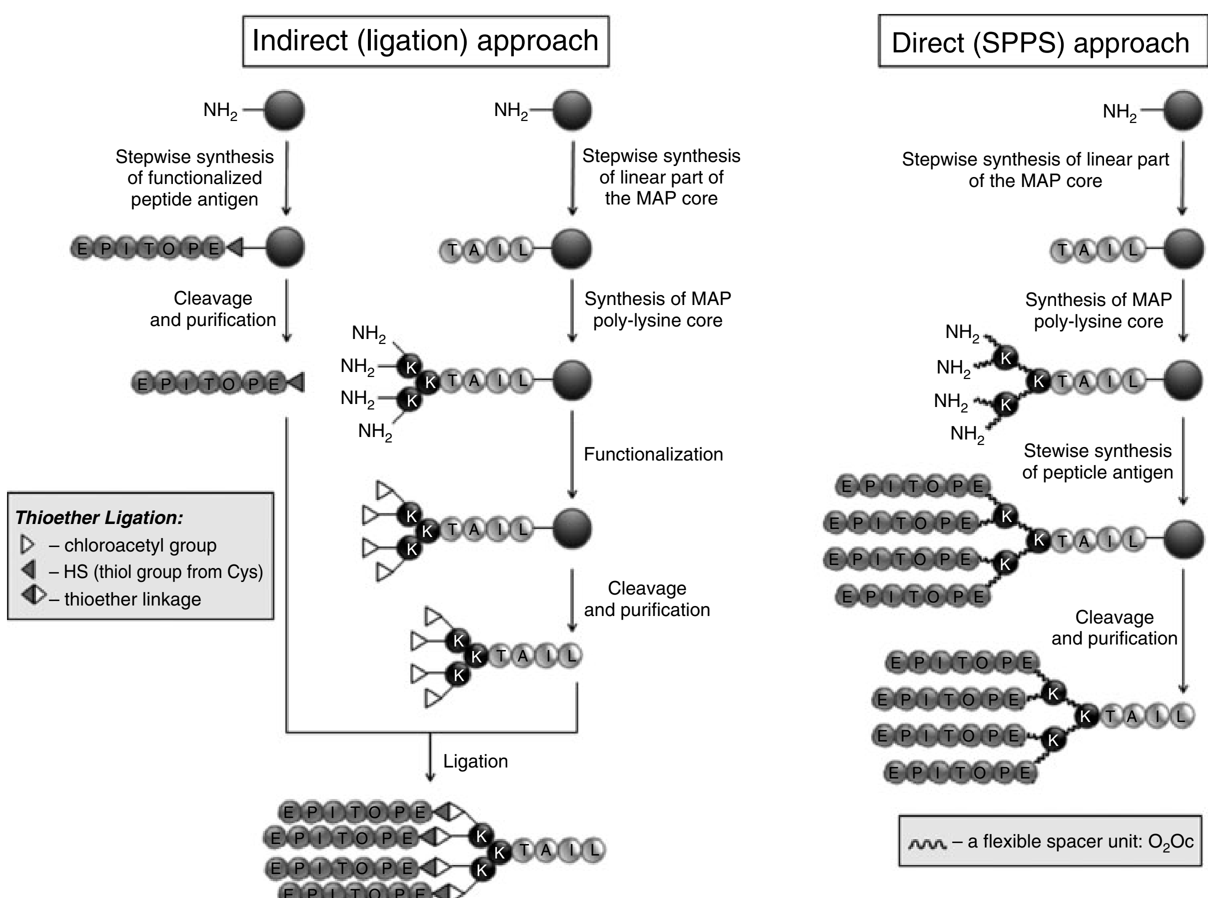

Figure 1. Reaction schemes for the two synthetic approaches (ligation in solution and fully stepwise) to constructs **1** – **3**.

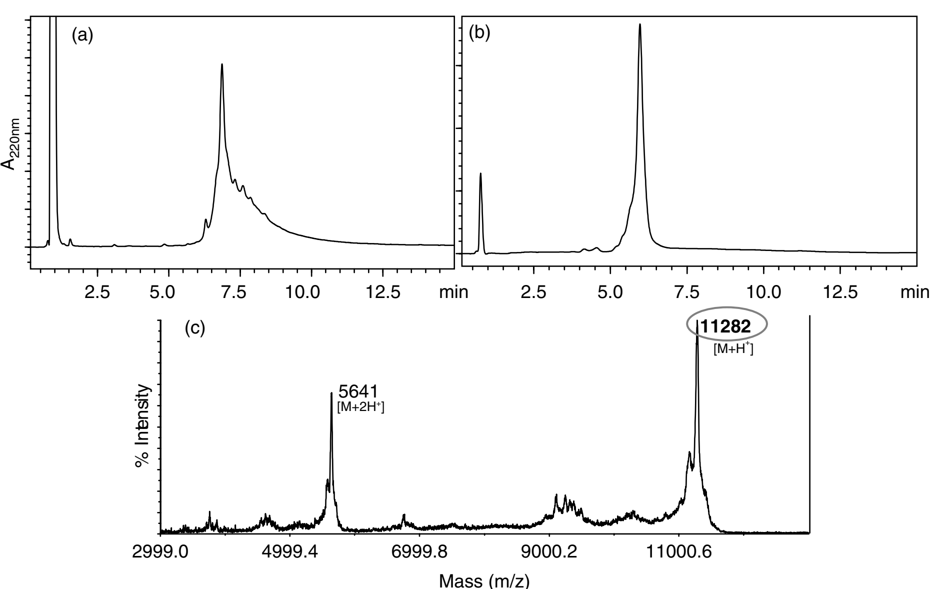

Figure 2. Analytical HPLC characterization of construct **1**: (a) synthetic product, 20–50% linear gradient of eluent B into A over 15 min. Solvent A: 0.045% (v/v) TFA in water, solvent B: 0.036% (v/v) TFA in acetonitrile. (b) Purified product, t_R = 5.5 min (elution conditions as above). (c) MALDI-TOF MS (expected mass, single- and double-charged ions).

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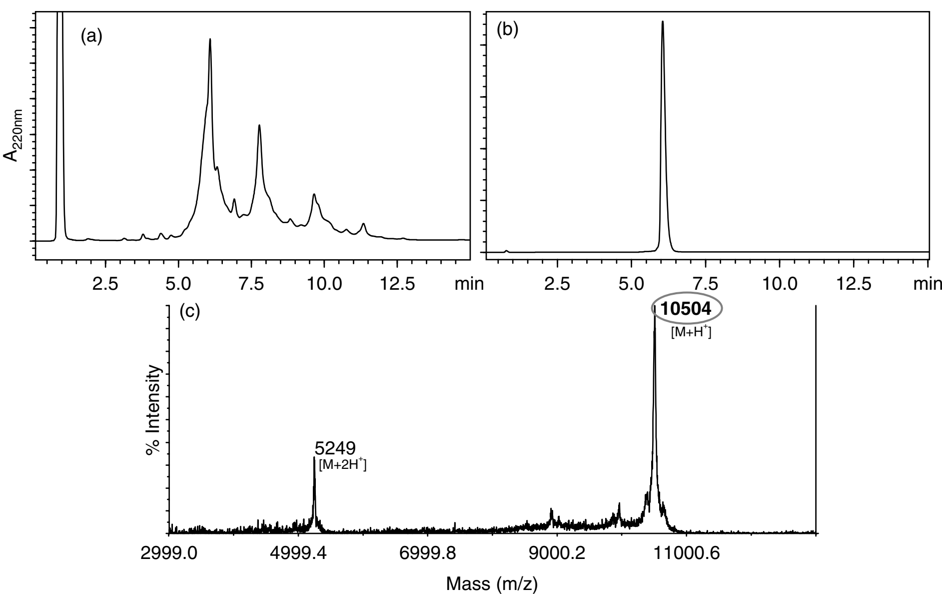

Figure 3. Analytical characterization of construct **2**: (a) synthetic product, 15–50% linear gradient of eluent B into A over 15 min. Solvents and elution conditions are as given in Figure 2. (b) Purified product, $t_R = 5.0$ min. (c) MALDI-TOF MS (expected mass, single- and double-charged ions).

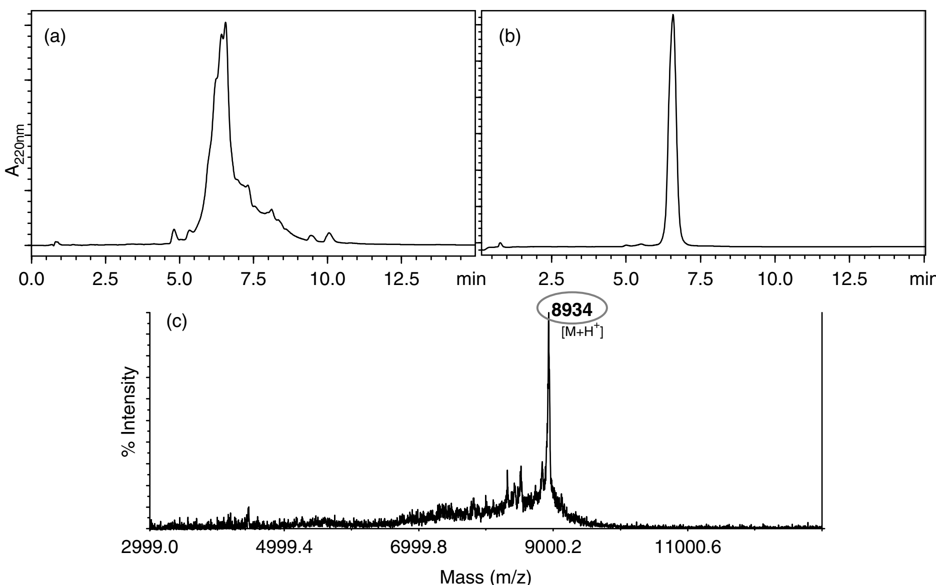

Figure 4. Analytical characterization of construct **3**: (a) synthetic product, 15–40% linear gradient of eluent B into A over 15 min. Solvents and elution conditions are as given in Figure 2 (b) Purified product, $t_R = 6.2$ min. (c) MALDI-TOF MS (expected mass, single- and double-charged ions).

purity by analytical HPLC were pooled and lyophilized. The purified peptides (linear or dendrimeric) were further characterized for identity by MALDI-TOF MS in a Voyager DE-STR instrument (Applied Biosystems) operating in the linear mode and using α-hydroxycinnamic acid matrices.

Immunization

To evaluate the immune response induced by the dendrimeric peptides, four groups of six domestic pigs were placed in four different sections of the CReSA BSL3 animal house. Three groups were inoculated twice, at days 0 and 21, by intramuscular injection of a 1.4-mg dose of constructs **1, 2**, and **3** (Table 1), respectively. Each peptide dose was dissolved in 1 ml of physiological serum and emulsified with an equal volume of Montanide V206 adjuvant (Seppic, Puteaux, France). The fourth group of animals was used as non-vaccinated control. Ten days after the last immunization, all pigs were challenged with a lethal dose (10^5 $TCID_{50}$) of CSFV (strain Margarita) [15]. Clinical signs of disease (fever, anorexia, diarrhea, petechiae, nervous disorder, prostration) were scored daily for the 15 days following challenge infection (d.p.c.). Score values ranged from 0 (no clinical signs) to 6 (major clinical signs). In addition, pigs were bled weekly to monitor the induction of specific neutralizing antibodies.

Assays

Peptide-specific antibodies were detected by indirect ELISA. Neutralizing antibodies were determined in a peroxidase-linked

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neutralization assay (NPLA) [46] and titers were expressed as the reciprocal dilution of serum that neutralized 100 $TCID_{50}$ of strain Margarita in 50% of the culture replicates. Evaluation of γ-interferon (IFN-γ)-producing cells was performed by enzyme-linked immunosorbent spot (ELISPOT) as described [47].

Results and Discussion

Immunogen Design and Synthesis

The general structure of the three CSFV dendrimeric (B_4T) peptide constructions used as immunogens is shown in Table 1. The constructions are designed to display four copies of B-cell epitopes from the E2 protein in a single molecule [construct **1**: $(694-712)_4$, construct **2**: $(712-728)_4$, construct **3**: $(829-842)_4$,], joined to a T-cell epitope from the NS2-3 protein (1446–1460) through a Lys tree [1,8] and also two additional Lys residues defining a putative cleavage site for cathepsin D [4], a protease suggested to be involved during *in vivo* major histocompatibility complex class II antigen processing [48]. Two synthetic approaches to the dendrimeric constructions were used. In line with our previous expertise in MAP synthesis, a convergent approach (Figure 1, left panel) was first chosen for B_4T, based on the chemoselective thioether ligation [8] of (i) a tetravalent peptide reproducing the T-cell epitope, *N*-terminally elongated with two Lys (cathepsin D site) plus three more Lys residues making up the dendrimeric core, of which the two outer residues had their α- and ε-amino groups functionalized as 2-chloroacetyl (ClAc) derivatives; (ii) the three B-cell epitope peptides, acetylated at the *N* terminus and *C*-terminally elongated with a Cys residue. The chemical ligation at pH 7 was monitored by HPLC and MALDI-TOF MS. Several drawbacks were found with this method because the linear peptides were difficult to obtain in desirable amounts and purity, and even when available they were quite insoluble in solvents useful for the conjugation process. The difficulties encountered in these three syntheses are not infrequent [49], and in our case they eventually led to the convergent approach being discarded in favor of stepwise SPPS (Figure 1, right panel). Previous work in our laboratory had demonstrated that the introduction of flexibilizing spacer units (e.g. aminohexanoic acid, Ahx) at each branch of the B_4T dendrimer substantially increased the quality of the end products [49]. In our case, and given the considerable difficulties in solubility observed for the individual B epitopes, we deemed it wise to optimize the global water solubility of the constructs by inserting PEG-like O_2Oc flexible spacer units (instead of Ahx) between the Lys core and the B epitopes. Like PEG, O_2Oc has a dynamic conformation and is well hydrated in aqueous media, which should result in improved water solubility of the resulting PEG-like molecule [50]. Additional synthetic improvements included the use of ChemMatrix solid support [51,52] and double couplings throughout the buildup of the B epitope, deemed advisable in view of the difficulties observed in their assembly as linear peptides for the ligation approach (data not shown). Despite these preventive measures, the synthetic process was not devoid of complications, to judge from the crude products obtained after TFA cleavage (Figures 2(a), 3(a), and 4(a)). Thus, for constructs **1** and **3**, the main peaks observed in the preparative HPLCs were either accompanied by a substantial satellite (crude **1**) or suggested microheterogeneity (crude **3**). For construct **2**, substantial levels of two slower eluting by-products (partially characterized as Fmoc adducts) accompanied the main peak. Difficult end products like these should be expected to be more the norm than the exception in the synthesis of large MAP systems of this type, given the potential synthetic pitfalls posed by multimeric targets of such structural complexity, although analytical details tend to be minimal, if any, in most accounts of MAP synthesis [49]. Nonetheless, even with complex crudes such as those of constructs **1**–**3**, HPLC purification allowed us to satisfactorily obtain homogeneous products (*ca* 90% by analytical HPLC for construct **1**, >90% for constructs **2** and **3**, Figures 2(b), 3(b), and 4(b)). MALDI-TOF MS analysis (Table 1 and Figures 2(c), 3(c), and 4(c)) showed a difference of 25–44 Da between theoretical $(M + H^+)$ and experimental masses. As spectra were acquired in the linear TOF mode, such differences were within expectations (resolution is insufficient for telling apart $[M + H^+]$ from $[M + Na^+]$ or $[M + K^+]$ satellites) and the purified products were thus judged to be satisfactory for the immunization experiments.

Vaccination Trial

The dendrimeric B_4T-type constructs **1**–**3** were tested as vaccine candidates in experimental immunization trials of domestic pigs. The aim of the trials was to characterize the B- and T-cell responses against CSFV in its natural host (pig) and to evaluate the protective response induced by the constructs. Each of the **1**–**3** dendrimers was administered to six animals at a 1.4-mg intramuscular dose, with an equal boost dose and serum conversion controls by ELISA 14 d.p.i. All three immunogens induced, from 14 d.p.i. on, ELISA-detectable humoral responses that for construct **1** (B epitope from the B–C domain) translated into globally higher titers than those for constructs **2** and **3** (Figure 5).

At 36 d.p.i., all the 18 vaccinated animals as well as 6 unvaccinated controls were challenged with a dose of CSFV that is known to have lethal effects [15], and were monitored for clinical signs of the disease during the following 15 days. At 3 d.p.c., the three control animals developed typical clinical signs of CSF, which reached severe enough levels (scores >5) to justify euthanasia between 6 and 13 d.p.c. In contrast, the immunized animals, overall, showed better clinical profiles, particularly the group that was administered with construct **1**, in which pigs 1 and 6 had the lowest clinical scores of the entire trial (Table 2). These two animals had also, at 13 d.p.i., developed neutralizing antibody titers well above the 1 : 50 value that is considered significant in terms of protection against CSFV [46,53–55]. For a few other animals

Table 2. Neutralizing peroxidase-linked assay (NPLA) titers and clinical scores at 13 days after CSFV challenge infection

		NPLA titers		
Dendrimer	Pig[a]	Margarita strain	Alfort strain	Score of clinical signs
1	1	1 : 160	1 : 20	0
	6	1 : 80	1 : 10	1
2	7	0	0	5
	8	0	0	5
	9	0	0	5
	12	1 : 20	1 : 20	2
3	15	1 : 20	0	2
	17	1 : 20	1 : 10	2
Control	21	0	0	6

[a] Vaccinated and non-vaccinated pigs not listed above were humanely euthanized when showing first signs of nervous disorder.

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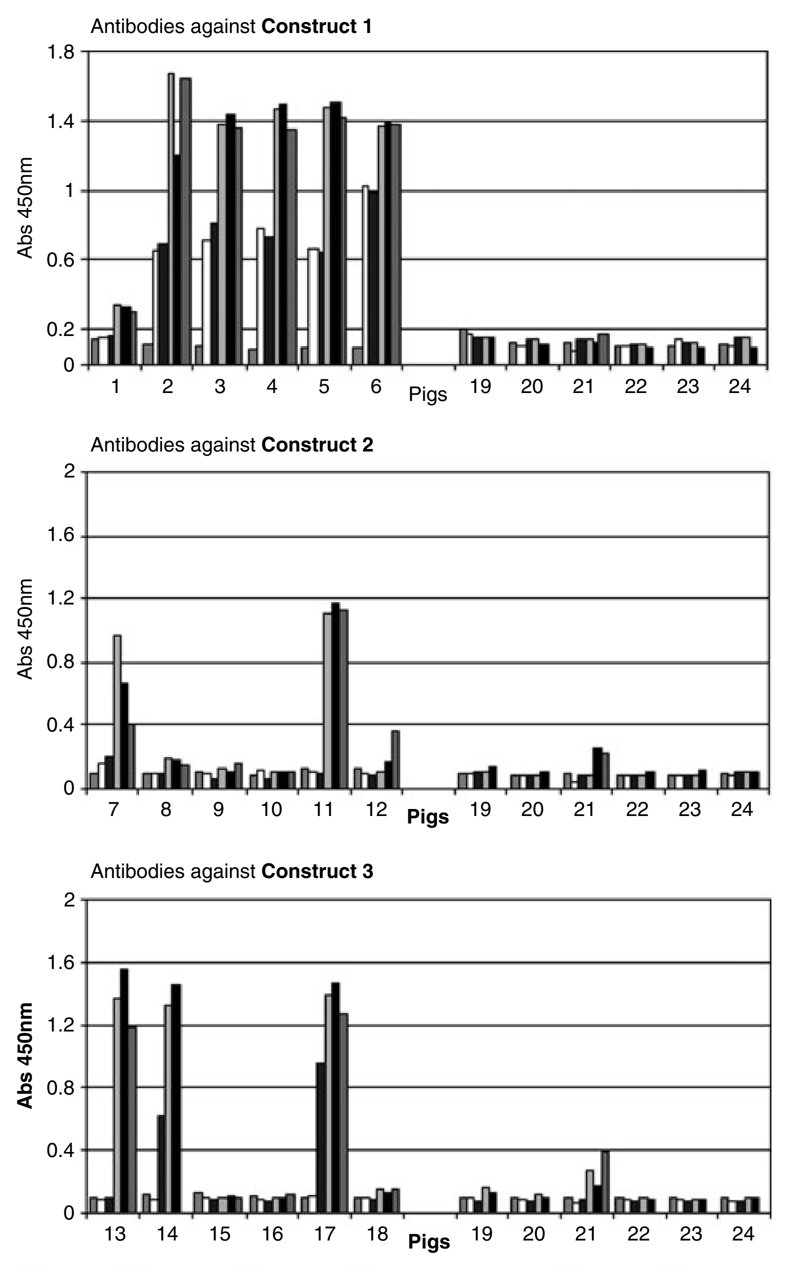

Figure 5. Antibody responses after vaccination with synthetic dendrimeric peptide constructs and challenge with CSFV. Pigs 1–6, 7–12 and 13–18 were vaccinated with constructs **1**, **2** and **3**, respectively; pigs 19–24 were non-vaccinated controls. Absorbance values above 0.2 were considered positive.

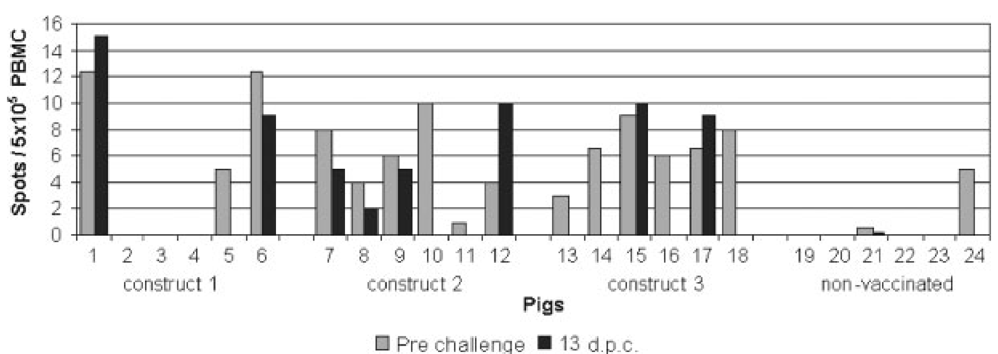

Figure 6. Specific CSFV IFN-γ-producing cells after challenge in vaccinated and non-vaccinated animals (stimulated with CSFV).

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that were vaccinated with peptides **2** and **3** (Figure 5), some neutralization was also observed, though with titers below 1 : 50 (Table 2) that correlated with their worse clinical scores. It must be kept in mind that the specific pathogenicity of CSFV, particularly its immunosuppressive effects, typically ensures a fatal outcome for any infection. Therefore, the finding of substantial neutralizing antibody levels after a lethal dose of CSFV is characteristically interpreted as a telling sign that the animal has successfully built up an effective immune response that may eventually help it survive the infection.

A third observation corroborating the superior immunogenic properties of construct **1** (again on pigs 1 and 6) was provided by ELISPOT analysis, which showed these two animals to have the highest values of IFN-γ-producing cells after CSFV challenge (Figure 6). The induction of specific IFN-γ-secreting cells is known to have a characteristic effect against CSFV infection [15,47], possibly by contributing to an early control of CSFV replication until a neutralizing, eventually protective, antibody response builds up [47]. In any event, it seems clear that, in addition to a globally higher antibody response, dendrimer **1** (by means of its T-cell epitope sequence and/or otherwise) can induce an efficient T-cell response in pigs 1 and 6, which eventually translates into protection against CSFV. IFN-γ-producing cells were also found by ELISPOT in some pigs that were immunized with dendrimer **2** or **3**, but always at much lower levels than that found for animals in the construct **1** group.

In conclusion, on the basis of these preliminary results, construct **1** appears as a promising CSFV vaccine candidate. Its partial (2/6) protecting effect is fully consistent with the neutralizing antibody titers elicited by the two protected animals, and coherent with the induction of anti-peptide antibodies, and of IFN-γ-producing cells even in the absence of neutralizing antibodies before challenge.

Acknowledgements

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