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7 **PSEUDORABIES VIRUS GLYCOPROTEIN B CAN BE USED**
8 **TO CARRY FOOT AND MOUTH DISEASE ANTIGENS IN**
9 **DNA VACCINATION OF SWINE**

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13 Running title: anti-FMDV responses after PrV-gB / FMDV
14 BT injection
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43 **Abstract**

44

45 To evaluate the feasibility of using Pseudorabies Virus (PrV) glycoprotein B (gB) as a carrier
46 of Foot and Mouth Disease Virus (FMDV) antigens in DNA immunization, FMDV B- and T-
47 cell epitopes were inserted either between the 2 B-cell epitopes of the N-term subunit of PrV
48 gB (BT-PrVgB-N term construct) or within the B-cell epitope of the C-term subunit of PrV
49 gB (BT-PrVgB-C term construct). Two animal experiments were performed, each with 3
50 injections of plasmids 2 weeks apart, followed by a booster inoculation of peptides
51 corresponding to the FMDV epitopes. Control groups of pigs were injected with plasmids
52 encoding either PrV gB or FMDV BT, or with empty pcDNA3. The results of both assays
53 were combined. Significant titers of FMDV neutralizing antibodies were detected after the
54 peptides boost in groups injected with BT-PrVgB-C term. Insignificant amounts were
55 detected in groups injected with BT-PrVgB-N term and FMDV BT constructs. PBMCs from
56 the BT-PrVgB-N term groups, isolated after the peptide boost injection, produced IFN- γ and
57 IL-4 mRNAs in vitro when stimulated with FMDV peptides. This was not observed with the
58 other groups. These results imply that PrV gB can be used to carry FMDV antigens in a DNA
59 vaccine.

60

61

62 **Keywords:** Foot and mouth disease virus; Pseudorabies virus glycoprotein B; B- and T-cell
63 epitopes; carrier of antigens; DNA vaccination

64

65 **1. Introduction**

66 Foot and Mouth Disease Virus (FMDV) is the etiological agent of an important disease of
67 livestock. FMD is highly contagious and affects cloven-hoofed animals, mostly cattle, swine,
68 sheep and goats. FMDV belongs to the Aphthovirus genus of the Picornaviridae family and is
69 classified into 7 serotypes (Bachrach, 1968; Rodrigo and Dopazo, 1995; Sobrino et al., 2001).
70 One strategy employed to control disease propagation consists of regular vaccination with an
71 inactivated whole virus antigen combined with an adjuvant (Barteling and Vreeswijk, 1991;
72 Grubman, 2005; Saiz et al., 2002) and has resulted in eradication of the disease in some parts
73 of the world (particularly Western Europe) (Sobrino et al., 2001). Pigs were protected against
74 experimental FMDV challenges 4 days after vaccination (Salt et al., 1998) but no antibodies
75 were detected at this time point. A Th1/Th2 balanced immune response was characterized 14
76 to 21 days after emergency vaccination (Barnard et al., 2005). As these vaccines have certain
77 drawbacks (Cox et al., 2003; Grubman and Baxt, 2004; King et al., 1981), much effort has
78 been made to develop efficient alternative vaccines, such as proteins, peptides, replicating
79 vectors or DNA vaccines (Grubman, 2005). In the latter case, plasmids encoding large
80 fragments of an FMDV genome-like P1-2A3C3D construct (Cedillo-Barron et al., 2001), VP1
81 (Li et al., 2007; Park et al., 2006; Xiao et al., 2007) or FMDV B and T cell epitopes (Borrego
82 et al., 2006; Cedillo-Barron et al., 2003; Fan et al., 2007; Wong et al., 2002; Zhang et al.,
83 2003) were tested. Considerable progress has been made to enhance DNA vaccination
84 efficacy but at least 2 to 3 doses of plasmids still need to be injected (Borrego et al., 2006;
85 Cedillo-Barron et al., 2001; Chen and Shao, 2006; Li et al., 2006).

86 In contrast, a single injection of the Pseudorabies Virus (PrV) specific DNA vaccine induced
87 immune responses against PrV and clinical protection against an experimental lethal PrV
88 infection (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). Among the 3 PrV
89 glycoproteins encoded by the DNA vaccine, PrV gB is of particular interest. This is a 913-

90 amino acid protein which contains a transmembrane domain and a furin cleavage site. This
91 protein is highly immunogenic since 2 B-cell epitope sites (aa 59-126 and 214-279) have been
92 identified in the N-term subunit and one B-cell epitope site (aa 540-734) in the C-term subunit
93 site (Zaripov et al., 1999; Zaripov et al., 1998). Furthermore, sequences allowing PrV-gB
94 endocytosis (aa 884-913), PrV-gB cell to cell spread (aa 824-854) and incorporation of this
95 glycoprotein into virions (aa 854-913) have been well documented (Nixdorf et al., 2000).
96 Thus, as PrV-gB is able to go both inside and outside cells, the opportunities for this
97 glycoprotein to encounter an efficient immune cell may be increased.

98 Therefore, the aim of the present study was to determine the feasibility of using PrV-gB to
99 carry FMDV epitopes in a DNA vaccine. The strategy differs from the one previously
100 published for BHV-1 gB (Keil et al., 2005) in that the carried antigens are not released from
101 gB but benefit from the immune properties of gB by staying attached to this glycoprotein. The
102 FMDV B epitope of VP1 is reported to elicit the production of neutralizing antibodies
103 (Francis et al., 1987). The T cell epitope of VP4 is capable of assisting a B-cell epitope when
104 in tandem (Blanco et al., 2000). These different items i.e. the FMDV type C, isolate C-8Sc1,
105 B-cell epitope (aa 133-156 of VP1) fused to the T-cell epitope (aa 20-34 of VP4), which have
106 been previously evaluated in mice (Borrego et al., 2006), were here inserted into PrV-gB. The
107 method used to insert the FMDV epitopes was therefore based on information in the
108 literature. The protein is known to contain 3 B cell epitopes which means that these sites are
109 recognized by the immune system and are therefore situated on the protein surface. Two
110 constructs were evaluated. The first was obtained by inserting FMDV BT between the 2 B-
111 cell epitopes of the N-term subunit of PrV-gB. The second was obtained by inserting the
112 FMDV epitopes into the B-cell epitope of the C-term subunit of PrV-gB. The immune
113 responses against FMDV obtained with these constructs were compared to those obtained
114 with a plasmid encoding the FMDV B- and T-cell epitopes. As the immune potentials of these

115 constructs are unknown, the likelihood of measuring subsequent events was maximized by
116 administering 3 injections of plasmids 2 weeks apart followed by a booster inoculation with
117 the corresponding B- and T-cell epitope peptides, 2 weeks after the last plasmids injection.
118 The immune responses against FMDV and PrV were analysed after each injection.

119

120 **2. Materials and methods**

121

122 **2.1 Construction and in vitro characterization of plasmids**

123 The pcDNA3 plasmid encoding PrV glycoprotein gB has already been constructed,
124 characterized and successfully used in our laboratory (Dory et al., 2005a; Dufour et al., 2000).

125 The pGEM-based plasmids encoding the fused FMDV type C, isolate C-8Sc1, B-cell epitope
126 (aa 133-156 of VP1) and T-cell epitope (aa 20-34 of VP4) were kindly provided by Belen
127 Borrego (INIA, Valdeolmos, Spain). The FMDV BT construct was subcloned into the
128 pcDNA3 expression cassette after *Bam*HI and *Not*I digestions. In order to insert the FMDV
129 BT sequence between the 2 B-cell epitopes of the N-term subunit of the PrV gB gene without
130 modifying the reading frame of gB, the BsiWI site (at the level of aa 187-189) was chosen
131 (Fig. 1). BsiWI restriction sites were introduced on the FMDV BT insert by performing PCR
132 on pGEM-FMDV BT with the following primers: 5'CGCATG**CGTACGG**CCGC3' and
133 5'ATTAGATC**CGTACG**TGGAGTT3' (BsiWI specific site is in bold and underlined). The
134 PCR product and pcDNA3-PrV gB were digested with BsiWI (New England Biolabs)
135 according to the manufacturer's instructions. After dephosphorylation of the digested
136 pcDNA3-PrV gB, the digested PCR product was inserted into the PrV gB gene. The resulting
137 plasmid was called BT-PrVgB–N term. In order to insert the FMDV BT sequence into the B-
138 cell epitope of the C-term subunit of the PrV gB gene without modifying the reading frame of
139 gB, we chose the FspAI restriction site (at the level of aa 682), which generates blunt ends.
140 Appropriate restriction sites generating blunt ends on the FMDV BT insert were produced by
141 performing a PCR on pGEM-FMDV BT with the following primers:
142 5'TGGAT**CCCGGG**CTACGAC3' containing a *Sma*I restriction site (in bold and underlined)
143 and 5'CTACATGG**AGTACT**TGGTACTG3' containing a *Sca*I restriction site (in bold and
144 underlined). The PCR product was first cloned into a TOPO-TA vector (Invitrogen). The
145 resulting vector was then digested with *Sma*I and *Sca*I and the insert transferred into

146 pcDNA3-PrV gB digested with *FspAI*. The resulting plasmid was called BT-PrVgB–C term.
147 Each construct was further characterized according to restriction patterns and sequencing (not
148 shown). Porcine kidney cell line 15 (PK15) was transfected with 2 µg of each plasmid by
149 using lipofectamine plus transfection reagent (Invitrogen, Gaithersburg, MD, USA) according
150 to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were stained
151 to reveal the expression of either PrV gB or FMDV BT. For PrV gB, the cells were
152 successively incubated with pig PrV-specific hyperimmune antiserum, with HRP-labeled
153 rabbit anti-swine IgG at 1:1000 (Sigma, Saint-Louis, MI, USA) and with the 3-amino-9-
154 ethylcarbazole peroxidase substrate (Serotec Ltd, Oxford, UK) according to the
155 manufacturer’s instructions (Fig. 1). For FMDV BT, the cells were successively incubated
156 with the monoclonal GB1 antibody at 1:100, with HRP-labelled rabbit anti-mouse IgG at
157 1:1000 (Dako, Trappes, France) and with the 3-amino-9-ethylcarbazole peroxidase substrate
158 (Serotec Ltd).

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

162

163 **2.2 Animal experiments**

164 Specific pathogen-free pigs were housed and treated in accordance with local veterinary
165 office regulations (Direction des Services Vétérinaires des Côtes d’Armor, France). In the
166 first assay, 5 groups of 4 pigs weighing 26.0 ± 0.7 kg at the 1st injection were used. Group 1
167 received the BT-PrVgB–N term-encoding plasmid. Group 2 received the BT-PrVgB–C term-
168 encoding plasmid. Group 3, which received uncarried FMDV BT, was injected with
169 pcDNA3-FMDV BT. Group 4 was injected with pcDNA3-PrV gB in order to evaluate the
170 immune responses against PrV induced by the unmodified PrV gB. Finally, the negative

171 control group 5 was injected with empty pcDNA3. All plasmids were co-injected with
172 pcDNA3-GM-CSF (previously used in our laboratory (Dufour et al., 2000)) as adjuvant and
173 administered 3 times by both intramuscular (i.m.) and intradermal (i.d.) routes at 2-week
174 intervals. One ml of sterile saline solution containing 150 µg of the plasmids of interest and
175 50 µg of pcDNA3-GM-CSF were injected by i.m. route in both sides of the neck using 0.8
176 mm x 40 mm needles. 0.25 ml of saline solution containing 150 µg of plasmids of interest and
177 50 µg of pcDNA3-GM-CSF were injected by i.d. route into the dorsal surface of both ears
178 using 0.45 mm x 12 mm needles. The i.d. injection was controlled by (i) parallel position of
179 the needle to the ear surface, (ii) high pressure applied to the syringe to inject the solutions
180 and (iii) the transient generation of white spots. The pigs then received 100 µg of the FMDV
181 T cell peptide and 100 µg of the FMDV B cell peptide, 2 weeks after the last plasmids
182 injection. A second assay, with 8 animals per group and 4 animals in the two control groups,
183 was carried out.

184 The pigs were carefully observed for any adverse reaction after injection. Body
185 temperature was measured daily and 4 hours after each injection. Relative daily weight gains
186 were determined (Stellmann et al., 1989) for each pig. Some pigs were sacrificed during or at
187 the end of the assays for ethical reasons. The injected areas were examined to see whether or
188 not the injection of plasmids or expression of the encoded proteins produced lesions. Other
189 organs were also examined.

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

194

195 **2.3 FMDV neutralizing antibodies (NAb).**

196 FMDV neutralizing antibody assays were carried out in 96-well plates as described in the OIE
197 Manual of Standards (OIE, 2000). Serial dilutions of sera were prepared in duplicate and 50µl
198 of each were added for 1 h to 50µl of 100 TCID₅₀ of the FMDV C1Noville strain. Cell
199 suspension was then added to each well and the plates were incubated at 37°C for 3 days. The
200 cells were fixed with formalin and stained with methylene blue. Titers were expressed as the
201 last serum dilution that inhibited viral replication in 50% of the wells.

202

203 **2.4 Determination of FMDV-specific serum antibodies.**

204 Anti FMDV type C antibodies were detected by applying a competitive Elisa test as described
205 by Mackay DK *et al* (Mackay et al., 2001). Briefly, 96-well, flat-bottomed plates (Maxisorp;
206 Nunc, Roskilde, Denmark) were coated with a rabbit anti FMDV C1 strain serum diluted in
207 carbonate/bicarbonate buffer pH 9.6 (Sigma, Saint Louis, MO, USA). After 3 washings with
208 PBS, the plates were incubated with FMDV antigen (C 1 Noville) diluted in PBS-tween 20
209 0.05% buffer supplemented with 10% bovine serum and 5% rabbit serum (blocking buffer)
210 for 1 h at 37°C. After washings, samples of pig sera diluted 1/5 in blocking buffer were added
211 in duplicate and a diluted guinea-pig anti type C was also added as a competitor in each well.
212 After washings and incubation with a HRP-conjugated anti guinea-pig serum, the reaction
213 was revealed with an OPD solution (Sigma). Results were expressed as the percentage
214 inhibition of the optical density (OD) obtained with the guinea-pig serum anti type C1 in the
215 antigen control well.

216

217

218 **2.5 PrV neutralizing antibodies (NAb)**

219 After complement inactivation (30 minutes at 56°C), 50 µL of 2-fold dilutions of serum were
220 incubated with 50 µL of 100 TCID₅₀ of NIA3 PrV strain in 5% CO₂ in 96-well plates for 1h at

221 37°C. 150 µL of PK15 cells (2.25×10^4 cells/150 µL) were then added and incubated in 5%
222 CO₂ for 5 days at 37°C.

223 NAb titers were expressed as the highest serum dilution inhibiting the cytopathic effect in 2
224 out of 4 wells containing the PrV-infected PK15 cell line.

225

226 **2.6 Determination of PrV-specific IgG1 and IgG2 serum antibodies (Ab)**

227 Anti-PrV IgG1 and IgG2 serum Ab titers were determined by indirect ELISA as previously
228 described (Dory et al., 2005b). Briefly, Maxisorb 96-well plates (Nunc, Naperville, IL) were
229 coated with PrV glycoproteins, kindly provided by J.C. Audonnet (Merial, Lyon, France), and
230 successively incubated with serial threefold dilutions of serum, with mouse anti-porcine IgG1,
231 IgG2 or total IgG (Serotec Ltd, Oxford, UK), with HRP-labeled rabbit anti-mouse IgG
232 (Jackson Laboratories, West Grove, Pennsylvania, USA) and finally with the peroxidase
233 substrate tetramethyl benzidine (Pierce, Rockford, IL, USA). The enzyme reaction was then
234 stopped by adding sulfuric acid. The ODs were measured at 450 nm. IgG1 and IgG2 titers
235 (\log_{10}) were obtained from the highest dilution that gave a higher OD value than the threefold
236 OD of a control serum from non-vaccinated and non-infected pigs.

237

238

239 **2.7 Quantification of porcine IFN- γ and interleukin 4 (IL-4) mRNA produced by** 240 **stimulated Peripheral Blood Mononuclear Cells (PBMC)**

241 PBMC were isolated from blood collected before plasmid injection, 2 weeks after each
242 plasmid injection and 1, 2 and 3 weeks after the peptides boost. PBMC were either incubated
243 *in vitro* for 16 hours with PrV strain NIA3 (multiplicity of infection: 1), with 5 ng of FMDV
244 T cell and B cell peptides, or with the RPMI culture medium alone. PBMC total RNA was
245 isolated using the 96 RNEasy kit (Qiagen, Hilden, Germany). Porcine IFN- γ and porcine IL-4

246 mRNA expressions were determined by quantitative real-time polymerase chain reaction
247 (PCR) using primers, probes and PCR conditions as previously described (Dory et al., 2005a).
248 Cytokine mRNA and β -actin mRNA threshold cycles (Ct) were determined simultaneously
249 for each sample and the relative quantities determined according to User Bulletin number 2,
250 ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The amount of cytokine
251 mRNA was standardized with the internal β -actin mRNA reference ($\Delta\text{Ct} = \text{cytokine Ct} - \beta$ -
252 actin Ct) and quantified in relation to the non-stimulated sample ($\Delta\Delta\text{Ct} = \Delta\text{Ct}$ of the
253 stimulated sample - ΔCt of the non-stimulated sample) according to equation $2^{-\Delta\Delta\text{Ct}}$.

254

255 **2.8 Statistical analysis**

256 The data were analysed using the nonparametric Mann-Whitney test (Mann and Whitney,
257 1947) included in the Systat 9 software (Systat Software, Inc., Point Richmond, CA, USA).
258 This test was applied because the generated data were few in number, did not present a
259 normal distribution and consisted of unpaired quantitative data.

260 The limit of significance was 0.05 for all comparisons.

261

262

263 **3. Results**

264 **3.1 Constructs**

265 Two plasmid constructs encoding PrV-gB/FMDV BT were obtained. Porcine PK15 cells
266 transfected with each construct were stained with a pig PrV hyper-immune serum and an
267 antibody directed against the B cell epitope of the FMDV used here (GB1) (Fig. 1). Each
268 PrV-gB based construct was detected by the PrV hyperimmune serum. Cells transfected with
269 the FMDV BT encoding plasmid were not detected by the GB1 anti-FMDV monoclonal
270 antibody, whereas under the same experimental conditions both PrV gB / FMDV BT chimeric
271 constructs were detected.

272

273

274 **3.2 Induction of immune responses against FMDV in pigs**

275 The plasmids were injected by i.m. and i.d. routes 3 times at 2-week intervals. Two weeks
276 after the last injection, individual FMDV B and T cell peptides were injected by the same
277 routes. The different injections were well tolerated. The animals grew normally and gained
278 approximately 1 kg per day in all groups in both assays. No fever peaks were observed 4
279 hours after any of the injections or during any of the daily measurements, except in 1 pig in
280 the BT-PrVgB-N term group of the 1st assay which had a body temperature of 40.3°C, 72
281 hours after the 3rd injection of the plasmids. This body temperature had returned to normal by
282 the next day. Several pigs in the second assay had to be euthanized for reasons unrelated to
283 immunization. One pig in the BT-PrVgB-N term group broke a hoof and had to be sacrificed
284 after the 3rd injection. One pig in the FMDV-BT group walked with a limp and was sacrificed
285 after the second injection and another, presenting a rectal prolapse, was euthanized one week
286 after the peptides injection. Finally, one aggressive pig in the group injected with empty-
287 pcDNA3 was euthanized after the peptides injection.

288 Experiments 1 and 2 were combined to determine the induction of immune responses, and the
289 average and standard deviation values were determined for each group. Insignificant amounts
290 of FMDV neutralizing antibodies were first detected 2 weeks after the second injection of
291 plasmids in the BT-PrVgB-C term group (Fig. 2). Statistically significant titers were only
292 observed in this group 1 and 3 weeks after the FMDV B and T peptides boost ($p < 0.05$). NAb
293 were also detected in the BT-PrV gB-C term and FMDV BT groups but the titers, in all cases,
294 were not statistically significant. Furthermore, NAb production in the FMDV BT group was
295 transient and could no longer be detected 3 weeks after the peptides boost, whereas it was still
296 observed in the BT-PrVgB-C term injected group. FMDV-specific antibodies were found at
297 the limit of detection in sera from 3 pigs in the BT-PrVgB-N term and in 2 out of 12 pigs in
298 the BT-PrVgB-C term injected groups. These antibodies were not detected in any of the other
299 groups.

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

311

312 **3.3 Induction of immune responses against PrV in pigs**

313 PrV neutralizing antibodies were produced in significant amounts 1 week after the third
314 injection of pcDNA3 PrV-gB (Fig. 4) and remained at a significant level until the end of the
315 assay. Significant amounts of PrV NAb were produced in the BT-PrVgB- N term and C term
316 groups, 2 and 3 weeks after the third plasmids injection. Nevertheless, except for the BT-PrV
317 gB-N term group 3 weeks after the third injection, the NAb titers were significantly lower
318 than in the PrV gB injected group. No significant NAb production was observed in the two
319 groups that received FMDV BT / PrV gB chimeric constructs, from the fourth week after the
320 last plasmids injection. Significant amounts of PrV-specific IgG1 were first detected 1 week
321 after the second plasmids injection in the groups of pigs injected with PrV gB or BT-PrVgB-
322 N term constructs ($p < 0.05$). IgG1 production was then maintained at a significant level until
323 the end of the assay. In contrast, production in the BT-PrV gB-C term group was
324 systematically and significantly lower from the first week after the second injection until the
325 end of the assay ($p < 0.01$).

326 IFN- γ and IL-4 mRNAs production was observed in PrV-stimulated PBMCs from pigs
327 injected with PrV gB, BT-PrV gB-N term and BT-PrV gB-C term constructs from week 2
328 after the second injection to week 2 after the third injection of plasmids ($p < 0.05$) (Fig. 5).
329 From week 1 to week 3 after the FMDV B and T peptides injection, all 3 groups produced
330 significant amounts of IFN- γ mRNA except for the Bt-PrvV gB-C term group at weeks 2 and
331 3, the BT-PrV gB-N term group at week 2 and the PrV gB group at week 3. The same was
332 true for IL-4 mRNA production, except for the BT-PrV gB-C term group at weeks 1 and 2
333 after the peptides boost, and for the BT-PrV gB-N term group at week 2. No production of
334 IFN- γ and IL-4 RNAs was observed in the other 2 groups throughout the assay.

335

336 **4. Discussion**

337 The production of an efficient DNA vaccine against FMDV represents a challenge for the
338 scientific community. Such a vaccine would provide an attractive alternative to the
339 conventional inactivated FMDV vaccine which often requires 3 injections of plasmids
340 (Bergamin et al., 2007; Cedillo-Barron et al., 2001; Cedillo-Barron et al., 2003; Li et al.,
341 2006). A prime-boost strategy, (Li et al., 2008) using inactivated FMDV as a booster to
342 significantly improve the efficacy of FMDV DNA vaccine, has recently been published. (Li et
343 al., 2008) The results reflect the difficulty of generating a powerful DNA vaccine against
344 FMDV that confers immunity after 1 or 2 injections of plasmids, as with anti-PrV vaccination
345 (Gravier et al., 2007). Our goal in this study was to see if the glycoprotein B of PrV could
346 serve as a carrier of FMDV antigens in a DNA vaccine. This glycoprotein was selected
347 because it is already used in a successful one-shot DNA vaccine combination against PrV-
348 infection in swine (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). It is also an
349 immunogenic protein (Zaripov et al., 1999; Zaripov et al., 1998) associated with functional
350 domains that enable it to be internalized and to go outside the cell (Nixdorf et al., 2000). It
351 might therefore be possible to take advantage of these characteristics to carry and present
352 foreign antigens. A modified gB of Bovine Herpesvirus 1 (BHV-1) was recently used to
353 transport foreign proteins (Keil et al., 2005). The transport and release of foreign proteins
354 inserted between the furin sites, was in fact facilitated by inserting a second furin cleavage
355 site into this glycoprotein. The concept in the present study is different since gB is expected
356 not only to transport, but also to present foreign antigens. FMDV B and T cell epitopes have
357 already been tested in DNA vaccines or recombinant vaccines to immunize mice or pigs
358 against FMDV. When mice were injected 3 times with a plasmid encoding these epitopes, no
359 neutralizing antibodies were produced and no viremia was present in half of them post-
360 challenge (Borrego et al., 2006). Fusion of these epitopes to a signal peptide produced NAb in

361 1 out of 4 mice. Co-injection of the fungus *Agaricus blazei murill* (Chen and Shao, 2006) or
362 fusion to swine IgG (Wong et al., 2002) were shown to enhance DNA vaccination against
363 FMDV in mice or pigs, respectively. Finally, the titers of neutralizing antibodies in pigs
364 injected twice with a recombinant adenovirus expressing FMDV B and T cell epitopes were
365 between 4 and 16, and 3 out of 5 pigs were protected (Du et al., 2007). These small FMDV B
366 and T cell epitopes were thus able to induce immunization and/or protection against FMDV,
367 even though the induced humoral immune responses were low or undetectable. In our carrier
368 study, 1 FMDV B-cell epitope (aa 133-156 of VP1) fused with 1 FMDV T-cell epitope (aa
369 20-34 of VP4) (Borrego et al., 2006) was inserted at 2 different sites on PrV-gB. The insertion
370 strategy was based on the knowledge gained from the functional studies of the B-cell epitopes
371 in PrV.

372 The three-dimensional structure of PrV gB has not been determined, but the X-ray structure
373 of gB ectodomain from Herpes simplex virus 1 (HSV-1) is available (Heldwein et al., 2006).
374 PrV and HSV-1 gB share 50% identity, and the high sequence conservation strongly suggests
375 that the PrV gB adopts a structure similar to the one reported for HSV-1. FMDV-BT was
376 inserted in PrV-gB between the two B-cell epitopes located close to the N-terminus of gB
377 (BT-PrV gB-N term), and in the B-cell epitope located in the region preceding C-terminus
378 (BT-PrV gB-C term). The residues in HSV-1 gB, which correspond to the FMDV-BT
379 insertion sites in PrV gB, were identified from the alignment of the PrV and HSV-1 gB
380 sequences. The locations of the PrV insertion sites were then mapped on the HSV-1 gB
381 structure as shown in Figure 6. For the BT-PrV gB-N term construct, the corresponding HSV-
382 1 gB insertion site, residue Y179, is part of the fusion loop of HSV-1 gB. Fusion loops are
383 sequences rich in hydrophobic residues and are typically buried within the protein or in a
384 membrane, suggesting that the N-terminal FMDV-BT epitope may not be fully exposed in
385 PrV-gB. The limited accessibility of the epitope in BT-PrV gB-N term could be one of causes

386 of the inefficient recognition by immune system and low antibody titers. For the BT-PrV gB-
387 C term construct, the homologous insertion site in HSV-1 gB is residue H657, which is
388 located in an exposed beta-strand of domain IV. Based on the HSV-1 gB structure, the C-
389 terminal FMDV-BT epitope would likely localize to the surface of the protein, consistent with
390 its availability for recognition by immune system.

391 The results presented here show that FMDV-BT epitopes could not be detected in vitro by the
392 GB1 monoclonal antibody unless they were carried by PrV-gB. In fact, positively stained
393 cells could be visualised after transfection by either one of the PrV-gB constructs carrying
394 the FMDV-BT epitope. Moreover, PrV-gB was strongly expressed and detected when a pig
395 PrV-hyperimmune serum was used. This implies that multiple epitopes were recognized,
396 whether they were near to the insertion sites or not, suggesting that some of them were not
397 perturbed by the insertion. These findings demonstrate that the concept of PrV-gB as a carrier
398 of FMDV epitopes is feasible, at least in vitro. The immunization potentials of our constructs
399 were evaluated after each of the 3 plasmids injections, as in other studies of DNA vaccination
400 against FMDV. A FMDV peptides boost was then added to observe the production of
401 immune responses against FMDV. The two assays (12 pigs per BT-PrV gB-C term, BT-PrV
402 gB-N term and BT groups or 8 pigs per PrV gB and empty pcDNA3 groups in total) were
403 combined and the averages of the two assays are presented. Significant induction of immune
404 responses against FMDV were observed only after the FMDV B and T peptides boost in the
405 groups primed with plasmids encoding chimeric PrV gB / FMDV BT constructs. This was not
406 observed in the groups that were 3 times injected with empty plasmids or plasmids encoding
407 PrV gB before injection of FMDV B and T peptides. In fact, in these last cases pigs received
408 FMDV antigens only once. FMDV-specific serum antibodies were faintly detected in 5 of the
409 24 pigs that received the FMDV BT / PrV-gB constructs. No specific antibodies were
410 detected in any of the other groups. Significant NAb production against FMDV was only

411 found in the BT-PrVgB-C term construct groups. Non-significant production was detected in
412 the BT-PrV gB-N term group. Although less NAb was produced than with DNA vaccines
413 containing larger FMDV constructs, such as P1-2A3C3D (Cedillo-Barron et al., 2001), the
414 amounts were similar to previous studies with these small epitopes. Borrego *et al.* found that
415 only 1 out of 31 mice produced NAb after 3 injections of DNA vaccines encoding FMDV B
416 and T cell epitopes (Borrego et al., 2006). Pigs injected twice with rAdV expressing FMDV
417 BT and GM-CSF produced NAb titers between 4 and 16 (Du et al., 2007). It is important to
418 note that the B-cell epitope from the CS8 strain encoded by the vaccine differed in 2 amino
419 acids from the one obtained from the C1 Noville strain used in the neutralizing assay. One of
420 these amino acids is located in an area (aa 146-156) important for NAb production (Francis et
421 al., 1987). It is therefore possible that the NAb produced was less able to inhibit C1 Noville
422 during *in vitro* replication and that the NAb has thus been under-estimated. Furthermore, the
423 conformations of the FMDV B and T cells may be modified by their insertion into PrV gB,
424 and the induction of immune responses may potentially be perturbed and decreased. Another
425 important element in protection against FMDV is the cellular immune response (Borrego et
426 al., 2006; Sobrino et al., 2001). The BT-PrVgB-N term group seemed to favour the
427 production of IFN- γ mRNA (a marker of the cellular immune response) by PBMCs stimulated
428 by FMDV B and T cell epitope peptides. Significant amounts of IL-4 mRNA (a marker of
429 induction of humoral immune responses) were detected in the same group. These results
430 suggest that both cellular and humoral immune responses were induced in this group, as in
431 efficient emergency FMDV vaccination (Barnard et al., 2005). However, the detection of
432 cytokine mRNAs does not necessarily imply that the corresponding proteins are induced.
433 These results should therefore be interpreted with caution. Barnard *et al.* showed that the
434 production of cytokine mRNAs by FMDV-stimulated PBMCs from FMDV vaccinated pigs
435 was correlated, in most cases, with the production of cytokines (Barnard et al., 2005). In some

436 rare cases, the cytokines were not detected, whereas the mRNAs were. We can therefore be
437 relatively confident of the results interpretation. Furthermore, the results presented here are
438 not predictive of the ability of the constructs to protect pigs against FMDV infection. This can
439 only be evaluated experimentally. Previous studies showed that although these FMDV B and
440 T cell epitopes induced no or only low titers of NAb, they were able to protect mice (Borrego
441 et al., 2006) and pigs (Du et al., 2007) against FMDV challenges. Other examples of
442 protection in the presence of low titers of FMDV NAb are reported in the literature (Sobrino
443 et al., 2001).

444

445 The immune responses to PrV-gB were equivalent to those previously reported after 3
446 injections of plasmids in pigs (van Rooij et al., 2000). Insertion of the FMDV epitopes into
447 the B-cell epitope of the C-terminal region of PrV-gB, which is described as a strong
448 conformational B-cell epitope with neutralizing activity (Zaripov et al., 1998), greatly reduced
449 the humoral immune response against the PrV studied here. In contrast, insertion of the
450 FMDV epitopes between the 2 B-cell epitopes close to the N-terminus of PrV-gB, described
451 as linear epitopes inducing low antibody responses (Zaripov et al., 1998), abolished or
452 strongly reduced the production of PrV NAb, but not of IgG1 and IgG2. Our constructs could
453 be used to study the influence of modifications of certain important functional sites on the
454 induction of immune responses against FMDV and PrV i.e. the carboxy-terminal part of PrV-
455 gB (Nixdorf et al., 2000) PrV-gB endocytosis (aa 884-913), PrV-gB cell to cell spread (aa
456 824-854) and incorporation of this glycoprotein into virions (aa 854-913).

457 It might be possible to use these constructs to generate a DNA vaccine against FMDV and
458 PrV. The BT-PrVgB-C term construct would probably be unable to protect against PrV
459 infection, due to dramatic attenuation of the immune responses against PrV, but it could be
460 useful when pigs have to be free of PrV-antibodies, as in areas where this virus has been

461 eradicated. The BT-PrVgB–N term construct greatly attenuated the production of PrV specific
462 NAb. Its use in protecting against PrV-infection should be experimentally evaluated as
463 protection against PrV has already been observed in the absence of detectable induction of
464 PrV-specific NAb and with similar levels of IgG1 and IgG2 to those in the present study
465 (Gravier et al., 2007).

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467

468 **5. Conclusion**

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

485

486

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488

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498

499 **Figure legends**

500 **Figure 1: constructs** Porcine PK15 cells were transfected with pcDNA3 based plasmids
501 encoding PrV-gB, BT-PrV gB N term, BT-PrV gB C term or FMDV BT. Twenty-four hours
502 later, cells were stained with pig PrV hyperimmune serum or anti-FMDV B epitope GD1
503 monoclonal antibody as described in materials and methods.

504
505 **Figure 2: Anti-FMDV neutralizing antibodies** Anti-FMDV neutralizing antibodies before
506 the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated
507 with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of
508 experiments 1 and 2 \pm SD are shown for each group indicated in the legend box .

509 *a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups

510 *b: $p < 0.05$ compared to the PrV gB, Empty pcDNA3 and FMDV BT groups

511
512 **Figure 3: IFN- γ and IL4 mRNA relative expressions by FMDV B and T cell peptides**
513 **stimulated PBMCs**

514 The cells were isolated before the first injection and after each of the 3 injections of plasmids
515 (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an
516 arrow). The cells were then incubated with FMDV B and T peptides or with culture medium
517 for 16 hours. IFN- γ and IL4 mRNA relative expressions were determined. Average titers of
518 experiments 1 and 2 \pm SD are shown for each group indicated in the legend box .

519 *a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups

520 *b: $p < 0.05$ compared to all the other groups

521 *c: $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups

522 *d: $p < 0.05$ compared to the PrV gB, BT-PrV gB-N term and Empty pcDNA3 groups

523
524 **Figure 4: Anti-PrV neutralizing, IgG1 and IgG2 antibodies production.**

525 Sera from pigs in both assays were collected before the first injection and after each of the 3
526 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P,
527 indicated with an arrow). Average titers of experiments 1 and 2 \pm SD are shown for each
528 group indicated in the legend box .

529 *a: $p < 0.05$ compared to all the other groups

530 *b: $p < 0.05$ compared to the BT-PrV gB-C term, FMDV BT and Empty pcDNA3 groups*c:

531 $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups

532 **: $p < 0.01$ compared to the PrV gB and BT-PrV gB-N term groups

533
534 **Figure 5: IFN- γ and IL4 mRNA relative expressions by PrV stimulated PBMCs**

535 The cells were isolated before the first injection and after each of the 3 injections of plasmids
536 (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an
537 arrow). The cells were then incubated with live PrV or with culture medium for 16 hours.
538 IFN- γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1
539 and 2 \pm SD are shown for each group indicated in the legend box.

540 * $p < 0.05$ compared to the FMDV BT and Empty pcDNA3 groups

541
542
543 **Figure 6: Location of the PrV gB FMDV epitope insertion sites mapped on the structure**

544 **of HSV-1 gB. A)** Ectodomains of HSV-1 gB form trimers. Each monomer is colored as blue,
545 green or red (left panel), and a space-filled model of the gB trimeric surface is shown on the
546 right. Y167 and H657 are the HSV-1 residues that correspond to the insertion sites of the

547 FMDV epitopes in PrV gB. HSV-1 gB fusion loops, which are the residues proposed to insert
548 into the target membrane during fusion, are marked. **B, C)** Only domains I and IV,
549 respectively, are shown for clarity, and insertion sites Y167 and H657 are labelled. Residue
550 Y167, which would correspond to the N-term FMDV epitope insertion, seems less accessible
551 than the fully exposed H657, which is analogous to the position where the C-term FMDV
552 epitope was added. This suggests that the latter position might be a better insertion target.

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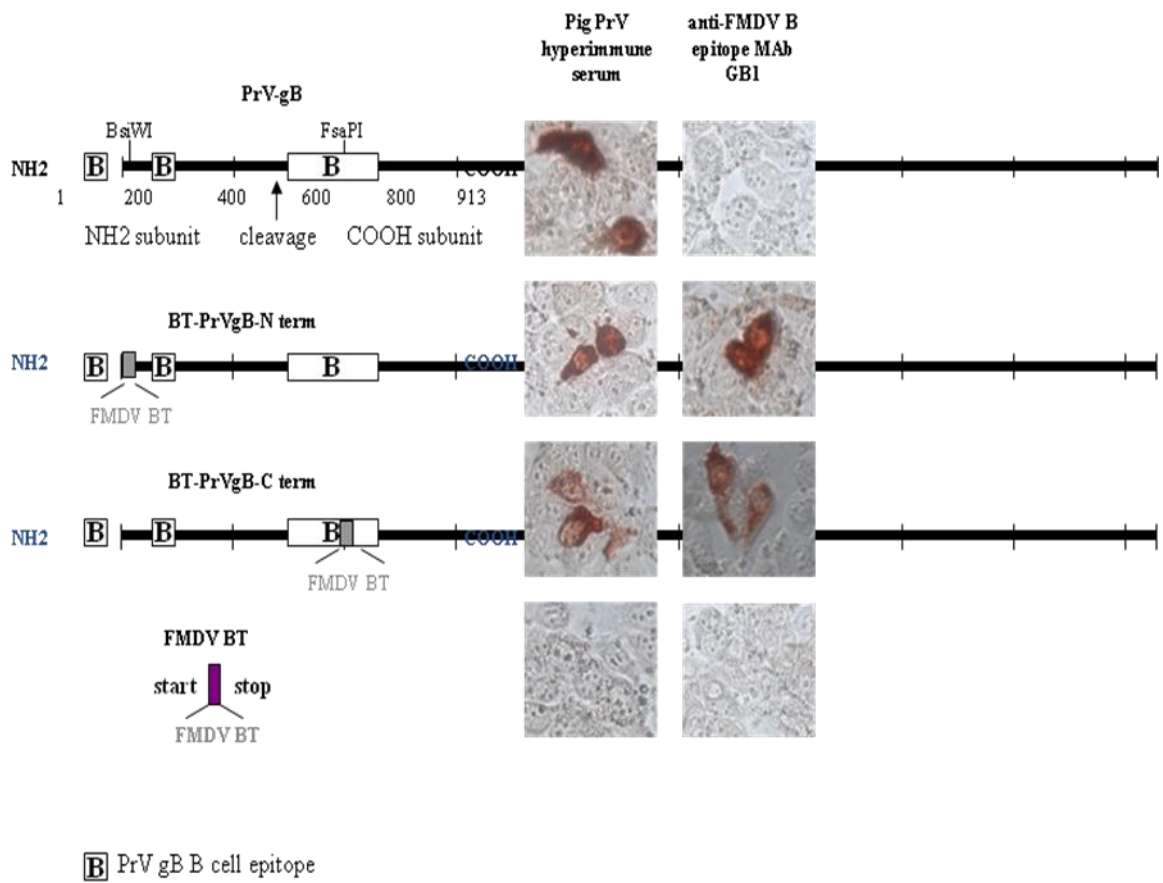


Figure 1: constructs

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 materials and methods.

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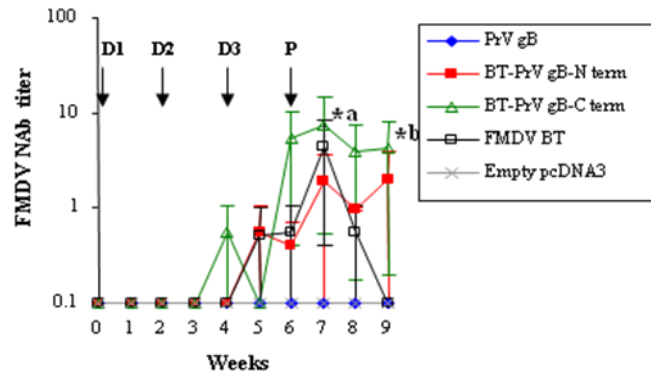


Figure 2: Anti-FMDV neutralizing antibodies

Anti-FMDV neutralizing antibodies before the first injection and after each of the 3 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 SD 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

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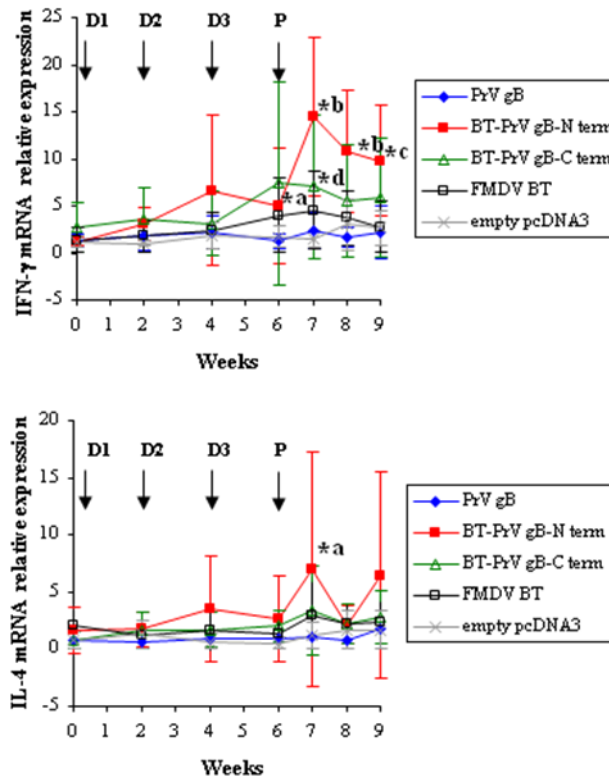


Figure 3: IFN- γ and IL4 mRNA relative expressions by FMDV B and T cell peptides stimulated PBMCs

The cells were isolated before the first injection and after each of the 3 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 or with culture medium for 16 hours. IFN- γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 \pm SD 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

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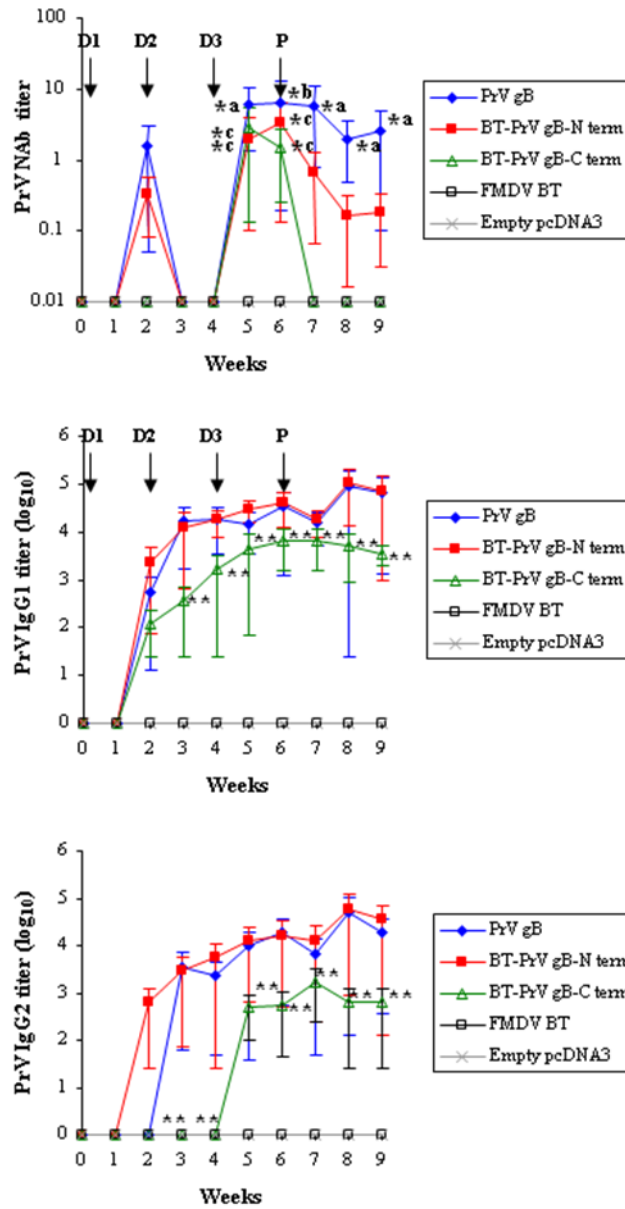


Figure 4: Anti-PrV neutralizing, IgG1 and IgG2 antibodies production.

Sera from pigs in both assays were collected before the first injection and after each of the 3 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 SD 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

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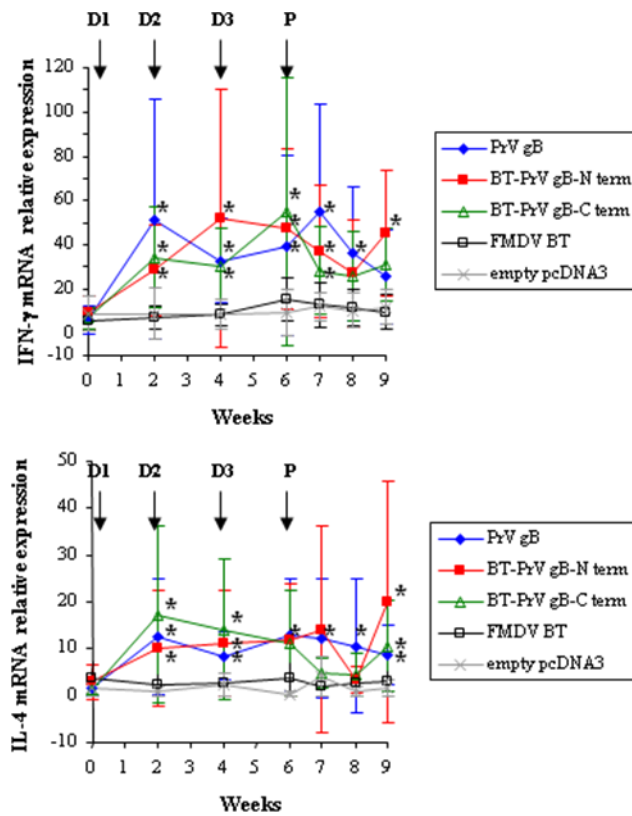


Figure 5: IFN- γ and IL4 mRNA relative expressions by PrV stimulated PBMCs

The cells were isolated before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were than incubated with live PrV or with culture medium for 16 hours. IFN- γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 \pm SD are shown for each group indicated in the legend box.

*p < 0.05 compared to the FMDV BT and Empty pcDNA3 groups

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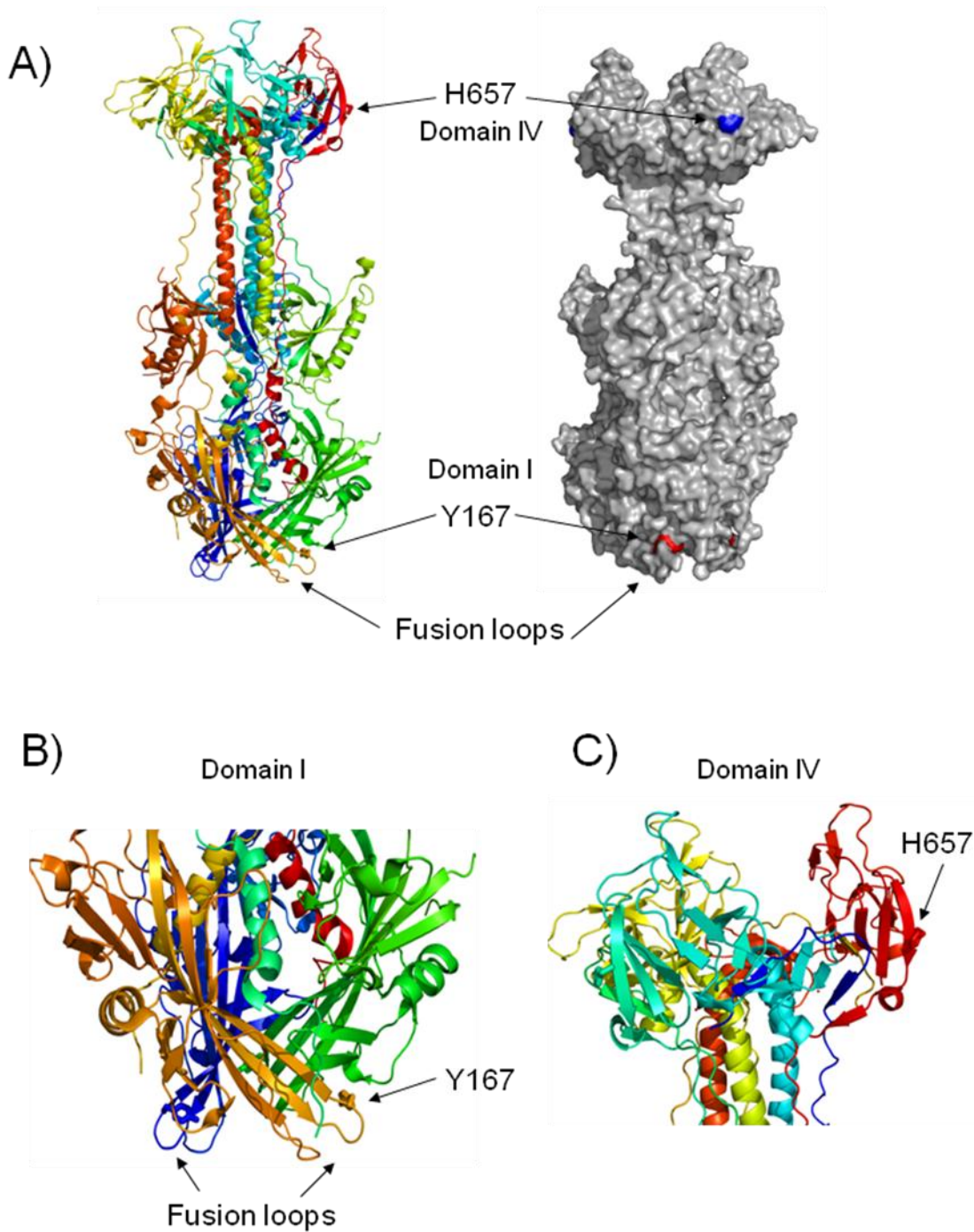


Figure 6: Location of the PrV gB FMDV epitope insertion sites mapped on the structure of HSV-1 gB. A) Ectodomains of HSV-1 gB form trimers. Each monomer is colored as blue, green or red (left panel), and a space-filled model of the gB trimeric surface is shown on the right. Y167 and H657 are the HSV-1 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, 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.