



Pseudorabies virus glycoprotein B can be used to carry foot and mouth disease antigens in DNA vaccination of pigs

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ARTICLE INFO

Article history:

Received 1 July 2008

Received in revised form 17 November 2008

Accepted 24 November 2008

Keywords:

Foot and mouth disease virus
Pseudorabies virus glycoprotein B
B- and T-cell epitopes
Carrier of antigens
DNA vaccination

ABSTRACT

To evaluate the feasibility of using pseudorabies virus (PrV) glycoprotein B (gB) as a carrier of foot and mouth disease virus (FMDV) antigens in DNA immunization, FMDV B- and T-cell epitopes were inserted either between the two B-cell epitopes of the N-term subunit of PrV-gB (BT-PrV-gB-N-term construct) or within the B-cell epitope of the C-term subunit of PrV-gB (BT-PrV-gB-C-term construct). Two animal experiments were performed, each with three injections of plasmids 2 weeks apart, followed by a booster inoculation of peptides corresponding to the FMDV epitopes. Control groups of pigs were injected with plasmids encoding either PrV-gB or FMDV-BT, or with empty-pcDNA3. The results of both assays were combined. Significant titers of FMDV neutralizing antibodies were detected after the peptide boost in groups injected with the BT-PrV-gB-C-term construct. Insignificant amounts were detected in groups injected with the BT-PrV-gB-N-term and FMDV-BT constructs. PBMCs from the BT-PrV-gB-N-term groups, isolated after the peptide boost injection, produced IFN- γ and IL-4 mRNAs *in vitro* when stimulated with FMDV peptides. This was not observed with the other groups. These results imply that PrV-gB can be used to carry FMDV antigens in a DNA vaccine.

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1. Introduction

Foot and mouth disease virus (FMDV) is the etiological agent of an important disease of livestock. FMD is highly contagious and affects cloven-hoofed animals, mostly cattle, swine, sheep and goats. FMDV belongs to the Aphthovirus genus of the Picornaviridae family and is classified into seven serotypes (Bachrach, 1968; Rodrigo and Dopazo, 1995; Sobrino et al., 2001). One strategy employed to control disease propagation consists of regular vaccination with an inactivated whole virus antigen combined with an adjuvant (Barteling and Vreeswijk, 1991; Grubman, 2005; Sáiz et al., 2002) and has resulted in eradication of the disease in some parts of the world (particularly Western Europe) (Sobrino et al., 2001). Pigs were protected against experimental FMDV challenges 4 days after vaccination (Salt et al., 1998), but no antibodies were detected at this time point. A Th1/Th2 balanced immune response was characterized 14–21 days after emergency vaccination (Barnard et al., 2005). As these vaccines have certain drawbacks (Cox et al., 2003; Grubman and Baxt, 2004; King et al., 1981), much effort has been

made to develop efficient alternative vaccines, such as proteins, peptides, replicating vectors or DNA vaccines (Grubman, 2005). In the latter case, plasmids encoding large fragments of an FMDV genome-like P1-2A3C3D construct (Cedillo-Barrón et al., 2001), VP1 (Li et al., 2007; Park et al., 2006; Xiao et al., 2007) or FMDV B- and T-cell epitopes (Borrego et al., 2006; Cedillo-Barrón et al., 2003; Fan et al., 2007; Wong et al., 2002; Zhang et al., 2003) were tested. Considerable progress has been made to enhance DNA vaccination efficacy but at least two to three doses of plasmids still need to be injected (Borrego et al., 2006; Cedillo-Barrón et al., 2001; Chen and Shao, 2006; Li et al., 2006).

In contrast, a single injection of the pseudorabies virus (PrV)-specific DNA vaccine induced immune responses against PrV and conferred clinical protection against an experimental lethal PrV-infection (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). Among the three PrV glycoproteins encoded by the DNA vaccine, PrV-gB is of particular interest. This is a 913-amino acid protein which contains a transmembrane domain and a furin cleavage site. This protein is highly immunogenic since two B-cell epitope sites (aa 59–126 and 214–279) have been identified in the N-term subunit and one B-cell epitope site (aa 540–734) in the C-term subunit site (Zaripov et al., 1998, 1999). Furthermore, sequences allowing PrV-gB endocytosis (aa 884–913), PrV-gB cell-to-cell spread

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(aa 824–854) and incorporation of this glycoprotein into virions (aa 854–913) have been well documented (Nixdorf et al., 2000). Thus, as PrV-gB is able to get both inside and outside cells, the opportunities for this glycoprotein to encounter an efficient immune cell may be increased.

Therefore, the aim of the present study was to determine the feasibility of using PrV-gB to carry FMDV epitopes in a DNA vaccine. The strategy differs from the one previously published for BHV-1 gB (Keil et al., 2005) in that the antigens are not released from gB but benefit from the immune properties of gB by staying attached to this glycoprotein. The FMDV B epitope of VP1 is reported to elicit the production of neutralizing antibodies (Francis et al., 1987). The T-cell epitope of VP4 is capable of assisting a B-cell epitope when in tandem (Blanco et al., 2000). These different items, i.e. the FMDV type C, isolate C-8Sc1, B-cell epitope (aa 133–156 of VP1) fused to the T-cell epitope (aa 20–34 of VP4), which have been previously evaluated in mice (Borrego et al., 2006), were now inserted into PrV-gB. The method used to insert the FMDV epitopes was therefore based on information in the literature. The protein is known to contain three B-cell epitopes which means that these sites are recognized by the immune system and are therefore situated on the protein surface. Two constructs were evaluated. The first was obtained by inserting FMDV-BT between the two B-cell epitopes of the N-term subunit of PrV-gB. The second was obtained by inserting the FMDV epitopes into the B-cell epitope of the C-term subunit of PrV-gB. The immune responses against FMDV obtained with these constructs were compared to those obtained with a plasmid encoding the FMDV B- and T-cell epitopes. As the immune potentials of these constructs are unknown, the likelihood of measuring subsequent events was maximized by administering three injections of plasmids 2 weeks apart followed by a booster inoculation with the

corresponding B- and T-cell epitope peptides, 2 weeks after the last plasmids injection. The immune responses against FMDV and PrV were analysed after each injection.

2. Materials and methods

2.1. Construction and in vitro characterization of plasmids

The pcDNA3 plasmid encoding PrV glycoprotein gB has already been constructed, characterized and successfully used in our laboratory (Dory et al., 2005a; Dufour et al., 2000). The pGEM-based plasmids encoding the fused FMDV type C, isolate C-8Sc1, B-cell epitope (aa 133–156 of VP1) and T-cell epitope (aa 20–34 of VP4) were kindly provided by Belen Borrego (INIA, Valdeolmos, Spain). The FMDV-BT construct was subcloned into the pcDNA3 expression cassette after BamHI and NotI digestions. In order to insert the FMDV-BT sequence between the two B-cell epitopes of the N-term subunit of the PrV-gB gene without modifying the reading frame of gB, the BsiWI site (at the level of aa 187–189) was chosen (Fig. 1). BsiWI restriction sites were introduced on the FMDV-BT insert by performing PCR on pGEM-FMDV-BT with the following primers: 5'CGCATG**CGTACGG**CCGC3' and 5'ATTAGAT**CCGTACG**TGGAGTT3' (BsiWI specific site is in bold and underlined). The PCR product and pcDNA3-PrV-gB were digested with BsiWI (New England Biolabs) according to the manufacturer's instructions. After dephosphorylation of the digested pcDNA3-PrV-gB, the digested PCR product was inserted into the PrV-gB gene. The resulting plasmid was called BT-PrV-gB-N-term. In order to insert the FMDV-BT sequence into the B-cell epitope of the C-term subunit of the PrV-gB gene without modifying the reading frame of gB, we chose the FspAI restriction site (at the level of

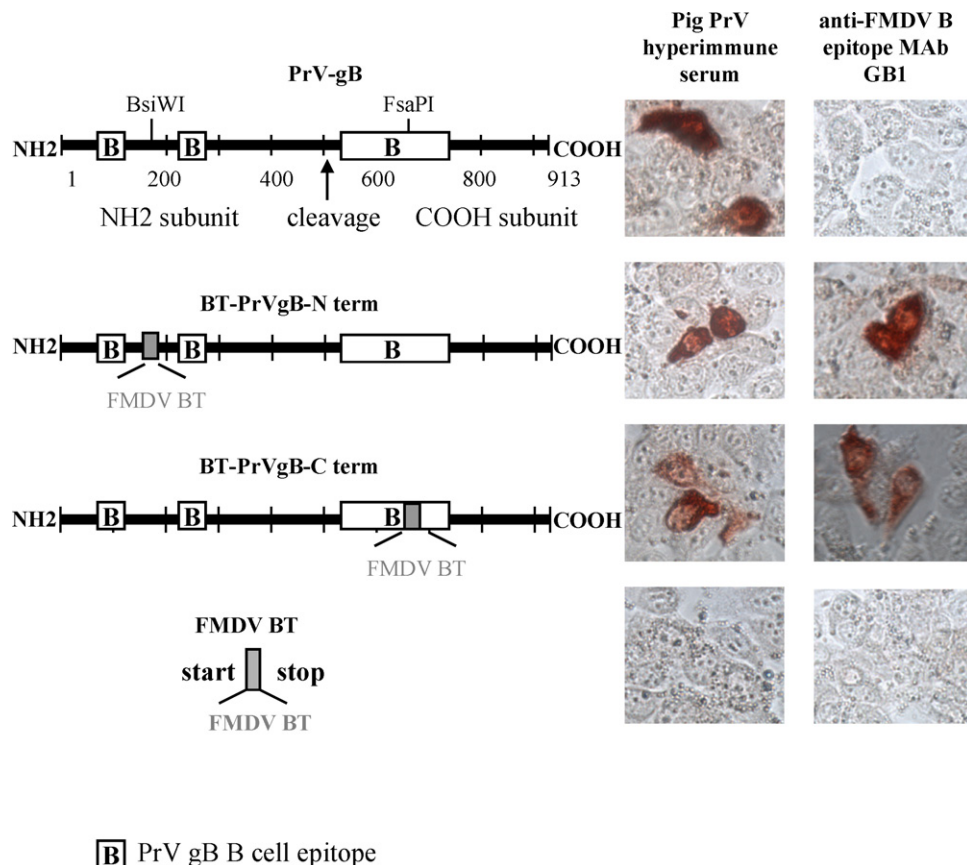


Fig. 1. Constructs: porcine PK15 cells were transfected with pcDNA3-based plasmids encoding PrV-gB, BT-PrV-gB N-term, BT-PrV-gB C-term or FMDV-BT. Twenty-four hours later, cells were stained with pig PrV hyperimmune serum or anti-FMDV B epitope GB1 monoclonal antibody as described in Section 2.

aa 682), which generates blunt ends. Appropriate restriction sites generating blunt ends on the FMDV-BT insert were produced by performing a PCR on pGEM-FMDV-BT with the following primers: 5'TGGAT**CCCGGCTACGAC**3' containing a *Sma*I restriction site (in bold and underlined) and 5'CTACATG**GAGTACTG**3' containing a *Sal*I restriction site (in bold and underlined). The PCR product was first cloned into a TOPO-TA vector (Invitrogen). The resulting vector was then digested with *Sma*I and *Sal*I and the insert transferred into pcDNA3-PrV-gB digested with *Fsp*AI. The resulting plasmid was called BT-PrV-gB-C-term. Each construct was further characterized according to restriction patterns and sequencing (not shown). The porcine kidney cell line 15 (PK15) was transfected with 2 µg of each plasmid by using lipofectamine plus transfection reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were stained to reveal the expression of either PrV-gB or FMDV-BT. For PrV-gB, the cells were successively incubated with pig PrV-specific hyperimmune antiserum, with HRP-labelled rabbit anti-swine IgG at 1:1000 (Sigma, St. Louis, MO) and with the 3-amino-9-ethylcarbazole peroxidase substrate (Serotec Ltd., Oxford, UK) according to the manufacturer's instructions (Fig. 1). For FMDV-BT, the cells were successively incubated with the monoclonal GB1 antibody at 1:100, with HRP-labelled rabbit anti-mouse IgG at 1:1000 (Dako, Trappes, France) and with the 3-amino-9-ethylcarbazole peroxidase substrate (Serotec Ltd.).

Each plasmid was individually introduced into the *Escherichia coli* Top10 strain, and amplified and purified using the EndoFree plasmid Mega kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Animal experiments

Specific pathogen-free pigs were housed and treated in accordance with local veterinary office regulations (Direction des Services Vétérinaires des Côtes d'Armor, France). In the first assay, five groups of four pigs weighing 26.0 ± 0.7 kg at the first injection were used. Group 1 received the BT-PrV-gB-N-term-encoding plasmid. Group 2 received the BT-PrV-gB-C-term-encoding plasmid. Group 3, which received uncarried FMDV-BT, was injected with pcDNA3-FMDV-BT. Group 4 was injected with pcDNA3-PrV-gB in order to evaluate the immune responses against PrV induced by the unmodified PrV-gB. Finally, the negative control group 5 was injected with empty-pcDNA3. All plasmids were co-injected with pcDNA3-GM-CSF (previously used in our laboratory (Dufour et al., 2000)) as adjuvant and injected three times by both intramuscular (i.m.) and intradermal (i.d.) routes at 2-week intervals. One milliliter of sterile saline solution containing 150 µg of the plasmids of interest and 50 µg of pcDNA3-GM-CSF were injected by i.m. route in both sides of the neck using $0.8 \text{ mm} \times 40 \text{ mm}$ needles. 0.25 ml of saline solution containing 150 µg of plasmids of interest and 50 µg of pcDNA3-GM-CSF were injected by i.d. route into the dorsal surface of both ears using $0.45 \text{ mm} \times 12 \text{ mm}$ needles. The i.d. injection was controlled by: (i) parallel position of the needle to the ear surface, (ii) high pressure applied to the syringe to inject the solutions and (iii) the transient generation of white spots. The pigs then received 100 µg of the FMDV T-cell peptide and 100 µg of the FMDV B-cell peptide, 2 weeks after the last plasmids injection. A second assay, with eight animals per group and four animals in the two control groups, was carried out.

The pigs were carefully observed for any adverse reaction after injection. Body temperature was measured daily and 4 h after each injection. Relative daily weight gains were determined (Stellmann et al., 1989) for each pig. Some pigs were sacrificed during or at the end of the assays for ethical reasons. The injected areas were examined to see whether or not the injection of plasmids or expression

of the encoded proteins produced lesions. Other organs were also examined.

For the antibodies determinations, sera were collected before the first plasmid injection, 1 and 2 weeks after each plasmid injection and 1, 2 and 3 weeks after the peptides boost. To isolate PBMCs, total blood samples were collected before the first plasmid injection, 2 weeks after each plasmid injection and 1, 2 and 3 weeks after the peptides boost.

2.3. FMDV neutralizing antibodies (NAbs)

FMDV neutralizing antibody assays were carried out in 96-well plates as described in the OIE Manual of Standards (OIE, 2000). Serial dilutions of sera were prepared in duplicate and 50 µl of each was added for 1 h to 50 µl of 100 CCID₅₀ of the FMDV C1Noville strain. Cell suspension was then added to each well and the plates were incubated at 37 °C for 3 days. The cells were fixed with formalin and stained with methylene blue. Titers were expressed as the highest serum dilution that inhibited viral replication in 50% of the wells.

2.4. Determination of FMDV-specific serum antibodies

Anti-FMDV type C antibodies were detected by applying a competitive ELISA test as described by Mackay et al. (2001). Briefly, 96-well, flat-bottomed plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with a rabbit anti-FMDV C1 strain serum diluted in carbonate/bicarbonate buffer pH 9.6 (Sigma). After three washings with PBS, the plates were incubated with FMDV antigen (C1 Noville) diluted in PBS-tween 20 0.05% buffer supplemented with 10% bovine serum and 5% rabbit serum (blocking buffer) for 1 h at 37 °C. After the washings, the samples of pig sera diluted one-fifth in blocking buffer were added in duplicate and a diluted guinea-pig anti-type C was also added as a competitor in each well. After washings and incubation with a HRP-conjugated anti-guinea pig serum, the reaction was revealed with an OPD solution (Sigma). Results were expressed as the percentage inhibition of the optical density (OD) obtained with the guinea-pig serum anti-type C1 in the antigen control well.

2.5. PrV neutralizing antibodies

After complement inactivation (30 min at 56 °C), 50 µl of twofold dilutions of serum were incubated with 50 µl of 100 TCID₅₀ of NIA3 PrV strain in 5% CO₂ in 96-well plates for 1 h at 37 °C. 150 µl of PK15 cells (2.25×10^4 cells/150 µl) were then added and incubated in 5% CO₂ for 5 days at 37 °C.

NAb titers were expressed as the highest serum dilution inhibiting the cytopathic effect in two out of four wells containing the PrV-infected PK15 cell line.

2.6. Determination of PrV-specific IgG1 and IgG2 serum antibodies (Abs)

Anti-PrV IgG1 and IgG2 serum Ab titers were determined by indirect ELISA as previously described (Dory et al., 2005b). Briefly, Maxisorb 96-well plates (Nunc, Naperville, IL) were coated with PrV glycoproteins, kindly provided by J.C. Audonnet (Merial, Lyon, France), and successively incubated with serial threefold dilutions of serum, with mouse anti-porcine IgG1 or IgG2 (Serotec Ltd., Oxford, UK), with HRP-labelled rabbit anti-mouse IgG (Jackson Laboratories, West Grove, Pennsylvania, PA) and finally with the peroxidase substrate tetramethyl benzidine (Pierce, Rockford, IL). The enzyme reaction was then stopped by adding sulfuric acid. The ODs were measured at 450 nm. IgG1 and IgG2 titers (log₁₀) were obtained from the highest dilution that gave a higher OD value

than the threefold OD of a control serum from non-vaccinated and non-infected pigs.

2.7. Quantification of porcine IFN- γ and interleukin 4 (IL-4) mRNA produced by stimulated peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood collected before plasmid injection, 2 weeks after each plasmid injection and 1, 2 and 3 weeks after the peptides boost. PBMCs were either incubated in vitro for 16 h with PrV strain NIA3 (multiplicity of infection: 1), with 5 ng of FMDV T-cell and B-cell peptides, or with the RPMI culture medium alone. PBMC total RNA was isolated using the 96 RNEasy kit (Qiagen, Hilden, Germany). Porcine IFN- γ and porcine IL-4 mRNA expressions were determined by quantitative real-time polymerase chain reaction (PCR) using primers, probes and PCR conditions as previously described (Dory et al., 2005a). Cytokine mRNA and β -actin mRNA threshold cycles (Ct) were determined simultaneously for each sample and the relative quantities determined according to User Bulletin number 2, ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The amount of cytokine mRNA was standardized with the internal β -actin mRNA reference (Δ Ct = cytokine Ct – β -actin Ct) and quantified in relation to the non-stimulated sample ($\Delta\Delta$ Ct = Δ Ct of the stimulated sample – Δ Ct of the non-stimulated sample) according to equation $2^{-\Delta\Delta$ Ct}.

2.8. Statistical analysis

The data were analysed using the nonparametric Mann-Whitney test (Mann and Whitney, 1947) included in the Systat 9 software (Systat Software, Inc., Point Richmond, CA). This test was applied because the generated data were few in number, did not present a normal distribution and consisted of unpaired quantitative data.

The limit of significance was 0.05 for all comparisons.

3. Results

3.1. Constructs

Two plasmid constructs encoding PrV-gB/FMDV-BT were obtained. Porcine PK15 cells transfected with each construct were stained with a pig PrV hyperimmune serum and an antibody directed against the B-cell epitope of the FMDV used here (GB1) (Fig. 1). Each PrV-gB-based construct was detected by the PrV

hyperimmune serum. Cells transfected with the FMDV-BT encoding plasmid were not detected by the GB1 anti-FMDV monoclonal antibody, whereas under the same experimental conditions both PrV-gB/FMDV-BT chimeric constructs were detected.

3.2. Induction of immune responses against FMDV in pigs

The plasmids were injected by the i.m. and i.d. routes three times at 2-week intervals. Two weeks after the last injection, individual FMDV B- and T-cell peptides were injected by the same routes. The different injections were well tolerated. The animals grew normally and gained approximately 1 kg/day in all groups in both assays. No fever peaks were observed 4 h after any of the injections or during any of the daily measurements, except in one pig in the BT-PrV-gB-N-term group of the first assay which had a body temperature of 40.3 °C, 72 h after the third injection of the plasmids. This body temperature had returned to normal by the next day. Several pigs in the second assay had to be euthanized for reasons unrelated to immunization. One pig in the BT-PrV-gB-N-term group broke a hoof and had to be sacrificed after the third injection. One pig in the FMDV-BT group walked with a limp and was sacrificed after the second injection and another, presenting a rectal prolapse, was euthanized 1 week after the peptides injection. Finally, one aggressive pig in the group injected with empty-pcDNA3 was euthanized after the peptides injection.

Experiments 1 and 2 were combined to determine the induction of immune responses, and the average and standard deviation val-

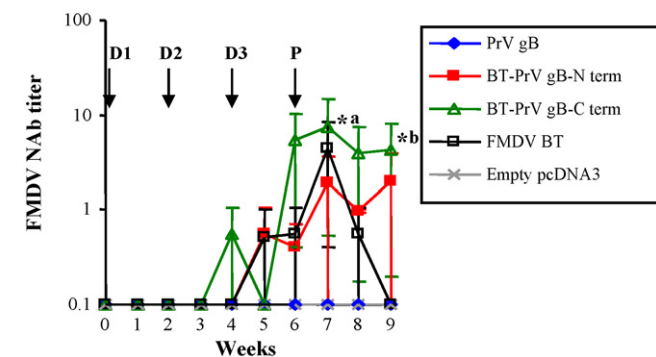


Fig. 2. Anti-FMDV neutralizing antibodies: anti-FMDV neutralizing antibodies before the first injection and after each of the three injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 \pm S.D. are shown for each group indicated in the legend box. *a, $p < 0.05$ compared to the PrV-gB and empty-pcDNA3 groups. *b, $p < 0.05$ compared to the PrV-gB, empty-pcDNA3 and FMDV-BT groups.

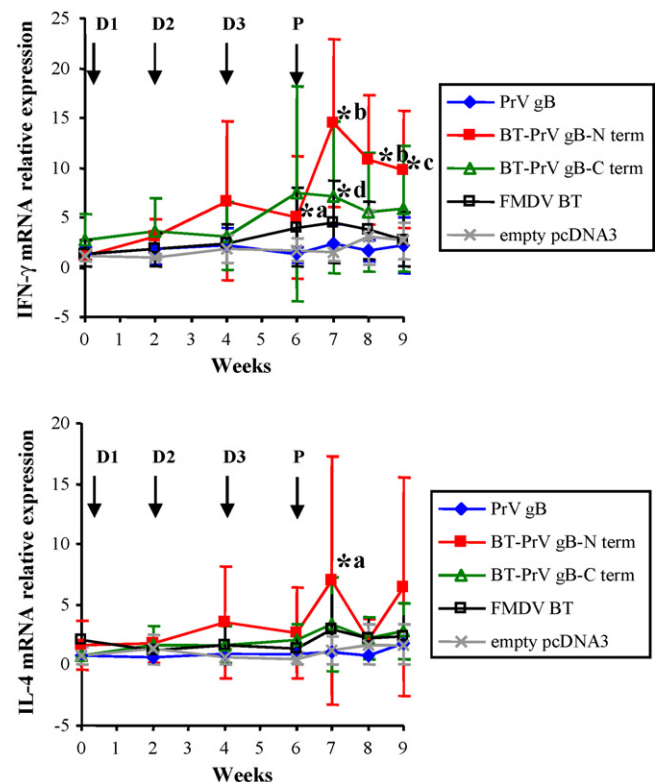


Fig. 3. IFN- γ and IL-4 mRNA relative expressions by FMDV B- and T-cell peptides stimulated PBMCs: the cells were isolated before the first injection and after each of the three injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were then incubated with FMDV B and T peptides for 16 h. IFN- γ and IL-4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 \pm S.D. are shown for each group indicated in the legend box. *a, $p < 0.05$ compared to the PrV-gB and empty-pcDNA3 groups. *b, $p < 0.05$ compared to all the other groups. *c, $p < 0.05$ compared to the PrV-gB, FMDV-BT and empty-pcDNA3 groups. *d, $p < 0.05$ compared to the PrV-gB, BT-PrV-gB-N-term and empty-pcDNA3 groups.

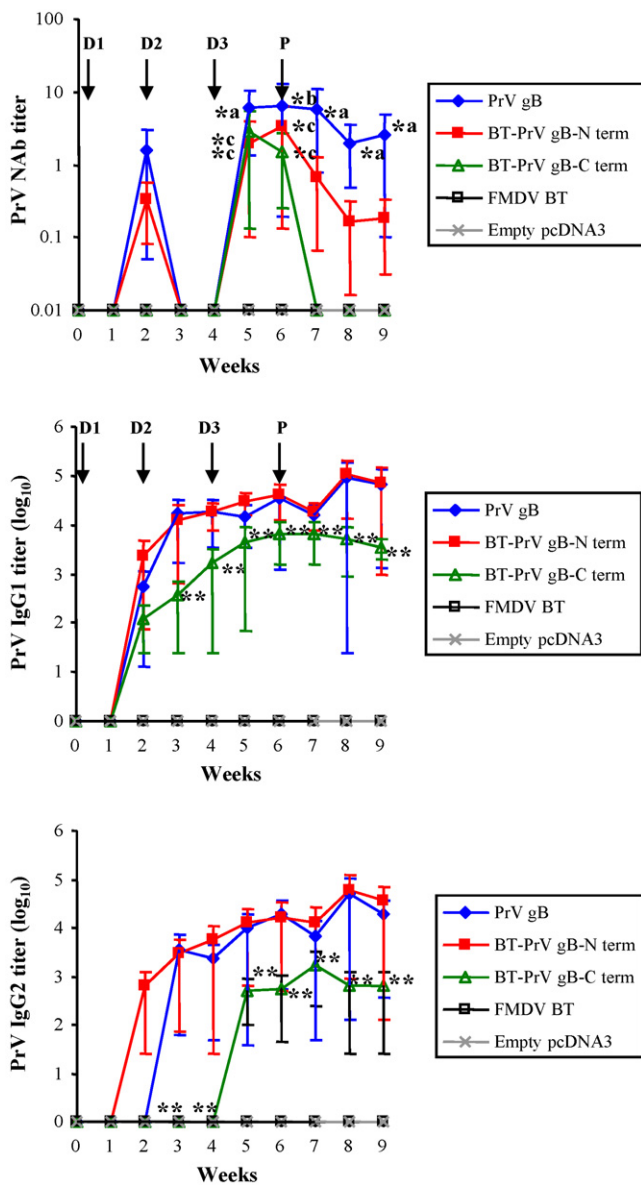


Fig. 4. Anti-PrV neutralizing, IgG1 and IgG2 antibodies production: sera from pigs in both assays were collected before the first injection and after each of the three injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 \pm S.D. are shown for each group indicated in the legend box. *a, $p < 0.05$ compared to all the other groups. *b, $p < 0.05$ compared to the BT-PrV-gB-C-term, FMDV-BT and empty-pcDNA3 groups. *c, $p < 0.05$ compared to the PrV-gB, FMDV-BT and empty-pcDNA3 groups. ** $p < 0.01$ compared to the PrV-gB and BT-PrV-gB-N-term groups.

ues were determined for each group. Insignificant amounts of FMDV neutralizing antibodies were first detected 2 weeks after the second injection of plasmids in the BT-PrV-gB-C-term group (Fig. 2). Statistically significant titers were only observed in this group 1 and 3 weeks after the FMDV B and T peptides boost ($p < 0.05$). NAb were also detected in the BT-PrV-gB-C-term and FMDV-BT groups but the titers, in all cases, were not statistically significant. Furthermore, NAb production in the FMDV-BT group was transient and could no longer be detected 3 weeks after the peptides boost, whereas it was still observed in the BT-PrV-gB-C-term injected group. FMDV-specific antibodies were found at the limit of detection in sera from 3 pigs in the BT-PrV-gB-N-term and in 2 out of 12 pigs in the BT-PrV-gB-C-term injected groups. These antibodies were not detected in any of the other groups.

IFN- γ has several immunoregulatory roles and effector functions involved in Th1-responses and IL-4 plays a key role in Th2-responses (Finkelman et al., 1988; Wood and Seow, 1996). The PBMCs isolated from all these pigs were restimulated in vitro by incubation with FMDV B- and T-cells peptides. Significant levels of IFN- γ mRNA were detected after the peptides boost in the BT-PrV-gB-N-term injected group ($p < 0.05$) (Fig. 3). IFN- γ mRNA was not detected in the other groups during the first assay. Significant levels were also found in the BT-PrV-gB-N-term group 1 week after the peptides boost ($p < 0.05$), but production was still significantly lower than in the BT-PrV-gB-C-term group ($p < 0.05$). All the other groups remained negative. Significant amounts of IL-4 mRNA were only detected in the BT-PrV-gB-N-term group 1 week after the FMDV B and T peptides boost ($p < 0.05$), but not in any of the other groups or in the second assay (Fig. 3).

3.3. Induction of immune responses against PrV in pigs

PrV neutralizing antibodies were produced in significant amounts 1 week after the third injection of pcDNA3-PrV-gB (Fig. 4) and remained at a significant level until the end of the assay. Significant amounts of PrV NAb were produced in the BT-PrV-gB-N-term and C-term groups, 2 and 3 weeks after the third plasmids injection. Nevertheless, except for the BT-PrV-gB-N-term group 3 weeks after the third injection, the NAb titers were significantly lower than in the PrV-gB injected group. No significant NAb production was observed in the two groups that received FMDV-BT/PrV-gB chimeric constructs, from the 4th week after the last plasmids injection. Significant amounts of PrV-specific IgG1 were first detected

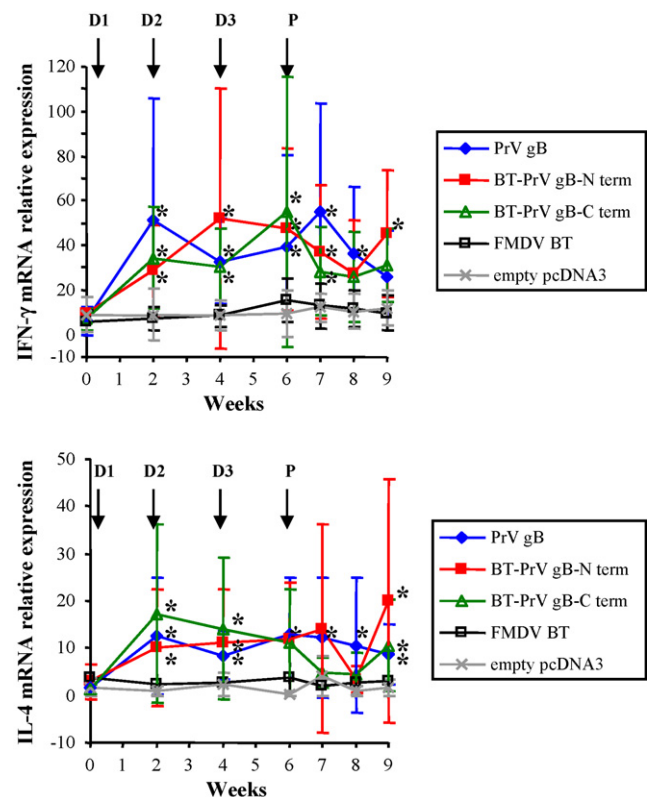


Fig. 5. IFN- γ and IL-4 mRNA relative expressions by PrV-stimulated PBMCs: the cells were isolated before the first injection and after each of the three injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were then incubated with live PrV for 16 h. IFN- γ and IL-4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 \pm S.D. are shown for each group indicated in the legend box. * $p < 0.05$ compared to the FMDV-BT and empty-pcDNA3 groups.

1 week after the second plasmids injection in the groups of pigs injected with PrV-gB or BT-PrV-gB-N-term constructs ($p < 0.05$). IgG1 production was then maintained at a significant level until the end of the assay. In contrast, production in the BT-PrV-gB-C-term group was systematically and significantly lower from the 1st week after the second injection until the end of the assay ($p < 0.01$).

IFN- γ and IL-4 mRNAs production was observed in PrV-stimulated PBMCs from pigs injected with PrV-gB, BT-PrV-gB-N-term and BT-PrV-gB-C-term constructs from week 2 after the second injection to week 2 after the third injection of plasmids ($p < 0.05$) (Fig. 5). From weeks 1 to 3 after the FMDV B and T peptides injection, all three groups produced significant amounts of IFN- γ mRNA except for the BT-PrV-gB-C-term group at weeks 2 and 3, the BT-PrV-gB-N-term group at week 2 and the PrV-gB group at week 3. The same was true for IL-4 mRNA production, except for the BT-PrV-gB-C-term group at weeks 1 and 2 after the peptides boost, and for the BT-PrV-gB-N-term group at week 2. No production of IFN- γ and IL-4 RNAs was observed in the other two groups throughout the assay.

4. Discussion

The production of an efficient DNA vaccine against FMDV represents a challenge for the scientific community. Such a vaccine would provide an attractive alternative to the conventional inactivated FMDV vaccine. Yet FMDV DNA vaccines which often require three injections of plasmids (Bergamin et al., 2007; Cedillo-Barrón et al., 2001, 2003; Li et al., 2006). Boosting with an inactivated FMDV improved significantly the DNA vaccine efficacy (Li et al., 2008). These results reflect the difficulty of generating a powerful DNA vaccine against FMDV that confers immunity after one or two injections of plasmids, as with anti-PrV vaccination (Gravier et al., 2007). Our goal in this study was to see if the glycoprotein B of PrV could serve as a carrier of FMDV antigens in a DNA vaccine. This glycoprotein was selected because it is already used in a successful one-shot DNA vaccine combination against PrV-infection in swine (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). It is also an immunogenic protein (Zaripov et al., 1998, 1999) associated with functional domains that enable it to be internalized and to go outside the cell (Nixdorf et al., 2000). It might therefore be possible to take advantage of these characteristics to carry and present foreign antigens. A modified gB of bovine herpesvirus 1 (BHV-1) was recently used to transport foreign proteins (Keil et al., 2005). The transport and release of foreign proteins inserted between the furin sites was, in fact, facilitated by inserting a second furin cleavage site into this glycoprotein. The concept in the present study is different, since gB is expected not only to transport, but also to present foreign antigens. FMDV B- and T-cell epitopes have already been tested in DNA vaccines or recombinant vaccines to immunize mice or pigs against FMDV. When mice were injected three times with a plasmid encoding these epitopes, no neutralizing antibodies were produced and no viremia was present in half of them post-challenge (Borrego et al., 2006). Fusion of these epitopes to a signal peptide produced NAb in one out of four mice. Co-injection of the fungus *Agaricus blazei* Murill (Chen and Shao, 2006) or fusion to swine IgG (Wong et al., 2002) were shown to enhance DNA vaccination against FMDV in mice or pigs, respectively. Finally, the titers of neutralizing antibodies in pigs injected twice with a recombinant adenovirus expressing FMDV B- and T-cell epitopes were between 4 and 16, and 3 out of 5 pigs were protected (Du et al., 2007). These small FMDV B- and T-cell epitopes were thus able to induce immunization and/or protection against FMDV, even though the induced humoral immune responses were low or undetectable. In our carrier study, one FMDV B-cell epitope (aa 133–156 of VP1) fused with one FMDV T-cell epitope (aa 20–34 of VP4) (Borrego et al., 2006)

was inserted at two different sites on PrV-gB. The insertion strategy was based on the knowledge gained from the functional studies of the B-cell epitopes in PrV.

The three-dimensional structure of PrV-gB has not been determined, but the X-ray structure of gB ectodomain from herpes simplex virus 1 (HSV-1) is available (Heldwein et al., 2006). PrV and HSV-1 gB share 50% identity, and the high sequence conservation strongly suggests that the PrV-gB adopts a structure similar to the one reported for HSV-1. FMDV-BT was inserted in PrV-gB between the two B-cell epitopes located close to the N-terminus of gB (BT-PrV-gB-N-term), and in the B-cell epitope located in the region preceding the C-terminus (BT-PrV-gB-C-term). The residues in HSV-1 gB, which correspond to the FMDV-BT insertion sites in PrV-gB, were identified from the alignment of the PrV and HSV-1 gB sequences. The locations of the PrV insertion sites were then mapped on the HSV-1 gB structure shown in Fig. 6. For the BT-PrV-gB-N-term construct, the corresponding HSV-1 gB insertion site, residue Y179, is part of the fusion loop of HSV-1 gB. Fusion loops are rich in hydrophobic residues and are typically buried within the protein or in a membrane, suggesting that the N-terminal FMDV-BT epitope may not be fully exposed in PrV-gB. The limited accessibility of the epitope in BT-PrV-gB-N-term could be one of the causes of the inefficient recognition by immune system and low antibody titers. For the BT-PrV-gB-C-term construct, the homologous insertion site in HSV-1 gB is residue H657, which is located in an exposed beta-strand of domain IV. Based on the HSV-1 gB structure, the C-terminal FMDV-BT epitope would likely localize to the surface of the protein, consistent with its availability for recognition by the immune system.

The results presented here show that FMDV-BT epitopes could not be detected in vitro by the GB1 monoclonal antibody unless they were carried by PrV-gB. In fact, positively stained cells could be visualized after transfection by either one of the PrV-gB constructs carrying the FMDV-BT epitope. Moreover, PrV-gB was strongly expressed and detected when pig PrV-hyperimmune serum was used. This implies that multiple epitopes were recognized, whether they were near to the insertion sites or not, suggesting that some of them were not perturbed by the insertion. These findings demonstrate that the concept of PrV-gB as a carrier of FMDV epitopes is feasible, at least in vitro. The immunization potentials of our constructs were evaluated after each of the three plasmids injections, as in other studies of DNA vaccination against FMDV. A FMDV peptides boost was then added to observe the production of immune responses against FMDV. The two assays (12 pigs per BT-PrV-gB-C-term, BT-PrV-gB-N-term and BT groups or 8 pigs per PrV-gB and empty-pcDNA3 groups in total) were combined and the averages of the two assays are presented. Significant induction of immune responses against FMDV was observed only after the FMDV B and T peptides boost in the groups primed with plasmids encoding chimeric PrV-gB/FMDV-BT constructs. This was not observed in the groups that were three times injected with empty plasmids or plasmids encoding PrV-gB before injection of FMDV B and T peptides. In fact, in these last cases pigs received FMDV antigens only once. FMDV-specific serum antibodies were faintly detected in 5 of the 24 pigs that received the FMDV-BT/PrV-gB constructs. No specific antibodies were detected in any of the other groups. Significant NAb production against FMDV was only found in the BT-PrV-gB-C-term construct groups. Non-significant production was detected in the BT-PrV-gB-N-term group. Although less NAb was produced than with DNA vaccines containing larger FMDV constructs, such as P1-2A3C3D (Cedillo-Barrón et al., 2001), the amounts were similar to previous studies with these small epitopes. Borrego et al. found that only 1 out of 31 mice produced NAb after 3 injections of DNA vaccines encoding FMDV B- and T-cell epitopes (Borrego et al., 2006). Pigs injected twice with rAdV expressing FMDV-BT and GM-CSF produced NAb titers between 4 and 16 (Du et al.,

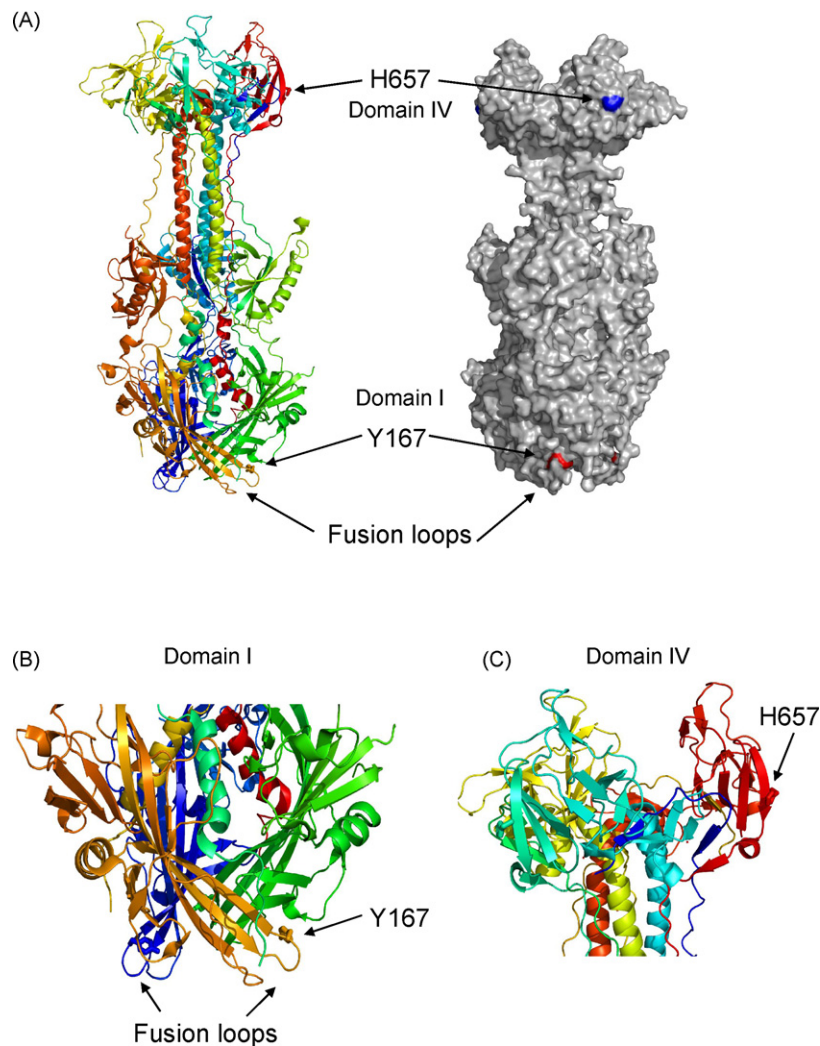


Fig. 6. Location of the PrV-gB FMDV epitope insertion sites mapped on the structure of HSV-1 gB: (A) ectodomains of HSV-1 gB form trimers. Each monomer is colored as blue, green or red (left panel), and a space-filled model of the gB trimeric surface is shown on the right. Y167 and H657 are the HSV-1 residues that correspond to the insertion sites of the FMDV epitopes in PrV-gB. HSV-1 gB fusion loops, which are the residues proposed to insert into the target membrane during fusion, are marked. (B and C) Only domains I and IV, respectively, are shown for clarity, and insertion sites Y167 and H657 are labelled. Residue Y167, which would correspond to the N-term FMDV epitope insertion, seems less accessible than the fully exposed H657, which is analogous to the position where the C-term FMDV epitope was added. This suggests that the latter position might be a better insertion target. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2007). It is important to note that the B-cell epitope from the CS8 strain encoded by the vaccine differed in two amino acids from the one obtained from the C1 Noville strain used in the neutralizing assay. One of these amino acids is located in an area (aa 146–156) important for NAb production (Francis et al., 1987). It is therefore possible that the NAb produced was less able to inhibit C1 Noville during in vitro replication and that the NAb has thus been underestimated. Furthermore, the conformations of the FMDV B- and T-cells may be modified by their insertion into PrV-gB, and the induction of immune responses may potentially be perturbed and decreased. Another important element in protection against FMDV is the cellular immune response (Borrego et al., 2006; Sobrino et al., 2001). The BT-PrV-gB-N-term group seemed to favor the production of IFN- γ mRNA (a marker of the cellular immune response) by PBMCs stimulated by FMDV B- and T-cell epitope peptides. Significant amounts of IL-4 mRNA (a marker of induction of humoral immune responses) were detected in the same group. These results suggest that both cellular and humoral immune responses were induced in this group, as in efficient emergency FMDV vaccination (Barnard et al., 2005). However, the detection of cytokine mRNAs does not necessarily imply that the corresponding proteins are

induced. These results should therefore be interpreted with caution. Barnard et al. showed that the production of cytokine mRNAs by FMDV-stimulated PBMCs from FMDV vaccinated pigs was correlated, in most cases, with the production of cytokines (Barnard et al., 2005). In some rare cases, the cytokines were not detected, whereas the mRNAs were. We can therefore be relatively confident of the results' interpretation. Furthermore, the results presented here are not predictive of the ability of the constructs to protect pigs against FMDV infection. This can only be evaluated experimentally. Previous studies showed that although these FMDV B- and T-cell epitopes induced no or only low titers of NAb, they were able to protect mice (Borrego et al., 2006) and pigs (Du et al., 2007) against FMDV challenges. Other examples of protection in the presence of low titers of FMDV NAb are reported in the literature (Sobrino et al., 2001).

The immune responses to PrV-gB were equivalent to those previously reported after three injections of plasmids in pigs (van Rooij et al., 2000). Insertion of the FMDV epitopes into the B-cell epitope of the C-terminal region of PrV-gB, which is described as a strong conformational B-cell epitope with neutralizing activity (Zaripov et al., 1998), greatly reduced the humoral immune response against

the PrV studied here. In contrast, insertion of the FMDV epitopes between the two B-cell epitopes close to the N-terminus of PrV-gB, described as linear epitopes inducing low antibody responses (Zaripov et al., 1998), abolished or strongly reduced the production of PrV NAb, but not of IgG1 and IgG2. Our constructs could be used to study the influence of modifications of certain important functional sites on the induction of immune responses against FMDV and PrV, i.e. the carboxy-terminal part of PrV-gB (Nixdorf et al., 2000) PrV-gB endocytosis (aa 884–913), PrV-gB cell-to-cell spread (aa 824–854) and incorporation of this glycoprotein into virions (aa 854–913).

It might be possible to use these constructs to generate a DNA vaccine against FMDV and PrV. The BT-PrV-gB-C-term construct would probably be unable to protect against PrV-infection, due to a dramatic attenuation of the immune responses against PrV, but it could be useful when pigs have to be free of PrV-antibodies, as in areas where this virus has been eradicated. The BT-PrV-gB-N-term construct greatly attenuated the production of PrV-specific NAb. Its use in protecting against PrV-infection should be experimentally evaluated, as protection against PrV has already been observed in the absence of detectable induction of PrV-specific NAb and with similar levels of IgG1 and IgG2 to those in the present study (Gravier et al., 2007).

5. Conclusion

In conclusion, the concept of a PrV-gB carrier of FMDV epitopes has been validated in the preliminary studies presented here. The levels of NAb produced against FMDV were similar to those reported in other studies involving immunization with FMDV B- and T-cell epitopes in which no NAb (Borrego et al., 2006) or titers between 4 and 16 (Du et al., 2007) were obtained. Nevertheless, strategies to improve immunization efficacy, based on co-injection of adjuvants, electroporation or changing the plasmid backbone, need to be evaluated. Other insertion sites on PrV-gB should also be tested, or other combinations of FMDV B- and T-cell epitopes should be inserted (Borrego et al., 2006). The nature of the immune response against FMDV depends on the site of insertion in PrV-gB. One construct, BT-PrV-gB-N-term, in which BT is inserted between 2 PrV-gB B-cell sites, seems to favor the induction of a balanced cytotoxic and humoral immune response against FMDV (Barnard et al., 2005). The other construct, BT-PrV-gB-C-term, in which FMDV-BT is inserted in a PrV-gB B-cell site, favors the induction of FMDV-specific NAb. Finally, and from a more fundamental point of view, it would be interesting to see how the PrV-gB sites involved in endocytosis and cell-to-cell spread (Nixdorf et al., 2000) influence the induction of immune responses with these different constructs.

Acknowledgments

The authors would like to thank Dr. Belen Borrego (INIA, Valdeolmos, Spain) for providing the plasmid encoding FMDV B- and T-cell epitopes, Dr. J.C. Audonnet (Merial, Lyon, France) for providing PrV glycoproteins and virulent PrV and Dr. F. Lefevre (Inra, Jouy-en-Josas, France) for providing the pcDNA3.1/GM-CSF plasmid. The authors are grateful to B. Jan and other persons on the staff of the healthy pigs production and experimentation section (Afssa, Ploufragan, France) for expert manipulation of the pigs and to A. Henry (Afssa, Ploufragan, France), F. Lebreton and C. Fays (Afssa, Maisons-Alfort, France) for their excellent technical assistance. This study was supported by grant QLK2-CT-2002-01204, *FMDnaVacc* project, from the European Commission.

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