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Original article

Genetic immunisation of cattle against Bovine herpesvirus 1: glycoprotein gD confers higher protection than glycoprotein gC or tegument protein VP8

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Abstract – Bovine herpesvirus 1 (BoHV-1) has frequently been used as a model for testing parameters affecting DNA immunisation in large animals like cattle. However, the selection of target antigens has been poorly studied, and most of the experiments have been conducted in mice. In the present study, we demonstrated in cattle that a DNA vaccine encoding BoHV-1 glycoprotein gD induces higher neutralising antibody titres than vaccines encoding BoHV-1 gC. Additionally, we show that a DNA vaccine encoding a secreted form of gD induces a higher immune response than a vaccine encoding full-length gD. However, the enhanced immunogenicity associated with the secretion of gD could not be extended to the glycoprotein gC. The current study also describes for the first time the development and the evaluation of a DNA vaccine encoding the major tegument protein VP8. This construct, which is the first BoHV-1 plasmid vaccine candidate that is not directed against a surface glycoprotein, induced a high BoHV-1 specific cellular immunity but no humoral immune response. The calves vaccinated with the constructs encoding full-length and truncated gD showed a non-significant tenfold reduction of virus excretion after challenge. Those calves also excreted virus for significantly ($p < 0.05$) shorter periods (1.5 days) than the non-vaccinated controls. The other constructs encoding gC and VP8 antigens induced no virological protection as compared to controls. Altogether the DNA vaccines induced weaker immunity and protection than conventional marker vaccines tested previously, confirming the difficulty to develop efficient DNA vaccines in large species.

BoHV-1 / DNA immunisation / gC / gD / VP8

1. INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is an alphaherpesvirus responsible for major economic diseases of cattle. It is the causative agent of infectious bovine rhinotracheitis (IBR), an infection of the upper respiratory tract associated with fever and drop of

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production before recovery, which generally occurs within a few days. IBR also facilitates superinfection of cattle by bacterial agents, leading to bronchitis and/or pneumonia that are frequently fatal if not treated [48]. Infectious balanoposthitis and infectious pustular vulvovaginitis are also due to BoHV-1 and are associated with reproductive disorders [58]. Besides the economic losses due to clinical consequences of infection with BoHV-1, impairment of international trade exchanges also represents a growing threat. For all of these reasons, several European countries have started BoHV-1 eradication programmes.

In the last decade, the goals of vaccination evolved with the growing trade restrictions associated with IBR. In addition to conferring good clinical protection, current vaccines must reduce virus excretion after infection and allow discrimination between vaccinated and infected animals [55]. Immunisation with plasmid vaccines expressing BoHV-1 glycoproteins has been studied extensively since a first experiment indicated that a plasmid encoding BoHV-1 gD was immunogenic in mice and cattle [10]. Further DNA vaccination experiments performed in mice and cattle have shown that a construct encoding a secreted form of BoHV-1 gD induces a higher immune response than membrane-associated full-length gD [4, 54]. Different routes of DNA administration to cattle have been tested such as intradermic or intramuscular injection [10, 17, 54] and biolistic delivery (gene-gun) of gold particles coated with DNA vaccines [6, 33, 34]. Mucosal administration of plasmid DNA via a gene-gun [34] or into suppositories [35] has also been shown to induce systemic as well as mucosal immunity.

Most subunit vaccines developed against BoHV-1 have focussed on the glycoproteins gB, gC and gD. These proteins are located on the surface of the virus and have both functional and immunological characteristics that render them attractive for vaccine development. They are the immunodominant antigens of BoHV-1 since they are the target of most neutralising antibodies [1, 37, 51] and the major antigens of the cellular immune response mounted after infection [11, 19, 26, 39]. Glycoprotein gD is an essential protein of BoHV-1 that plays critical functions for the viral entry process. In addition to its role as a secondary attachment factor [37], gD mediates the penetration of BoHV-1 into host cells [8, 14] and the spread of virus by cell fusion [47]. The glycoprotein gC is a non-essential protein of BoHV-1 [32]. However, the binding of gC to heparin-like moieties on host cells is considered as the major mechanism of virus attachment [31, 32, 38] and gC has been shown to be necessary for efficient virus replication in vivo [30].

The VP8 protein is the most abundant component of BoHV-1 virions [7]. It is located between the nucleocapsid and the envelope, in the matrix called tegument [37]. The tegument protein VP8 is poorly recognised by the sera of cattle infected once, but subsequent exposures to BoHV-1 enhance the titres of VP8-directed antibodies [50]. With glycoprotein gD, VP8 is the BoHV-1 protein that stimulates the highest proliferation of lymphocytes isolated from infected cattle [19, 53] indicating that it is an important target of the cellular immune response.

Vaccination with naked DNA offers several advantages over conventional immunisation strategies (for a review, see [2]). Plasmid DNA administration does not induce any adverse reactions and can therefore be administered to young or immunocompromised animals. Plasmid vaccines can be combined to produce multivalent vaccines [5] and they allow modulation of the immune response in the required (Th1 or Th2) polarity [40, 41]. Multiple parameters such as the promoter of the plasmid, the presence of CpG motifs in the plasmid backbone, the cellular localisation of the protein and the administration route all influence the immune response and should be optimised for each vaccine. Moreover, the results obtained in mice are not always transposable to large animals like cattle since most of these parameters are

Table I. Primers and restriction sites used for cloning.

Construct		Primers[a]	Restriction[b]
pCIgC	dgCfrw	5' TTTGAATTC*C**GCCACC***ATG**GGCCCGCTGGGGCGA 3'	EcoRI
	dgCrev	5' AAAGAATTCGGGCCCCGCG**CTA**CAG 3'	EcoRI
pCItgC	dgCfrw	5' TTTGAATTC*C**GCCACC***ATG**GGCCCGCTGGGGCGA 3'	EcoRI
	dtgCrev	5' AAAGAATT**CTA**TCCGATTAGGCCGGGCGAG 3'	EcoRI
pCIgD	dgDfrw	5' TTTCTCGAG*CCACC***ATG**CAAGGGCCGACATTGGC 3'	XhoI
	dgDrev	5'AAAGAAT**TCA**CCCGGGCAGCGCGCT 3'	EcoRI
pCItgD	dgDfrw	5' TTTCTCGAG*CCACC***ATG**CAAGGGCCGACATTGGC 3'	XhoI
	dtgDrev	5' AAAGAATT**CTA**GGGCGTAGCGGGGGCGG 3'	EcoRI
pCDNA3.1-VP8-V5-HIS	dVP8frw	5' TTTCTCGA*GCCACC***ATG**GACGCCGCTAGGGATG 3'	XhoI
	dVP8rev2	5' AGATATCAG**CTA**ATCAGCTGCGGCCGCCCAGGCGC 3'	PvuII
pCDNA3.1-VP8	dVP8frw	5' TTTCTCGA*GCCACC***ATG**GACGCCGCTAGGGATG 3'	HindIII[c]
	dVP8rev2	5' AGATATCAG**CTA**ATCAGCTGCGGCCGCCCAGGCGC 3'	EcoRV

[a] The underlined nucleotides correspond to the restriction site used for cloning. Nucleotides presented in italic and bold corrrespond to the Kozak consensus motif and the ATG initiation or stop codon, respectively.
[b] Restriction endonucleases used for cloning.
[c] The restriction site HindIII originates from the pCRII vector.

species-specific [3]. In the present study, we first compared in cattle the efficacy of constructs coding for two major glycoproteins of BoHV-1, namely gC and gD. We also compared in cattle the immune response generated by recombinant plasmids expressing secreted forms of gC and gD to that induced by constructs expressing native, membrane-bound gC and gD. Finally, we report the development of the first IBR DNA vaccine that is not directed against a glycoprotein of BoHV-1 but against the major tegument protein VP8.

2. MATERIALS AND METHODS

2.1. Cells and virus

Madin Darby bovine kidney (MDBK; ATCC CCL-22) cells were grown in minimum essential medium (MEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS, Invitrogen), 10 mg/L gentamycin and 1×10^6 IU/L penicillin. COS-7 cells (ATCC CRL-1651) were grown in DMEM 4500 mg/L glucose (Invitrogen) supplemented with 10% FCS, 10 mg/L gentamycin and 1×10^6 IU/L penicillin. Virus neutralisation assays were performed with the Belgian strain BV158 characterised earlier as BoHV-1.1 [44]. The same strain BV158 was used to prepare the recall antigen for cellular immunity assays. The challenge infection was performed with BoHV-1.1 strain Lam kindly provided by Dr F. Rijsewijk (ID-DLO, The Netherlands).

2.2. Plasmids

Viral DNA were purified from BV158 infected MDBK cells using Chelex-100 (Bio-Rad Laboratories, Eke, Belgium) as described by Santurde et al. [45]. Primers (Tab. I) for PCR amplification of the genes coding for gC [dgCfrw + dgCrev], gD [dgDfrw + dgDrev] and VP8 [DVP8frw + DVP8rev2] were designed from the published sequence of BoHV-1 (accession number NC_001847). The sequences coding for truncated forms of gC (tgC, amino acids 1–473) and gD (tgD, amino acids 1–355) were generated by PCR amplifications with primer pairs dgCfrw + dtgCrev

and dgDfrw + dtgDrev, with both reverse primers containing a stop codon upstream of the transmembrane anchor sequences. An optimal context for initiation of translation was generated by including the consensus motif GCCACC [21, 22] in every forward primer and restriction sites were added at the 5' extremity of all primers for cloning.

All PCR amplifications were performed with high fidelity Taq DNA polymerase (Platinum Pfx DNA Polymerase, Invitrogen) in the presence of 1X enhancer solution and 100 pmol of each primer. The PCR products were then digested with the restriction endonucleases (Roche Diagnostics, Brussels, Belgium) listed in Table I before purification on 1% agarose gel eletrophoresis and gel extraction with QIAquick gel extraction kit (Qiagen Benelux, Venlo, The Netherlands). Purified PCR products were ligated with T4 DNA ligase (Roche Diagnostics) in linearised pCI (Promega, Leiden, the Netherlands) or pCDNA3.1V5-HIS (Invitrogen) vectors, generating pCIgC, pCItgC, pCIgD, pCItgD and pCDNA3.1VP8-V5-HIS. After insertion in the pCRII vector by TA-cloning (Invitrogen), the VP8 PCR product was also subcloned in the HindIII – EcoRV sites of pCDNA3.1 (Invitrogen).

The sequence of all cloned PCR products was determined with an automatic sequencer (ABI Prism 310, Applied Biosystems, Lennik, Belgium) using fluorescent deoxynucleoside triphosphates. Large scale production of plasmid DNA was finally purified from *Escherischia coli* (strain DH5α, Invitrogen) using anion exchange columns (Plasmid Giga Kits, Qiagen Benelux). The DNA concentration of all productions was spectroscopically determined before storage at –20 °C. The A260/280 ratios of each plasmid production always reached 1.8 and successful transfection was performed with each production.

2.3. Transient expression of gD, tgD, gC, tgC and VP8

COS-7 cells were transfected in 24-well plates with 0.4 µg of plasmid DNA and 3 µg of Lipofectamine reagent (Invitrogen) per well in serum-free Optimem medium (Invitrogen) without antibiotics as recommended by the manufacturer. Protein expression was assessed 24 h after transfection by performing immunoperoxydase monolayer assays (IPMA) according to Kramps et al. [23]. The glycoproteins gC and gD were detected with the serum of a calf immunised 4 times at intervals of 1 month with IBR gE deleted marker vaccines [49] and with an anti-bovine IgG whole molecule antibody from rabbit linked to peroxydase (Sigma-Aldrich, Bornem, Belgium). The tegument protein VP8 was labelled with the serum from a rabbit vaccinated 4 times by a gene-gun with pCDNA3.1VP8 and then by an anti-rabbit IgG antibody linked to peroxydase. The fusion protein VP8-V5 was labelled with a monoclonal antibody (Mab) directed against the V5 epitope (Invitrogen) and then with an anti-mouse IgG antibody linked to peroxydase (Sigma-Aldrich). When required, permeabilisation was avoided during IPMA by fixing live cells in 4% paraformaldehyde and by avoiding detergent in all buffers.

Fine localisation of the proteins expressed by transfected cells was determined by confocal microscopy as described elsewhere [56]. For this purpose, gC, gD and their truncated counterparts were labelled with polyclonal sera collected from mice immunised twice with pCIgC or pCIgD (diluted 200-fold) followed by an anti-mouse IgG antibody from sheep linked to fluorescein (Sigma-Aldrich). Detection of the fusion protein VP8-V5 was performed with the commercial anti-V5 Mab (Invitrogen) followed by an anti-mouse IgG antibody from goat conjugated to phycoerythrine (Dako, Heverlee, Belgium).

2.4. Western blot analysis

The supernatants of COS-7 transfected with pCIgC, pCItgC, pCIgD or pCItgD was concentrated about 50 times on microcon-YM30 (Millipore, Brussels, Belgium), migrated on reducing 8% SDS-PAGE and then transferred on nitrocellulose membranes

(Bio-Rad laboratories). The proteins were then labelled with the sera from a polyvaccinated calf diluted 500-fold [49] and then with an anti-bovine IgG antibody conjugated to alkaline phosphatase (Sigma-Aldrich). Revelation was finally performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Sigma-Aldrich).

2.5. Immunisation of cattle

Twenty-four calves between 4 and 10 weeks-old were purchased in IBR-free Belgian farms. They were randomly distributed among 6 groups of 4 animals and kept by pairs in identically conditioned stables. The animals from groups A to E were intradermally vaccinated 3 times, 4 weeks apart, with pCIgD, pCItgD, pCIgC, pCItgC or pCDNA3.1VP8. Each vaccine dose consisted of 1 mL normal saline (0.15 M NaCl) that contained 500 μg of plasmid DNA and that was administrated intradermally into 4 sites in the necks of the cattle. Saline buffer was administrated to the animals of group F, which were used as non-vaccinated controls. The experimental design and all manipulations of the calves were performed according to European ethic rules after the approval of the local ethic committee.

2.6. Serological analysis

Blood samples were collected every week and serum was prepared by centrifugation before heat-inactivation at 56 °C for 30 min. Virus neutralising antibody titres (VNT) were determined in a microtitration system as previously described [20]. BoHV-1-specific antibody titres were determined with a home-made indirect ELISA system proven to be efficient in a compared ring trial [24, 42]. Briefly, a BoHV-1 antigen and a control preparation were prepared by sonicating lysates of BV158-infected or mock-infected MDBK cells before an incubation of 2 h at RT in 0.5% non-ionic detergent IGEPAL CA-630 (Sigma-Aldrich). Plates were coated for 24 h at 4 °C with the antigen or the control preparation diluted 500-fold in 50 mM sodium carbonate buffer pH 9.6. Serum were serially two-fold diluted in PBS PH 7.2 containing 0.5% Tween-80, 0.1 M NaCl and 5% horse serum and incubated in the plates for 1 h at 37 °C. The plates were washed with PBS 1% Tween-80 before an incubation of 1 h at 37 °C with anti-bovine whole IgG antibody from rabbit conjugated to horseradish peroxydase (Sigma-Aldrich). After additional wash, colour development was performed by adding TMB (Sigma-Aldrich) and the reaction was stopped with 1 N sulphuric acid. The BoHV-1 specific antibody titres were determined as the reciprocal dilutions with a difference between the OD_{450} antigen and the OD_{450} control of 0.4.

2.7. Evaluation of cellular immunity

Gamma-interferon (IFN-γ) production was measured after a 24 h in vitro stimulation of heparinised blood with BoHV-1 antigen or control preparation [15, 20]. The amounts of IFN-γ in the plasma were quantified with the *Bovine γ-interferon EASIA* (Biosource Europe, Nivelles, Belgium) and the results were expressed as stimulation indexes (SI) corresponding to the optical density (OD) obtained with the BoHV-1 antigen divided by the OD obtained with the control preparation. Samples with an OD above 0.2 after stimulation with the control preparation were considered non-interpretable. The respective OD and SI cut-off values 0.2 and 1.6 were calculated as three standard deviations (SD) above the mean OD and SI values of all calves before vaccination.

The BoHV-1 specific lymphocyte proliferation was assessed after a 7-day stimulation of ten-fold diluted blood with BoHV-1 antigen and control preparation [20]. The samples with a mean cpm (quadruplicate) above 1500 after stimulation with the control preparation were considered as non-interpretable. The lymphocyte proliferation was considered significant when the mean

cpm of BoHV-1-antigen stimulated blood was above 1500 and when the SI calculated by dividing the cpm obtained with the BoHV-1 antigen by the cpm measured after incubation with the control preparation was equal or superior to 2.8 (mean + 3 × SD of the values observed before vaccination).

2.8. Challenge inoculation and virus isolation

Four weeks after the third immunisation, all calves were challenge-infected intranasally with 1×10^7 $TCID_{50}$ wild-type BoHV-1 strain Lam. Nasal secretions were sampled according to standardised procedures during 15 consecutive days after the challenge infection and virus titres were determined by incubating serial ten-fold dilutions of the nasal swabs in culture medium with MDBK cells for 3 days at 37 °C and 5% CO_2 according to Kerkhofs et al. [20].

2.9. Statistical analysis

The data collected in the six groups were compared to each other by one-way analysis of variance (ANOVA) and by the Tukey test with a family error rate of 0.05. Comparison of the four constructs coding for gC or gD were performed by two-way analysis of variance with the gene (gC or gD) as the first factor and the form of the glycoprotein (full-length or truncated) as the second factor. Logarithmic transformation was applied to the VNT, the ELISA titres, the IFN-γ SI, the lymphocyte proliferation cpm and the titres of virus excreted after the challenge infection in order to fulfill the conditions of variance homogeneity (checked by the Bartlett's test) and normality (assessed by the Ryan and Joiner test).

3. RESULTS

3.1. Construction and in vitro characterisation of the expression vectors

The genes coding for BoHV-1 full-length gC, gD and VP8 and the truncated sequences encoding secreted forms of gC (tgC) and gD (tgD) were cloned from field isolate BV158 either in pCI or in pCDNA3.1 and pCDNA3.1-V5-HIS. All constructions were sequenced and proven to be correct. In the pCIgC, pCItgC, pCIgD and pCItgD, the predicted amino acids sequences were 100% identical to the published gC and gD sequences of the reference strain Cooper. Interestingly, the amino acid sequence of VP8 from the strain BV158 (submitted under accession number AY530215) differed significantly (92 and 91% identity respectively) from the sequences published for the strains Cooper and P8-2. Most differences were clustered within five areas covering the amino acids 33–47, 366–376, 382–399, 406–421 and 615–633. The signification of these highly variable regions within VP8 is currently unknown.

The expression and the cellular distribution of gC, tgC, gD, tgD and VP8 was assessed in COS-7 cells transfected with each recombinant plasmid. Confocal microscopy and immunoperoxydase monolayer assays (IPMA) detected both full-length and truncated forms of glycoproteins gC and gD as well as the tegument protein VP8 (Fig. 1). The glycoproteins gC and gD were mainly detected in the cytoplasmic membranes. Truncated gC and gD were absent from cellular membranes but displayed a pattern characteristic of secreted proteins with a localisation in the Golgi apparatus and in export vesicles dispersed through the whole cell. VP8 was exclusively found in the nucleus but was nearly excluded from the nucleolus (Fig. 1).

IPMA performed on non-permeabilised cells allowed further characterisation of tgC and tgD. The full-length but not the truncated glycoproteins were detected at the surface of non-permeabilised cells (Fig. 2A), indicating that the truncations of gC and gD abrogated their localisation in the cytoplasmic membrane. Western blot analysis of 50-fold concentrated supernatants from COS-7 cells transfected with pCIgC, pCItgC, pCIgD or pCItgD finally confirmed the

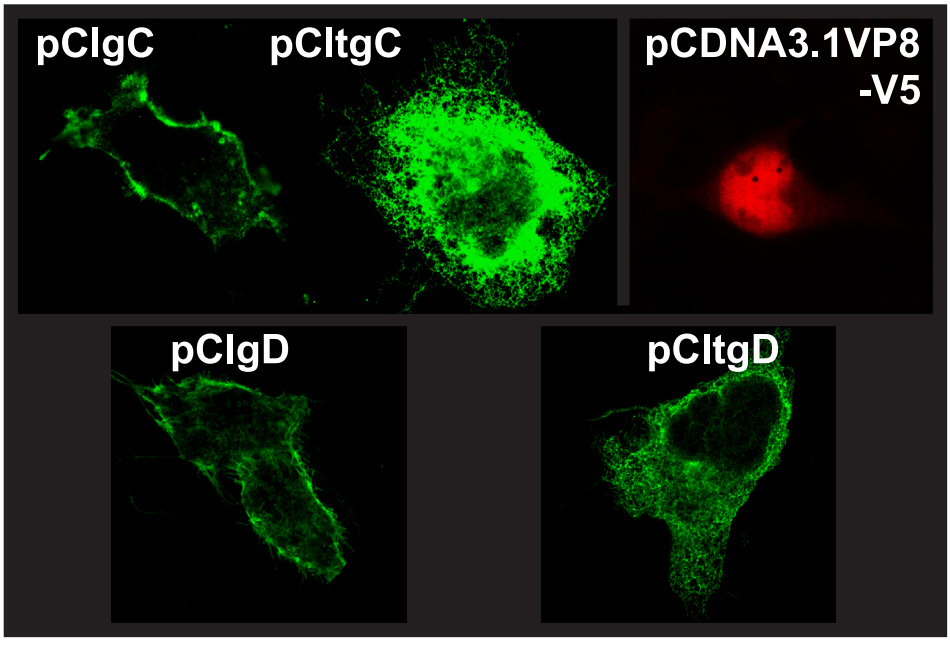

Figure 1. Confocal microscopy observation of COS-7 cells transfected with pCIgC, pCItgC, pCDNA3.1VP8-V5, pCIgD or pCItgD. Negative controls included gC, gD and V5-specific labelling of cells transfected with empty plasmids and labelling of transfected cells either with the sera of unvaccinated mice or in the absence of the conjugates. They were not shown since they gave no signal (black picture) (see www.edpsciences.org/vetres for a colour version of this figure).

secretion of the truncated glycoproteins. The detection of the truncated forms in the supernatant of COS-7 cells transfected with pCItgC or pCItgD combined to the lack of full-length gC and gD in the supernatant of cells transfected with pCIgC or pCIgD (Fig. 2B) demonstrated that the deletion of the transmembranous anchor resulted in efficient secretion of tgC and tgD.

3.2. In vivo characterisation of the plasmid constructs

To determine whether the constructs were immunologically active, groups of three Balb/c mice were injected three times intramuscularly one month apart with 20 µg of either pCIgC, pCItgC, pCIgD, pCItgD, pCDNA3.1-VP8 or pCI. The dilutions of individual sera were tested by performing IPMA on MDBK cells infected with BoHV-1 (strain Lam) or mock-infected MDBK as the control. The sera of all mice vaccinated with pCIgC, pCItgC, pCIgD or pCItgD detected BoHV-1 antigens at dilutions ranging from 1/100 to 1/200 after the first injection and at higher dilutions after the second and the third administrations. These sera proved to be specific for BoHV-1 since they detected proteins with a size corresponding respectively to gC and gD when performing immunoblot with clarified supernatant from MDBK cells infected with BoHV-1 strain Lam (data not shown). In contrast the sera collected in all mice injected with pCDNA3.1-VP8 or pCI failed to detect BoHV-1 antigens even after three administrations.

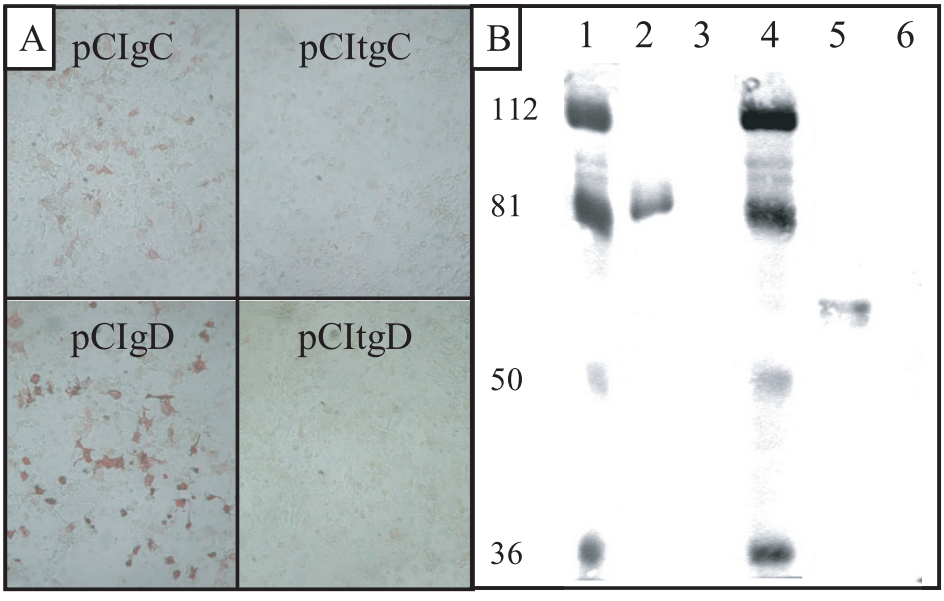

Figure 2. (A) Immunoperoxydase monolayer assay performed on non-permeabilized COS-7 cells transfected with pCIgC, pCItgC, pCIgD or pCItgD. (B) Western blot analysis of concentrated supernatants collected from cells transfected with pCItgC (lane 2), pCIgC (lane 3), pCItgD (lane 5) or pCIgD (lane 6). Lanes 1 and 4 correspond to the molecular weight marker "Prestained SDS-PAGE Standard Low Range" (Bio-Rad Laboratories), whose molecular weights in kD are indicated on the left (see www.edpsciences.org/vetres for a colour version of this figure).

3.3. Efficacy of the plasmid vaccines in cattle

Among the 16 calves vaccinated with pCIgC, pCItgC, pCIgD or pCItgD, respectively 11 and 15 developed BoHV-1 specific antibodies after the second and the third intradermic immunisations whereas no calf vaccinated with pCDNA3.1VP8 or injected with saline seroconverted before the challenge (Fig. 3). After 2 and 3 vaccinations, the calves vaccinated with pCItgD or pCIgC showed higher mean ELISA titres than the calves vaccinated with pCIgD or pCItgC (Fig. 3), but one-way analysis of variance (ANOVA) indicated that only the differences between tgD and gD were significant (data not shown). After challenge, the ELISA titres of the calves vaccinated with pCIgC, pCItgC, pCIgD or pCItgD reached significantly higher levels than those of the controls, indicating that vaccination with those plasmids had efficiently primed the humoral immune response. In contrast, the ELISA titres recorded in the animals vaccinated three times with pCDNA3.1VP8 were similar to those observed in control calves, suggesting that no humoral priming had occurred in VP8 immunised calves.

Two to three vaccinations were also necessary to induce neutralising antibodies in all the calves vaccinated with pCIgD or pCItgD, while respectively 2/4, 1/4 and 0/4 calves vaccinated with pCIgC, pCItgC and pCDNA3.1VP8 showed significant VNT after the third immunisation (Fig. 4). At this time, the constructs encoding gD or tgD induced significantly higher virus neutralising antibody titres (VNT) than the constructs encoding gC or tgC or VP8 (Fig. 4).

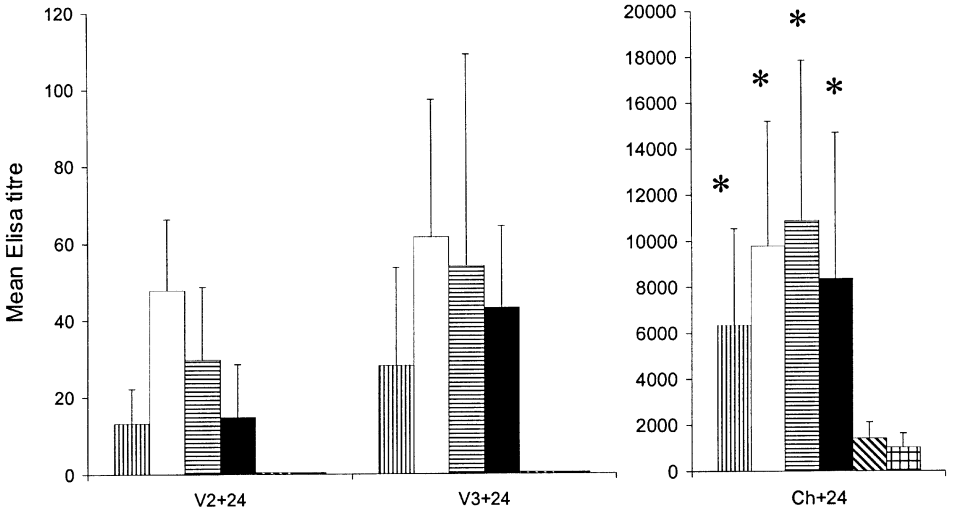

Figure 3. Mean antibody titres (n = 4) 24 days after the second (V2) or after the third vaccination (V3) and after the challenge inoculation (Ch). Calves were vaccinated with pCIgD (vertical hatching), pCItgD (white), pCIgC (horizontal hatching), pCItgC (black), pCDNA3.1VP8 (diagonal hatching) or received saline (squared). Note that different scales are used on both parts of the graph. Antibody titres were measured 24 days after each vaccination and after challenge to detect the maximum effect associated with these events. No BHV-1 specific antibodies were detectable after the first vaccine administration. The bars represent the standard deviation of the means. Before challenge inoculation, the means of the vaccinated animals could not be compared to those of the control groups since all control calves had nul values leading to rejection of the application conditions of analysis of variance. After challenge, the stars indicate the means that differ significantly from those of the controls (Tukey test, family error rate = 0.05).

Virus replication that occurred after the challenge infection induced a rise of the VNT of all calves. The calves vaccinated with pCIgD or pCItgD showed the highest neutralising antibody titres after challenge. The VNT of the calves vaccinated with pCIgC or pCItgC reached intermediate levels while those recorded in the calves vaccinated with pCDNA3.1VP8 were the lowest and were similar to the mean VNT of the controls.

In both cases, truncation of the coding sequences of gC and gD resulted in secreted proteins instead of the membrane-anchored native proteins. However, the truncation of gC and gD had a different effect on their immunogenicity since secreted gD was associated with significantly enhanced VNT and ELISA titres as compared to full-length gD while secreted gC induced lower VNT and ELISA titres than membrane-bound gC (Figs. 3 and 4). When performing two-way ANOVA (1st factor = gC vs. gD; 2nd factor = full-length vs. truncated form), a significant interaction detected between both factors (data not shown) statistically confirmed that the deletion of the transmembranous anchors had a significant opposite effect on the immunogenicity of the constructs coding for gC or gD.

Before the challenge infection, the BoHV-1 specific lymphocyte proliferation and IFN-γ production of all calves vaccinated with pCIgD, pCItgD, pCIgC or pCItgC or injected with saline remained below the cut-off (Figs. 5 and 6). On the contrary, all calves vaccinated with pCDNA3.1VP8 developed significant lymphocyte proliferation and IFN-γ production after two to three vaccinations. Challenge infection was followed

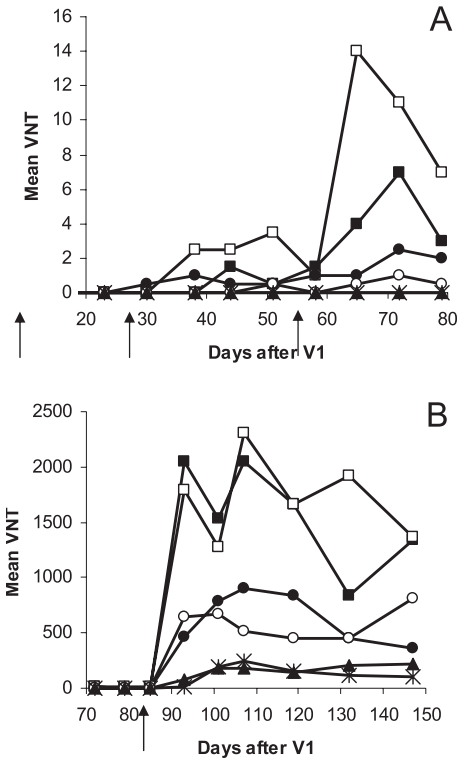

Figure 4. Mean Virus neutralising antibody titres ($n = 4$) before (A) and after the challenge inoculation (B). The calves were vaccinated with (■) pCIgD, (□) pCItgD, (●) pCIgC, (○) pCItgC, (▲) pCDNA3VP8 or (✳) saline. Note that different scales are used on both parts of the graph. Arrows of figure A represent the three vaccinations whereas arrow of figure B indicates the time of challenge inoculation.

by a transient period during which all calves showed significant IFN-γ production and lymphocyte proliferation. The cellular immunity of most calves then decreased rapidly but unequally among the various groups. Seven weeks after the challenge, only the two groups that had been vaccinated with pCIgC or pCDNA3.1VP8 showed mean IFN-γ production and lymphocyte proliferation significantly higher ($p < 0.05$) than that recorded in the control groups.

All calves excreted virus for several days after the challenge infection. The calves vaccinated with pCIgD or with pCItgD showed over 10 times less virus excretion than the control calves (Tab. II). These calves also excreted virus for significantly shorter periods as compared to the controls. On the contrary, the calves vaccinated with pCIgC, pCItgC or pCDNA3.1VP8 showed almost no reduction of virus excretion amount and duration as compared to the control calves. Two-way ANOVA indicated that the calves vaccinated with the gD and tgD constructs excreted significantly ($p < 0.05$) lower amounts of virus for significantly ($p < 0.05$) shorter periods than the calves vaccinated with pCIgC or pCItgC (Tab. II).

4. DISCUSSION

Despite the intensive use of the BoHV-1 model for testing several parameters affecting DNA vaccination in cattle and mice, the data relative to the choice of the viral target antigens of such vaccines are scarce. Moreover, the design of efficient DNA vaccines should be performed directly in the host species since the results obtained in mice are not always transposable to cattle [3]. The final goal of this study was therefore to compare in a same experiment performed in cattle the immunogenicity of recombinant plasmids expressing 3 major proteins of BoHV-1, namely gC, gD and VP8. In COS-7 cells transfected with pCIgC and pCIgD, the glycoproteins gC and gD had a membranous localisation similar to that reported in bovine cells [14, 37, 47]. VP8 had a nuclear localisation that was also consistent with the previously reported localisation of VP8 in BoHV-1 infected cells [53] and the reported localisation of the homolog protein VP13/14 of HSV-1 in transfected mammalian cells [12]. As expected, truncated forms of the glycoproteins gC and gD localised in the secretory pathways and were found in the supernatant of transfected cells.

The only previous experiment comparing the immunogenicity of DNA vaccines

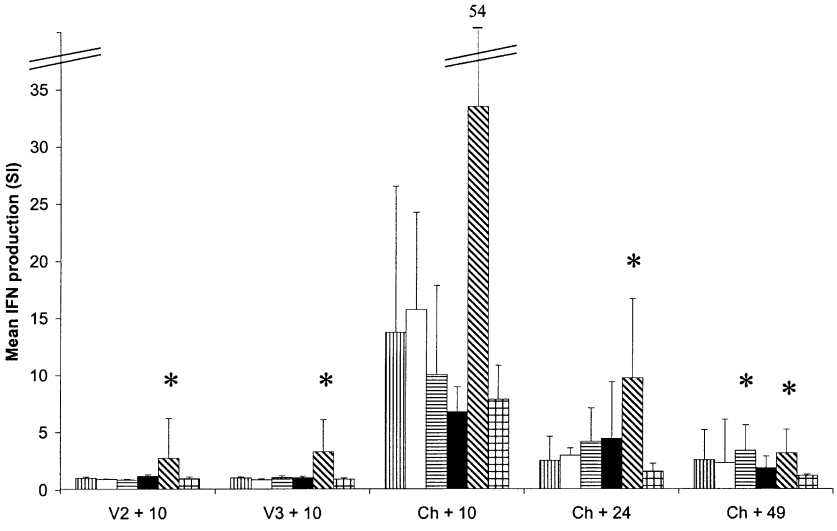

Figure 5. Mean IFN-γ production ($n = 4$) 10 days after the second (V2) or after the third vaccination (V3) and 10 days, 24 days or 49 days after the challenge infection (Ch). Calves were vaccinated with pCIgD (vertical hatching), pCItgD (white), pCIgC (horizontal hatching), pCItgC (black), pCDNA3.1VP8 (diagonal hatching) or received saline (squared). Expressed as stimulation index as described in materials and methods. The bars represent the standard deviation of the means. The stars indicate the means that differ significantly from that of the controls (Tukey test, family error rate = 0.05).

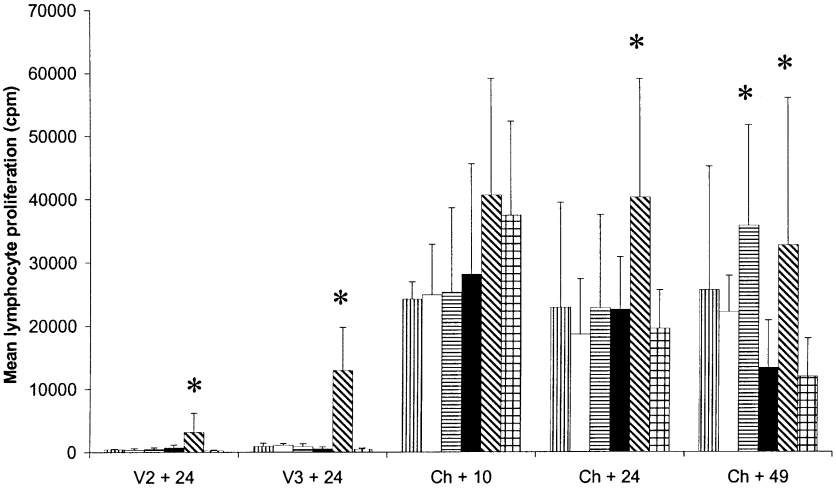

Figure 6. Mean lymphocyte proliferation ($n = 4$) 24 days after the second (V2) or after the third vaccination (V3) and 10 days, 24 days or 49 days after the challenge infection (Ch). Calves were vaccinated with pCIgD (vertical hatching), pCItgD (white), pCIgC (horizontal hatching), pCItgC (black), pCDNA3.1VP8 (diagonal hatching) or received saline (squared). The bars represent the standard deviation of the means. The stars indicate the means that differ significantly from those of the controls (Tukey test, family error rate = 0.05).

Table II. Virus excretion after the challenge infection: mean total amount of virus excreted and mean duration in days.

Vaccine	Mean Virus excretion	
	$\log_{10}(TCID_{50}/50\ \mu L)$	Duration
gD[a]	6.6	5.8*
tgD	7.2	6.3*
gC	8.3	7.3
tgC	7.9	7.3
VP8	8.3	7.8
Control	8.3	7.8
gD[b]	6.9$	6.0$
gC	8.1	7.3

[a] One-way analysis of variance. The stars indicate the means that differ significantly from the mean of the control group (Tukey test, family error rate = 0.05).
[b] Comparison of the contructs encoding for gC and gD by a two-way analysis of variance: effect associated with the glycoproteins independantly from the expression form (full-length or truncated). The $ indicates that the differences between the two glycoproteins differ significantly ($p = 0.05$).

coding for the glycoproteins gC and gD was performed in the mouse model. It showed that a construct expressing gD induced higher neutralising antibody titres than the one expressing gC [10]. In the current study performed in cattle, the four vaccines encoding full-length or truncated forms of the glycoproteins gC and gD induced the development of a low but statistically significant humoral immune response. Statistical analysis indicated that there were no significant differences between total IgG titres induced by both glycoproteins but that gD constructs elicited significantly higher virus neutralising antibody titres than pCIgC and pCItgC. This discrepancy between ELISA and virus neutralising titres could be attributed to the higher number of neutralising epitopes on gD as compared to gC [18, 37] and to the requirement of complement for the neutralising activity of most antibodies directed against gC [9, 13, 37]. There was no difference between both glycoproteins when considering the cellular immunity before the challenge, but the slower decrease of the anamnestic cellular immune response observed in the calves vaccinated with pCIgC suggested that this construct could have conferred a better priming of the cellular immune response than pCIgD. Indeed, such a "silent" priming that can only be detected after a boost with a different antigen is frequently observed in heterologous prime-boost strategies based on DNA vaccines [36, 46]. A BoHV-1 challenge inoculation is the most relevant test of vaccine efficacy and is the major asset of the experiments performed in cattle. Reduction of virus excretion after challenge was moderate (one logarithm and 1.5 days) in the calves vaccinated with the constructs encoding truncated or full-length gD but it was statistically significant as compared to calves immunised with pCIgC or pCItgC, which did not differ from the controls. This difference in virological protection suggests that the glycoprotein gD is a better vaccine candidate than gC when developing DNA vaccines, paralleling observations made with recombinant adenoviral vectors [16]. This observation also underlines the importance of vaccination-induced neutralising antibodies for reducing BoHV-1 excretion after infection [52].

To our knowledge, all DNA vaccines developed so far against BoHV-1 or the related HSV-1 and PRV-1 have only focused on surface glycoproteins. In this study we report for the first time the development and the evaluation of a DNA vaccine directed against a protein from the tegument of BoHV-1. The recombinant plasmid expressing VP8 elicited strong specific IFN-γ production and lymphocyte proliferation after two and three vaccinations but failed to induce any humoral immune response. Similarly, vaccination with a recombinant vaccinia virus expressing VP8 was not associated with the development of neutralising antibodies [25] while administration of adjuvanted purified VP8 induced both cellular and humoral immune response [53]. The failure to induce antibodies could reasonably be attributed to the intracellular

localisation of VP8 expressed by transfected cells, which accumulates in the nucleus and is therefore not accessible extracellularly. Despite their high BoHV-1 specific IFN-γ production and lymphocyte proliferation, the calves vaccinated three times with the pCDNA3.1VP8 showed no reduction of virus excretion as compared to the controls. This discrepancy between the cellular immune response and the virological protection after challenge contrasts with the results of studies performed with conventional vaccines [20, 57] but is in agreement with observations of BoHV-1 DNA immunisation studies using other viral antigens [33, 34, 43]. The viral target antigens of DNA vaccines are very limited as compared to those encountered in conventional vaccines and the protective cellular immunity may be restricted to only a few proteins of BoHV-1. Another hypothesis assuming that a humoral immune response has to be associated to cellular immunity to be correlated to protection [43] cannot be discarded.

Another goal of the present study was to compare the immunogenicity of secreted forms of the glycoproteins gC and gD to their native, membrane-anchored counterparts. Previous studies had shown that plasmid DNA encoding a secreted form of the glycoprotein gD elicited higher immune response than the same plasmid vector encoding membrane-anchored gD [27–29, 54]. Two hypotheses, which are not mutually exclusive had been proposed to explain this observation: the highest immunocompetency of the skin for antigens secreted extracellularly [28, 29, 54] and/or the decreased toxicity of secreted gD as compared to full-length gD [47]. To our knowledge, this phenomenon had not been further explained or studied for other antigens of BoHV-1. Here, we first confirmed that the intradermal administration of a construct encoding secreted gD induced significantly higher IgG and virus neutralising titres in cattle than a construct encoding full-length gD but we failed to confirm that truncated gD also elicited enhanced cellular immunity [54]. Although the deletion of the transmembranous anchor of gC also induced secretion of the truncated protein, it had a slight adverse effect on both IgG and virus neutralising titres. Moreover the anamnestic cellular immune response was higher in the calves vaccinated with pCIgC than in those immunised with pCItgC, suggesting that the priming could have been less efficient in the calves vaccinated with pCItgC. We therefore concluded that the enhanced immunogenicity associated with secretion of the glycoprotein gD could not be extended to the glycoprotein gC. This suggests that the adjuvant effect associated with secretion of gD could be restricted to this protein instead of being a common property of BoHV-1 glycoproteins.

In the past, we conducted extensive experiments on conventional attenuated and inactivated marker vaccines [20, 49]. By successively combining an attenuated and an inactivated preparation in a single immunisation protocol, we notably enhanced the efficacy of vaccination strategies against IBR. In the present study, we describe the characterisation and the evaluation of several DNA vaccines in cattle in similar conditions. Although these DNA vaccines conferred an immune response comparable to that described by other groups [17, 54], they induced weaker immune response and virological protection than the best protocol developed previously with conventional marker vaccine [20]. Therefore, a further experiment will aim at optimising some of the parameters known to modulate the efficacy of DNA vaccines such as the adjuvancy of immunostimulatory CpG motifs in the plasmid backbone [43]. Since the construct encoding truncated gD was associated with a high humoral immune response while pCDNA3.1VP8 induced a high cellular immune response an additional experiment will also test the efficacy of a vaccine simultaneously combining both constructs. The DNA vaccines reported here efficiently primed the humoral (gC and gD) or cellular (VP8) arms of the immune response of cattle as indicated by the high anamnestic immune response observed after challenge.

Since DNA vaccines are associated with many advantages that render them very attractive for performing priming [2], further studies will also be performed to determine whether the DNA vaccines reported here could be used for developing prime-boost strategies that could bring additional assets to vaccination against IBR.

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