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Vesicular Stomatitis Virus glycoprotein G carrying a tandem dimer of Foot and Mouth Disease Virus antigenic site A can be used as DNA and peptide vaccine for cattle

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ABSTRACT

Effective Foot and Mouth Disease Virus (FMDV) peptide vaccines for cattle have two major constraints: resemblance of one or more of the multiple conformations of the major VP1 antigenic sites to induce neutralizing antibodies, and stimulation of T cells despite the variable bovine-MHC polymorphism. To overcome these limitations, a chimeric antigen was developed, using Vesicular Stomatitis Virus glycoprotein (VSV-G) as carrier protein of an in tandem-dimer of FMDV antigenic site A (ASA), the major epitope on the VP1 capsid protein (aa 139–149, FMDV-C3 serotype). The G-ASA construct was expressed in the Baculovirus system to produce a recombinant protein (DEL BAC) (cloned in pCDNA 3.1 plasmid) (Invitrogen Corporation, Carlsbad, CA) and was also prepared as a DNA vaccine (pC DEL). Calves vaccinated with both immunogens elicited antibodies that recognized the ASA in whole virion and were able to neutralize FMDV infectivity *in vitro*. After two vaccine doses, DEL BAC induced serum neutralizing titers compatible with an "expected percentage of protection" above 90%. Plasmid pC DEL stimulated FMDV specific humoral responses earlier than DEL BAC, though IgG1 to IgG2 ratios were lower than those induced by both DEL BAC and inactivated FMDV-C3 after the second dose. DEL BAC induced FMDV-specific secretion of IFN-γ in peripheral blood mononuclear cells of outbred cattle immunized with commercial FMDV vaccine, suggesting its capacity to recall anamnestic responses mediated by functional T cell epitopes. The results show that exposing FMDV-VP1 major neutralizing antigenic site in the context of N-terminal sequences of the VSV G protein can overcome the immunological limitations of FMDV-VP1 peptides as effective protein and DNA vaccines for cattle.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most economically and socially devastating diseases affecting animal agriculture throughout the world (Rodriguez and Gay, 2011). FMD is highly contagious and affects wild and domestic cloven-hoofed animals. The causative agent is Foot-and-Mouth Disease Virus (FMDV) which belongs to the genus *Aphthovirus* in the family *Picornaviridae* (Grubman and Baxt, 2004). Due to the devastating economical consequences that could arise from an FMD outbreak, this disease is considered a potential bioterrorism weapon (Hietela and Ardans, 2003).

Vaccination against FMDV is a major strategy to control the disease during an outbreak and in endemic areas. Current FMDV vaccines are serotype-specific and consist of inactivated virus formulated in oil or aluminum hydroxide adjuvants (Doel, 2003). Although these vaccines can induce strong humoral protective immunity, there are major drawbacks associated with their use, namely, the requirement of propagating virulent virus in containment facilities (Barteling and Vreeswijk, 1991) and the associated risk of escape from manufacturing sites (Strohmaier et al., 1982), the limited shelf life and the relatively short-term nature of protection (Grubman and Baxt, 2004; Rodriguez and Gay, 2011).

Much effort has been made to develop alternative vaccines that are both efficient and safe based on either recombinant proteins, peptides, replicating vectors or plasmid DNA (Grubman, 2005). DNA vaccines are particularly attractive for field application, mainly because pure DNA is stable and immune-stimulating, does not need a cold chain or adjuvant (Carvalho et al., 2009). Plasmids encoding large fragments of an FMDV genome-like P1-2A3C3D construct (Cedillo-Barrón et al., 2001; Li et al., 2006), VP1 (Li et al., 2007; Park et al., 2006) or FMDV B and T-cell epitopes (Borrego et al., 2006; Cedillo-Barrón et al., 2003; Dory et al.,

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2009a,b; Fan et al., 2007) have been tested in mice and swine. Considerable progress has been made to enhance DNA vaccination efficacy but at least two to three doses of plasmids are needed to achieve immunity, even in mouse models (Borrego et al., 2006).

Attempts to develop peptide FMD vaccines have been based on the B-cell target antigenic site A (ASA), spanning amino acids 139 through 149 of the capsid protein VP1 (G-H loop). ASA is exposed on the surface of the viral particle (Acharya et al., 1989; Bittle et al., 1982; DiMarchi et al., 1986; Pfaff et al., 1982), and neutralizing antibodies are mostly induced and targeted to the ASA of VP1 capsid protein. This sequence contains an arginine–glycine–aspartic acid (RGD) motif, highly conserved among the different FMDV serotypes and involved in the integrin-mediated attachment of FMDV virions to the cellular membrane (Baxt and Becker, 1990; Fox et al., 1989; Mason et al., 1994).

Crystalographic data demonstrated that the serotype C3-ASA sequence in solution has no unique but rather multiple conformations, each of which displays a particular immunogenicity (Harrison, 1989; Mateu et al., 1990). In fact, the limited capacity of linear synthetic peptides spanning the GH loop of VP1 to induce an effective antiviral response in cattle (Taboga et al., 1997) could be at least partially attributed to their inability to mimic one or more of the multiple conformations of the ASA present in the surface of the virion.

Efforts to improve and broaden VP1 G-H loop peptide immunogenicity entailed the association of ASA sequences to T helper (Th) epitopes able to amplify the antiviral response. A dendrimeric peptide that integrates ASA repeats, and Th epitopes enhanced the effectiveness of presentation to the porcine immune system of viral antigenic sites capable of stimulating B- and T-cell-specific lymphocytes (Cubillos et al., 2008). Earlier studies demonstrated that the inclusion of consensus residues into the hypervariable positions (“UBI peptide”) resulted in high level of protection in swine following FMDV 01 Taiwan challenge (Wang et al., 2002) but was unable to protect cattle at 3 weeks post-vaccination (Rodriguez et al., 2003). Failures to induce protection in cattle have been related to lower activation of naïve T cells due to the highly variable Bovine Major Histocompatibility Complex (MHC/BoLA) (Baxter et al., 2009; Leach et al., 2010).

In this study, a chimeric construct containing the first 193 aa of the ectodomain of the Vesicular Stomatitis Virus glycoprotein (VSV-G) harboring an in tandem dimer insertion of the FMDV-C3 ASA sequence was evaluated as peptide and DNA vaccine in cattle. ASA tandem dimer was inserted within the non-linear epitope IV of the VSV N-terminal region, a major disulfide bond-stabilized loop exposed in the surface of the protein (Grigera et al., 1992). Conformation of ASA in this particular display was found to mimic one or several of the alternative conformations of ASA in the virions, critical to induce infection blocking antibodies in mice (Grigera et al., 1996). This study shows that the association of ASA to N-terminal sequences of the VSV glycoprotein, a powerful inducer of neutralizing antibodies and protection in cattle (Glass, 2004; Grigera et al., 1996), can provide appropriate conformation and circumvent poor responses induced by ASA peptides themselves.

2. Materials and methods

2.1. Cells and viruses

Growth of COS and BHK cells in monolayer cultures have been described previously (Capozzo et al., 1997; Grigera et al., 1996, 1992). Conditions for infection, cloning, and harvesting of recombinant baculovirus grown in *Spodoptera frugiperda* (Sf9) cells were carried out as described before (Grigera et al., 1996). Purified VSV-G New Jersey (NJ) serotype Glycoprotein was kindly provided by Grigera et al. (1992).

FMDV-C3/Arg/85 whole particles used as target antigen for the enzyme-linked immunosorbent assay (ELISA), functional or vaccination experiments were purified from a vaccine Manufacturer’s inactivated antigen produced by the Frenkel or BHK method. Whole particles (140 S) were purified by a standard sucrose density gradient centrifugation method and selected fractions were ultra-centrifuged and pellet suspended in NET (sodium–EDTA–Tris buffer: 0.1 M NaCl, 0.004 M EDTA, 0.5 M Tris–HCl, pH 8) buffer to 1 μg/ml (Grubman et al., 1985).

2.2. Polyclonal and monoclonal antibodies

Anti-FMDV serotype C3/Arg/85 and anti-VSV-G NJ serotype polyclonal sera were prepared in rabbits (Grigera et al., 1996, 1992). Preparation and characterization of monoclonal antibody (MAb) directed to epitope IV of the VSV-NJ G protein (MAb 9) as well as Mabs used in Liquid Phase Blocking ELISA (LPBE) (Capozzo et al., 1997; Robiolo et al., 2010), have been described in detail elsewhere (Bricker et al., 1987; Freer et al., 1994). MAb against serotype C3-VP1 (MAb 43) used for Western blot was produced in our institute (Seki et al., 2009). Anti-bovine isotypes IgGl and IgG2 MAbs were purchased to VMRD (Veterinary Medical Research & Development, Pullman, WA) and Sigma (St. Louis, MO), respectively.

2.3. Chimeric constructs

Detailed construction of pC G (from VSV-NJ) and the construct containing a tandem dimer of the ASA endecapeptide ARRGDLAHLAT of the VP1 protein of the FMDV C3 serotype (comprises sequences of more than ten C3 isolates) inserted between G-gene codons 160 and 161 of truncated VSV-G (aa 193) have already been published (Grigera et al., 1996). This chimeric construct was subcloned in pC DNA 3.1 (Invitrogen, Gaithersburg, MD) between Eco RI y XbaI sites to produce pC DEL plasmid. Plasmids were purified using plasmid Mega kit (Qiagen, Hilden, Germany) columns according to the Manufacturer’s instructions, and prepared in sterile PBS to a final concentration of 1.5 mg/ml for cattle vaccination.

DEL BAC was produced in Sf21 cells grown in spinner flasks, infected (in serum-free medium) at a high MOI (5 PFU/cell) with recombinant BV (Grigera et al., 1996) and harvested 2–3 days later. Centrifuged-sonicated pellet was kept as the antigen source, from which chimeric proteins were purified by elution from anti-VSV G antibody–Sepharose columns as described previously (Grigera et al., 1996). DEL BAC was quantified by using a commercial protein assay method (Micro BCA, Pierce, Rockford, USA). Production yielded 32 ± 0.98 μg per 1×10^6 cells.

2.4. Transient expression in COS cells

Experimental conditions for expression of the plasmids in COS cells were identical to those already described (Grigera et al., 1992). Briefly, COS monolayers were cultured to 80% confluence and lipotransfected with 2 μg of plasmid pC DEL, pC G and pC DNA for 3 h using lipofectamine-plus transfection reagent (Invitrogen) according to the Manufacturer’s instructions. The cells were further incubated for another 6 h in the presence of medium containing 10% fetal calf serum and then labeled with [^{35}S] methionine (150 mCi/ml) for 1 h in methionine-free medium. After the labeling period, cells were washed with PBS, lysed in radio-immunoprecipitation assay buffer, and processed for immuno-precipitation (Grigera et al., 1992).

Immunofluorescent staining of transfected cells was performed on fixed COS cells following standard procedures and revealing the

expressed proteins with rabbit sera against VSV-G (1:500) followed by anti-rabbit FITC antibody (1:200; Sigma).

2.5. Immunization experiments

Animal procedures were performed according to standard guidelines of humane care and treatment of animals from the Animal Welfare Act, supervised by the local animal welfare committee. Four groups of 5 Hereford calves of about 5 months of age, sero-negative to FMDV by Liquid Phase Blocking ELISA (Section 2.9.3) and 3ABC ELISA (Robiolo et al., 2006) and also negative to VSV (by ELISA) were injected intramuscularly in the neck with 150 μg of pC DEL in 1 ml of PBS; 30 μg of DEL BAC protein in water-in-oil Marcol Montanide adjuvant (Seppic, Fairfield, NJ) 3 ml per dose, or 3 ml of Monovalent BEI-inactivated FMDV-C3 vaccine in the same oil adjuvant containing 5 μg per dose of 140S purified particles produced by the Frenkel method. Control groups (placebo) received 150 μg of pC DNA empty plasmid (2 animals) or 3 ml of PBS-oil Adjuvant (3 animals). Animals were immunized twice, at day 0 and 30 post vaccination (dpv). Blood samples were taken from the caudal vein at regular intervals after the immunizations (0, 15, 30, 45, 60 and 90 dpv). Two animals per group were followed up to 160 dpv.

2.6. Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using the discontinuous buffer system and 12% acrylamide gels. Equal amounts (50 ng) of FMDV 140S particles, purified VSV-G (Grigera et al., 1996) or DEL BAC were transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA) and blocked with 5% (wt/vol) non-fat dry milk in PBS-0.1% Tween 20 (PBS-T). Membranes were individually incubated for 1 h with the corresponding antisera or MAb, at pre-established dilutions, followed by 1 h incubation with the appropriate horseradish conjugate (1:5000, Amersham Biosciences, Piscataway, NJ). Bound conjugates were detected with ECL (chemiluminscence substrate) (Amersham Bio-sciences, Piscataway, NJ) Plus detection system (GE Healthcare Technologies, Waukesha, WI) or by 3,3′-Diaminobenzidine (DAB, Sigma).

For the immune-precipitation experiments, released immune-precipitated particles (50 ng FMDVi per treatment) incubated with pools of test sera (diluted 1:50) and protein–Sepharose were treated with reducing loading buffer, blotted to nitrocellulose membrane and assessed with MAb 43 against FMDV-C3 VP1 (1:100), revealed with anti-mouse Ig conjugate (Jackson Immunoresearch Laboratories, West Grove, PA) using chemiluminescence, as stated above.

2.7. Titration of neutralizing antibodies

Neutralizing antibodies present in serum of vaccinated cattle were titrated by plaque reduction assays of FMDV-C3 plated on BHK cell monolayers (OIE, 2008). Briefly, each serum was heated at 56 °C for 30 min to inactivate complement. A standard virus neutralization test was performed in 96-well plates by incubating twofold serial dilutions of each serum with 100 CID50 of FMDV-C3 for 30 min at 37 °C. Remaining viral activity was determined in 96-well plates containing monolayers of BHK-21 cells. Serum VNT (virus-neutralizing titer) titers were determined as the log10 serum dilution neutralizing 50% of the virus inoculum. Sera from calves inoculated with inactivated FMDV (FMDVi) were used as positive controls; sera from pre-immune and placebo-immunized animals served as negative controls. The table of correlation used in this study to calculate the expected percentage of protection (EPP) was the one currently applied by the Argentinean Sanitary Authority (Maradei et al., 2008; Robiolo et al., 2010).

2.8. ELISAs

2.8.1. ELISA to native and denatured virus

Anti-FMDV rabbit serum was used as capture antibody in a 1:5000 dilution in carbonate fixing buffer; this procedure was followed by washing with PBS, blocking with bovine serum albumin (BSA)-containing buffer, and incubating with appropriate dilutions of FMDV whole virions as the source of native viral antigen. When denatured antigen was used, FMDV preparations were treated with 3% SDS for 3 min at 100 °C (Grigera et al., 1996). Dilutions of FMDV-immune and control bovine sera were then added. The mixtures were incubated overnight at 4 °C, washed, and anti-mouse immunoglobulin–peroxidase conjugates (Jackson Immunoresearch) were added in 1:5000 dilution. The substrate used was ABTS/H_2O_2 (Sigma). After 15 min incubation, the reaction was stopped by the addition of 100 μl of 2% NaFl and absorbance was measured at 450 nm.

2.8.2. IgG subtype ELISA

Titration of bovine IgG1 and IgG2 was performed as described by Capozzo et al., 1997. Anti-FMDV isotype titers were defined as the reciprocal $\log_{10}$ of the highest dilution of the test sera able to give an optical density (OD) equal to 1.00 (OD = l).

2.8.3. Liquid-phase blocking sandwich ELISA (LPBE)

Details of LPBE performance, optimization, conditions and application have already been published (Capozzo et al., 1997; Periolo et al., 1993; Robiolo et al., 1995, 2010). Antibody titers were expressed as the reciprocal $\log_{10}$ of serum dilutions giving the 50% of the absorbance recorded in the virus control wells, without serum. EPP was calculated as stated in the National Law (SENASA, 2010).

2.10. Measurement of IFN-γ

Protocol to quantify IFN-γ from plasma of cultured blood has been described by Bucafusco et al. (2010), adapted from Palmer et al. (2006). Briefly, aliquots of 1.5 ml of whole blood were incubated for 24 h at 37 °C and 5% CO_2 with Pokeweed Mitogen (PWM) (Sigma, 10 μg/ml), purified inactivated FMDV-C3 (10 μg/ml), DEL BAC protein (10 μg/ml), a SF21-Baculovirus lysate expressing an unrelated protein (1×10^4 PFU per treatment, equivalent to the amount of particles present in DEL BAC lysate prior to column purification) or PBS as control. Stimulated plasma samples were stored at −20 °C until used. IFN-γ was quantified using a commercial ELISA (Bovigam®, Prionics, Zürich) according to the Manufacturer's instructions. Each trial included a standard curve of recombinant bovine IFN-γ (Serotec Ltd., Oxford, UK) ranging from 50 ng/ml to 195 pg/ml.

2.11. Modeling of G-ASA structure

VSV-NJ G sequence spanning aa 17–215 was modeled using VSV-G Indiana sequence as template. VSV-G Indiana protein has been crystallized: PDB ID: 2J6JA (Roche et al., 2007). Based on the template, aa sequence identity of VSV-G chimera was 61.7%. Structural identity of both sequences was acceptable. Modeling was performed in SWISS-MODEL Workspace server (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003) and images were generated with Jmol (http://www.jmol.org). ASA in the VSV-G chimera was compared to ASA in the native FMDV viral particles. A model of 12S unit of FMDV is shown in Fig. 4 (PDB ID

1FOD), from the crystallographic structure obtained by Logan et al. (1993).

2.12. Statistical analysis

Antibody titers measured at different time points in vaccinated and control animals were compared using *t* test or Mann–Whitney *U* test (if normality failed). Titers between two groups were compared Differences with *p* values over 0.05 were considered significant. Statistical analysis was performed using MedCalc 11.5.1 (Systat software).

3. Results

3.1. Expression of VSV/G-ASA chimera from pC DEL vector

The capacity of the plasmids to express the cloned constructs from the CMV promoter was evaluated in a transfection assay using COS cells. Plasmids pC DEL, pCG and empty backbone pC DNA plasmids were independently transfected as controls. Recombinant protein expression was evaluated by immunoprecipitation with both anti-G (Fig. 1A, lanes 1–3) and anti-FMDV rabbit polyclonal sera (lanes 4–6). Fig. 1A shows that anti-VSV G serum reacted with the G-ASA chimeric protein expressed from pCDEL (lane 1) and with VSV-G expressed by pCG (lane 2) while anti-FMDV polyclonal serum reacted only with the ASA containing construct expressed by pCDEL, as expected (lane 4). The presence of double bands correspond to differential glycosylation patterns as already described (Grigera et al., 1996, 1992).

Cellular localization of the proteins expressed by pC G and pC DEL was studied by immunofluorescence staining. Transfected COS cells revealed the perinuclear location of the chimeric protein expressed from pCDEL, which was retained in the cytoplasm, probably along the endoplasmic-reticulum due to the shape of the image (Fig. 1B). VSV-G expressing cells showed intense labeling of the protein associated to the plasma membrane, as previously described (Grigera et al., 2000; Katz et al., 1977).

3.2. T cell epitopes within DEL-BAC recall FMDV T-cell anamnestic responses

The functionality of T-cell epitopes within the chimera was assessed on peripheral blood cells from FMDV-vaccinated outbred cattle, sero-negative for VSV, by measuring their capacity to recall anamnestic T-cell responses. Blood samples from naïve and vaccinated cattle, immunized with multiple doses of tetravalent commercial vaccine, were stimulated *ex vivo* with the Baculovirus-expressed G-ASA protein DEL BAC, inactivated FMDV-C3 (FMDVi), an unrelated recombinant Baculovirus lysate or Pokeweed Mitogen (PWM, positive control). T-cell responses were determined by quantifying secreted IFN-γ by ELISA. Results are shown in Table 1. Cells from vaccinated animals induced high IFN-γ responses when stimulated with FMDVi. Naïve animals only reacted to PWM. DEL BAC induced production of IFN-γ in those cells obtained from FMDV-vaccinated animals, while no responses were measured in naive calves. Responses to the unrelated Baculovirus lysate were negligible. These results suggest that DEL BAC can recall anamnestic responses mediated by functional T-cell epitopes.

3.3. Chimeric constructs induce strong humoral immune responses in cattle

Four independent groups of five FMDV and VSV-seronegative calves each, were immunized with either two doses of 30 μg of DEL-BAC, 5 μg of FMDV-C3 (both antigens formulated in oil adjuvant), 150 μg of pC DEL in saline buffer or placebo (pC DNA and PBS-oil adjuvant). Serum samples were taken at the beginning of the experiment and every 15 days post vaccination, and specific

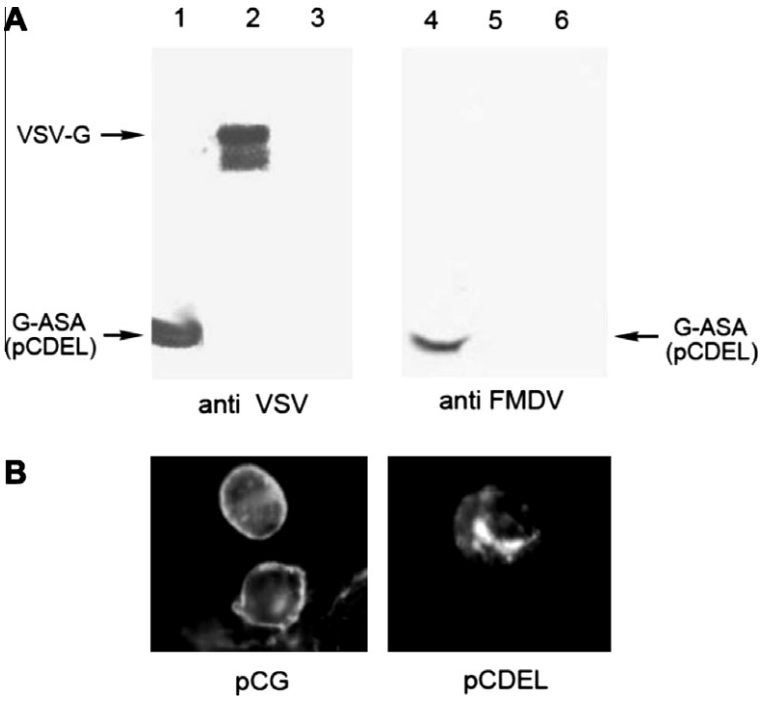

Fig. 1. Expression of the chimeric protein from pC DEL. (A) Immunoprecipitation analysis of COS cells transfected with pC DEL (lanes 1 and 4), pC G (lanes 2 and 5) or pC DNA empty plasmid (lanes 3 and 6), with rabbit anti-VSV-G or anti-FMDV polyclonal sera, as indicated. Positions of the proteins are indicated by arrows. (B) pC DEL and pC G transfected COS cells were stained by immunofluorescence with anti-VSV-G rabbit sera (diluted 1:100) followed by anti-rabbit FITC conjugate, to reveal the location of the recombinant protein within the cells.

Table 1
IFN-γ concentrations (in pg/ml) measured by Bovigam®. Aliquots of whole-blood samples (1.5 ml) obtained from naïve outbred cattle or FMDVi multi-vaccinated calves (that had received more than 3 commercial vaccine doses) were incubated overnight at 37 °C with different antigens as indicated. IFN-γ concentration was measured from stimulated plasma using BOVIGAM®. Upper and lower detection limits were 25,000.00 and 195.00 and pg/ml of IFN-γ, respectively.

	Bovine id	PWM[a]	FMDVi[b]	DEL BAC[c,*]	BAC[d]	NIL[e]
FMDV Vaccinated	33	20,914.95	4563.45	<195.00	<195.00	<195.00
	47	22,454.85	8233.57	237.91	<195.00	<195.00
	50	1418.60	366.20	<195.00	<195.00	<195.00
	30	>25,000.00	>25,000.00	578.80	340.00	<195.00
	641	21,208.82	237.91	555.29	<195.00	<195.00
	10	21,996.41	9465.58	990.25	<195.00	<195.00
	605	>25,000.00	>25,000.00	766.88	<195.00	<195.00
	108v	19,427.35	3429.10	435,05	<195.00	<195.00
	62	21,830.30	4372.85	409.35	<195.00	<195.00
Naive	128	21,208.83	<195.00	<195.00	<195.00	<195.00
	146	19,512.60	<195.00	<195.00	<195.00	<195.00

Treatments per sample (1.5 ml of blood):
[a] PWD, pockeweed mitogen (10 μg/ml) (PWM).
[b] FMDVi, 10 μg/ml of purified-inactivated C3 virus.
[c] Ten microgram pre milliliter of DEL BAC protein.
[d] BAC, a non-related Baculovirus Lysate (1×10^4 PFU).
[e] NIL, PBS, negative control.
[*] For FMDV vaccinated animals, differences between DEL BAC and NIL or BAC treatments were significant ($p > 0.05$).

humoral responses induced by the recombinant constructs were evaluated.

Protection against FMDV requires high levels of specific and neutralizing antibodies. Measurement of anti-FMDV antibodies levels by virus-neutralization tests and also by LPBE are a validated indirect way to estimate the protective potential of FMDVi vaccines as EPP, without the need of infecting animals. Neutralizing and LPBE titers have been correlated to *in vivo* protection against generalized foot infection for particular viruses from defined serotypes (Maradei et al., 2008; Robiolo et al., 1995; SENASA, 2010).

DEL BAC and pC DEL, reached neutralizing titers that correspond to an EPP of 92.83 ± 4.35% and 82.41 ± 12.05, respectively, at 90 days post vaccination (dpv). The inactivated vaccine gave a mean EPP of 99.82 ± 0.17. Fig. 2A shows the kinetics of anti-FMDV C3 neutralizing titers obtained from sera of immunized calves. Animals vaccinated with either pC DEL or DEL BAC elicited neutralizing titers that were significantly higher than those in the negative control groups ($p < 0.05$) at all time points. One dose of plasmid pC DEL induced higher neutralizing antibodies titers (EPP = 78.14 ± 4.38) than its Baculovirus-expressed counterpart at 15 dpv. At this early time point, neutralizing titers induced by pCDEL were not significantly different ($p > 0.05$) from to those elicited by the inactivated vaccine (Fig. 2A). At later time points, animals vaccinated with FMDVi elicited the highest neutralizing titers, 10 times over the other groups at 90 dpv.

Anti-FMDV serum titers were also determined by LPBE (Robiolo et al., 1995, 2010). Fig. 2B shows that the kinetics of the levels specific antibodies by LPBE follows a similar profile as the neutralizing assessment. pC DEL vaccinated animals elicited LPBE titers within the same level compared to FMDVi-immunized animals. Serum titers from DEL BAC vaccinated animals did not differ from negative controls at 30 dpv, while LPBE titers from pC DEL vaccinated cattle were significantly higher than negative controls at 15, 30 and 45 dpv ($p < 0.05$). LPBE titers were similar in pcDEL and DEL BAC vaccinated animals after 60 dpv. At that time point, 3 out of 5 animals inoculated with DEL BAC showed titers above 1.75 that remained among the same levels up to at least 160 dpv (data not shown). FMDVi vaccinated animals reached higher LPBE titers than the other groups.

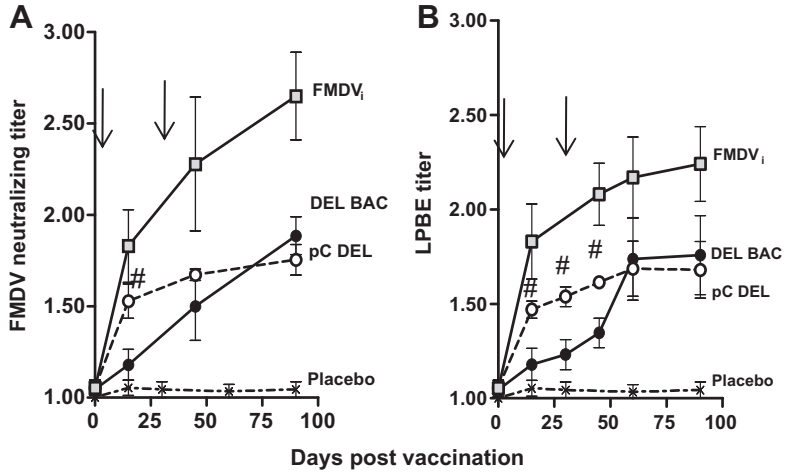

Fig. 2. Anti-FMDV specific antibodies. Graphs showing anti-FMDV neutralizing antibodies (A) and Liquid Phase Blocking ELISA (LPBE) titers (B) at the beginning of the experiment and after each of the two immunizations (indicated with arrows) of plasmid pC DEL (open circles), protein DEL BAC (black filled squares), inactivated FMDV (FMDVi; grey squares) or placebo (dotted line, filled circles). Mean titers of each animal group at the different time points ±SD are shown. $^{\#}p < 0.05$ compared to pre-immune values.

Table 2
Isotype immune responses. Mean anti-FMDV IgG1 and IgG2 antibodies titers ±SD and IgG1 to IgG2 ratio were determined for each group of vaccinated calves after the first injection (15 and 30 dpv) and after the booster (45, 60 and 90 dpv).

Vaccine	DPV	IgG1 titer	IgG2 titer	IgG1/IgG2
pC DEL	15	1.62 ± 0.32	1.54 ± 0.42	1.05
	30	1.60 ± 0.43	1.72 ± 0.41	0.93
	45	1.64 ± 0.14	2.08 ± 0.16	0.78
	60	1.63 ± 0.18	2.20 ± 0.17	0.74
DEL BAC	15	ND	ND	ND*
	45	1.54 ± 0.38	1.35 ± 0.31	1.14
	60	1.70 ± 0.36	1.43 ± 0.28	1.19
	90	1.80 ± 0.37	1.56 ± 0.29	1.15
$FMDV_i$	15	ND	ND	ND*
	45	2.30 ± 0.40	1.76 ± 0.18	1.31
	90	2.20 ± 0.23	1.48 ± 0.19	1.48

* ND, not determined.

FMDV responses biased towards the IgG1 isotype have been related to protection in vaccinated cattle challenged with infectious FMDV, even in the presence of low LPBE titers (Capozzo et al., 1997; Mulcahy et al., 1990). Table 2 shows anti-FMDV IgG1 and IgG2 isotypes levels in sera from vaccinated cattle (Table 2). As expected, animals immunized with inactivated Frenkel-produced FMDVi-C3 vaccine elicited higher IgG1 than IgG2 anti-FMDV-C3 titers at all time points. The IgG1/IgG2 ratio was also higher than in animals vaccinated with DEL BAC up to 90 dpv while calves vaccinated with pC DEL showed equivalent serum titers for both isotypes (IgG1/IgG2 = 1.05) at 15 dpv, but the response biased towards IgG2 after the second vaccine dose.

3.4. Sera from bovine immunized with VSV/G-ASA constructs recognized the native ASA within whole FMDV particles

We have previously shown that sera from DEL BAC-immunized mice reacted poorly with denatured VP1 in Western blots (Grigera et al., 1996) but efficiently with whole particles in an ELISA. We suggested that this differential reactivity was related to the ability of G-ASA chimeras to raise antibodies directed to one or several of ASA conformations in the native virus. Therefore, we tested the reactivity of sera from calves vaccinated with DEL BAC and pC DEL against native and denatured viral particles by ELISA (Fig. 3A).

Reactivity measured by ELISA revealed that sera from calves immunized with pC DEL and DEL BAC were less reactive to the denatured virus than to its native counterparts, while sera from animals immunized with inactivated FMDV (Fig. 3A), which have antibodies to several non conformational epitopes, showed no measurable differential reactivity between native and denatured virus. These results are in agreement with our previous findings using sera from DEL BAC and FMDVi immunized mice (Grigera et al., 1996).

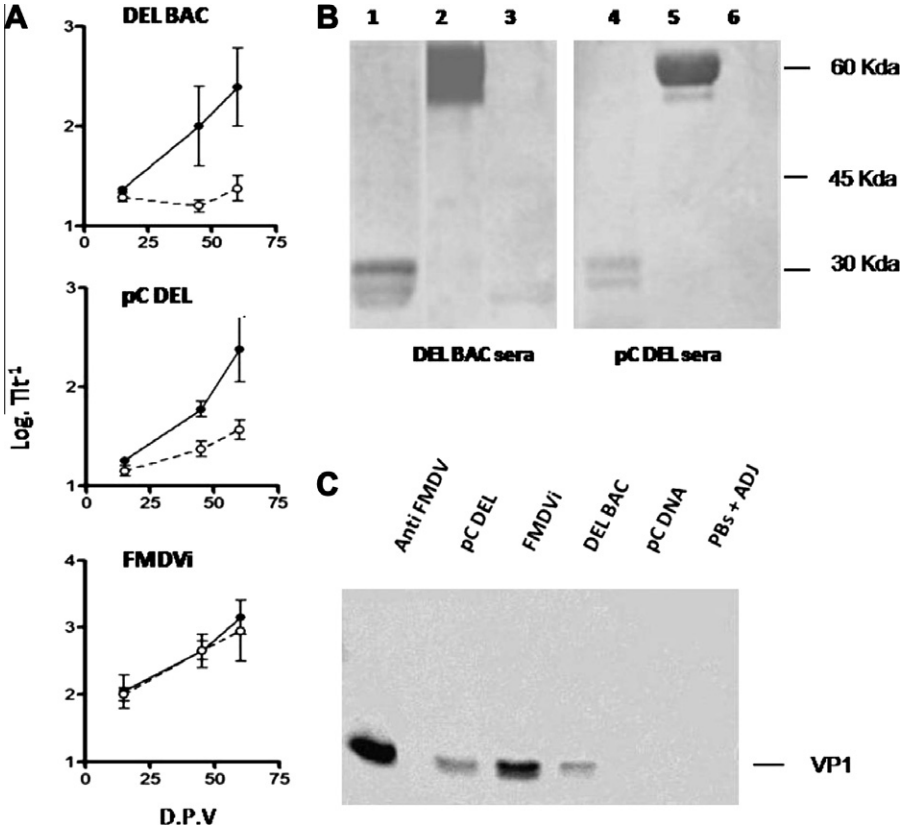

Fig. 3. Reactivity of sera from G-ASA vaccinated cattle to native or denatured FMDV. Comparative binding of antibodies in anti-DEL BAC, pC DEL and anti-FMDV serum to native and SDS-denatured FMDV as determined by ELISA (A), Western blot (B) and immunoprecipitation analysis (C). (A) Sera from pC DEL, DEL BAC and FMDVi vaccinated animals (as indicated) were individually titered against purified native or denatured FMDV particles (filled and open circles, respectively), at days 15, 45 and 60 days post vaccination. Mean serum titers ±SD are depicted. (B) DEL BAC (lanes 1 and 4), VSV-NJ G (lanes 2 and 5) and FMDV particles (lanes 3 and 6) were separated by SDS–PAGE (12% gel), electro blotted onto nitrocellulose paper and incubated with anti-DEL BAC or pC DEL pooled sera (diluted 1:50), as indicated. Reactivity with the respective antibodies was monitored by anti-bovine immunoglobulin G conjugated with peroxidase in the chemiluminescence signal development system. Molecular weight markers are indicated on the right. (C) Purified FMDV 140S native particles were incubated with a 1:50 dilution of rabbit anti-FMDV-C3 polyclonal sera (anti-FMDV) and pooled sera from pC DEL, DEL BAC, FMDVi, pC DNA and PBS/Adjuvant vaccinated animals, as indicated. Immune-precipitated particles were solved in SDS–PAGE and revealed with a MAb anti-FMDV-C3 VP1 by Western blot.

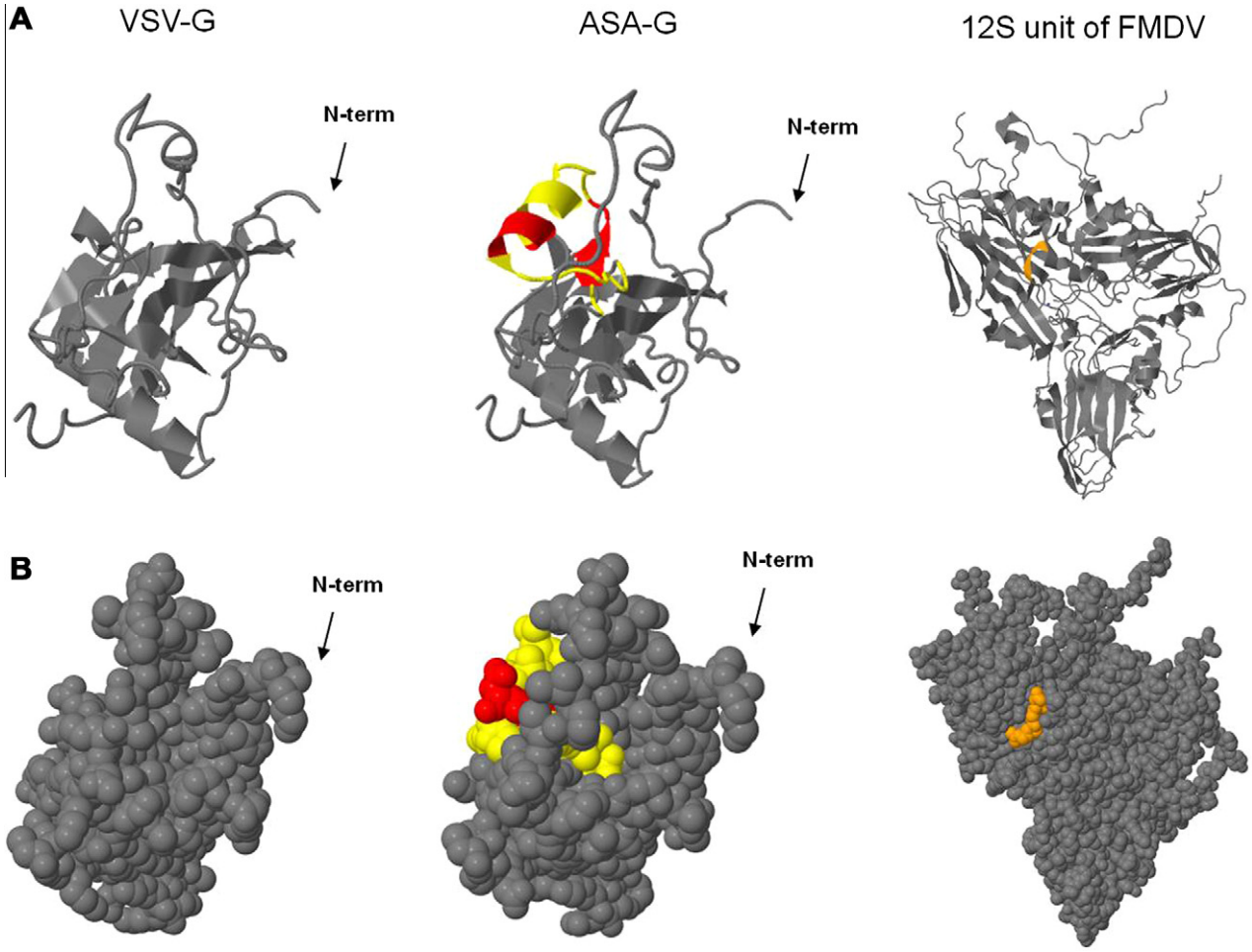

Fig. 4. Location of the FMDV-ASA dimer insertion mapped on the crystallography structure of VSV-G ectodomain. (A) Cartoon and (B) Spacefill stereoview of VSV-G, VSV-G chimera and 12S unit of FMDV model, as indicated. Residues that correspond to the insertion of the FMDV Antigenic Site A (ASA) in VSV-G epitope IV stabilized by disulfur bonds are highlighted in yellow. RGD motifs are highlighted in red (VSV-G chimera) and orange (12S unit of FMDV). ASA sequence appears exposed on VSV-G surface which would allow conformational flexibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sera from pC DEL and DEL BAC vaccinated calves bound to DEL BAC and VSV-G proteins in Western blot (Fig. 3B) but were less reactive to denatured VP1 (lane 6). Sera from FMDVi-vaccinated animals recognized both DEL BAC and FMDV proteins and did not bind VSV-G protein (data not shown). The preferential reactivity of sera from G-ASA vaccinated calves to native FMDV was also established by the immunoprecipitation assay shown in Fig. 3C that confirms the capacity of sera from either pC DEL, DEL BAC or FMDVi vaccinated cattle to efficiently immunoprecipitate whole FMDV native viral particles.

The location of the dimer-ASA insertion site was then mapped on the VSV-G ectodomain, and the structure of the G-ASA chimera was modeled based on crystallographic data and sequence analysis (shown in Fig. 4). Based on the template, aa sequence and structural identity of VSV-G were over the 60% SWISS-MODEL cut-off. The QMEAN score for secondary structure agreement were over 70% (Benkert et al., 2011). The modeled structure was consistent with the hypothesis that the RGD motif is exposed on the surface of the chimeric molecule (Fig. 4, highlighted in red). The dimeric ASA insert formed an α-helix with a short β-sheet (Fig. 4, panel B), leaving one of the RGD sequences exposed and the other bulked within VSV-G. The flexibility of FMDV antigenic site A harbored by the G-ASA chimera may be responsible for the FMDV-specific conformation-dependent antibodies induced by pC DEL and DEL BAC vaccination.

4. Discussion

Peptide and DNA vaccines have been explored previously as alternatives to the classical FMDV inactivated vaccines. Despite the initial success with a peptide containing ASA and a C-terminal region of VP1 (DiMarchi et al., 1986), peptides have not shown to induce protective responses against FMDV in cattle. Moreover, peptides containing the ASA coupled with T-helper cell sequences have been proven as efficient immunogens in swine but not in cattle (Rodriguez et al., 2003; Taboga et al., 1997); probably due to the great heterogeneity of bovine genetic components (Baxter et al., 2009; Glass, 2004; Leach et al., 2010). It is also believed that the inability of synthetic peptides to mimic the variable ASA conformations displayed on the surface of FMDV viral particles leads to a poor immunological response (Grigera et al., 1996; Kitson et al., 1991).

These obstacles were circumvented in this study by means of a recombinant chimeric antigen containing the first 193 aa of the N terminal sequences of the VSV-New Jersey glycoprotein (G) associated to a dimer of the main antigenic site of FMDV VP1 (ASA). The G-ASA construct expressed *ex vivo* (DEL BAC) or *in vivo* from a DNA vaccine (pC DEL) induced specific anti-FMDV FMDV-neutralizing antibodies in cattle, compatible with an "expected percentage of protection" above 80% (Maradei et al., 2008) after two doses, which is very high considering that only one FMDV antigenic site was

used. In this study, EPP calculation was applied only as a reference point of the magnitude of the humoral responses elicited against the ASA and not as an indicator of the protection status of DEL BAC and pC DEL vaccinated animals.

DEL BAC immunogen elicited high-titer humoral responses in calves immunized with two doses of 30 μg. Although cross comparisons with previous published data are difficult, the immunizing doses of DEL BAC are equivalent to about 3–5 μg of ASA sequence per dose; that is from 460 to 200 times lower than those reported before (Leach et al., 2010; Taboga et al., 1997). For instance, VP1 peptides have shown to be poorly immunogenic in cattle even at doses of 1 mg of peptide per animal (Taboga et al., 1997). Cubillos et al. (2008) showed that swine reacted well to dendrimeric peptides, composed of multiple copies of ASA and T-cell epitopes but 2 doses of 1.4 mg of peptide per animal were needed to achieve protection.

The aim of this study was to develop a model immunogen and get insights in how to overcome the poor immunogenicity elicited by the ASA peptide itself, considering that the same peptide is immunodominant when exposed by a flexible loop within the viral particle. Although direct *in vivo* protection was not assessed, the results presented here show that the use of VSV-G protein to harbor an ASA dimer can be a strategy to improve antibody responses to this antigenic site.

The IgG isotype profile induced and the booster of the total antibody titers after re-vaccination with the G-ASA constructs, indicate the presence of functional T-cell epitopes associated to the ASA. In addition, anamnestic T-cell responses could be evoked *in vitro* in blood cells from FMDV-vaccinated bovines (Table 1). These data suggest that T-cell epitope clusters from the VSV-G could be candidates to be included in peptide or DNA-based vaccines for cattle.

The kinetics of the immune response developed by the genetic immunogen (pC DEL) differed from that induced by its protein counterpart (DEL BAC). Single dose vaccination with pC DEL elicited specific neutralizing antibodies levels at 15 dpv that were significantly higher ($p > 0.05$) than those induced by DEL BAC, and comparable to those from inactivated virus vaccinated animals, enriched in the desirable IgG1 isotype (Capozzo et al., 1997; Mulcahy et al., 1990). After the second DNA-vaccine dose, higher levels of specific IgG2 were elicited, compatible with the Th1 type biased profile described for genetic immunogens (Taylor et al., 2005).

Better performance of DNA vaccines than peptide vaccines in young animals have already been described (Capozzo et al., 2006; Castro et al., 2009; Hamers et al., 2007; Jensen et al., 2009; Taylor et al., 2005). The efficiency of pC DEL immunogen to induce antibodies in calves may also been explained due to the fact that the chimeric proteins expressed from pC DEL accumulate within the transfected cells (Fig. 1B) and are probably released after cell death making them accessible to B-cells.

This study demonstrates that an efficient FMD-DNA vaccine, able to trigger specific FMD immunity after one dose of plasmid, can be developed by fusing FMDV sequences to a highly immunogenic carrier glycoprotein like the VSV-G.

Sera from DEL BAC- and pC DEL-vaccinated calves reacted strongly with native virus in ELISAs, efficiently immunoprecipitated whole FMDV particles (Fig. 3C) and neutralized viral infectivity in cell culture (Fig. 2). These results suggest that G-ASA constructs pC DEL and DEL BAC can expose an SDS-sensitive conformation of the ASA epitope that induces, and reacts with, antibodies that block virus infectivity. Modeling of the G-ASA protein based on crystallography data shows that at least one of the two ASA sequences can acquire an exposed configuration in the surface of the chimeric molecule (Fig. 4); supporting the conclusion that immunization of calves with G-ASA constructs induce antibodies against a restricted set of ASA conformations exclusively present in native viral particles.

The insertion of ASA as a tandem dimer results in one exposed ASA sequence (Fig. 4), whereas a single ASA insertion remains hidden within VSV-G structure (data not shown). Modeling data are in agreement with our previous publication, in which we showed that the insertion of only one ASA sequence within epitope IV of VSV-G was less immunogenic than the dimer insertion (Grigera et al., 1996). Our results are also in agreement with previous reports showing that ASA sequences are more immunogenic if presented as a linear dimer or tetramer, or as a part of a carrier protein (Brown, 1988; Dory et al., 2009a; Fan et al., 2007).

5. Conclusions

The immunogenicity of a VSV-G/FMDV-ASA chimera for cattle was confirmed by means of genetic and peptide vaccination. The improved immunogenicity of this construct can be explained by its capacity to mimic ASA native conformation(s) present on the surface of the virion and the presence of functional bovine T-cell epitopes.

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