Evaluation of safety and efficacy of DNA vaccines against bovine herpesvirus-1 (BoHV-1) in calves.

(Article begins on next page)
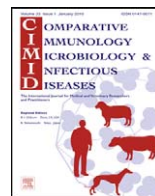
12 October 2022

Evaluation of safety and efficacy of DNA vaccines against bovine herpesvirus-1 (BoHV-1) in calves

S. Petrini [a,*], G. Ramadori [d], A. Corradi [c], P. Borghetti [c], G. Lombardi [a], R. Villa [a], E. Bottarelli [c], A. Guercio [e], A. Amici [d], M. Ferrari [b]

[a] Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Via G. Salvemini 1, 06126 Perugia, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Via A. Bianchi 7, 25124 Brescia, Italy
[c] Department of Animal Health, University of Parma, Via del Taglio 10, 43100 Parma, Italy
[d] Laboratory of Genetic immunization, Department of Molecular Cell and Animal Biology, University of Camerino, Via Camerini 1, 62032 Camerino, Italy
[e] Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, 90129 Palermo, Italy

ARTICLE INFO

ABSTRACT

Four DNA vaccines against BoHV-1 were evaluated for their efficacy in calves. Twelve animals were divided into four groups which were injected with four different DNA vaccines: pVAX-tgD (Vaccine A); pVAX-tgD co-immunised with pVAX-48CpG (Vaccine B); pVAX-UbiLacI-tgD-L (Vaccine C); pVAX-UbiLacI-tgD-L co-immunised with pVAX-48CpG (Vaccine D). Three additional calves were given the plasmid vector and served as controls. Ninety days after the first vaccination all calves were challenge infected with BoHV-1.

All animals developed a severe form of infections bovine rhinotracheitis. Only the calves given the pVAX-tgD co-immunised with pVAX-48CpG (Vaccine B) developed humoral antibodies against BoHV-1 between 56 and 90 days after the first vaccination, whereas in calves of other groups and in the controls, antibodies appeared only after the infection. In the calves vaccinated with either pVAX-tgD (Vaccine A) or pVAX-tgD combined with pVAX-48CpG (Vaccine B), BoHV-1-specific IFN-γ secreting cells were detected in PBMCs 90 days after the first vaccination and their number increased after challenge exposure. In the other groups the IFN-γ secreting cells were detected after virus infection and at low values.

1. Introduction

Bovine herpesvirus-1 (BoHV-1) is an important pathogen of cattle which, as well as causing severe respiratory disease, may also be responsible for several other affections, such us conjunctivitis, genital lesion, abortion, enteritis and encephalitis. The continuous circulation of the virus among the bovine population is the cause of serious economic losses for the cattle industry.

The protective measures aimed to control BoHV-1 infection include immunization with live attenuated or inactivated vaccines [1,2]. However, although those products have been partially successful, better and safer methods of immunization are needed. DNA immunization is an approach which could improve the safety and the efficacy of vaccination [3]. One of the first tests of the DNA immunization approach [4] demonstrated that inoculation of a plasmid encoding a viral gene into mouse resulted in expression of that gene in vivo. The use of DNA vaccination for BoHV-1 has shown promising results in mice [5] and partial success in calves [3]. In a previous study [6] we tested a candidate BoHV-1 DNA vaccine composed of a plasmid encoding epitopes of a single antigen encoded by the gD gene, with a truncated trans-membrane domain. Unfortunately, this vaccine did not protect the calves against infection with virulent BoHV-1.

* Corresponding author. Tel.: +39 0 734 621489; fax: +39 0 734 623449.
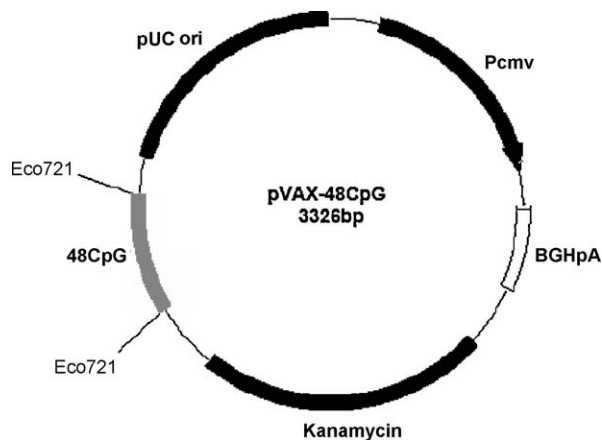E-mail address: s.petrini@izsum.it (S. Petrini).

Following on from study, here we evaluated the safety and efficacy of four BoHV-1 DNA vaccines, comprising combinations of plasmids encoding the truncated form of the gD gene including immunostimulatory cytidine-phosphate-guanosine (CpG) motifs contained in the oligodeoxyribonucleotide (ODN) 2135 and ubiquitin protein. Conjugation of the gD antigen with ubiquitin should target the endogenously synthesized antigen to the proteosome, resulting in enhanced MHC-I presentation.

2. Materials and methods

2.1. Virus

The Cooper strain 90/180 TN of BoHV-1 (subtype 1) [7] was selected for this study. The virus was used at the fourth passage on bovine embryo kidney (BEK) cell cultures at a titre of $10^{9.50}$ TCID$_{50}$/ml.

2.2. Plasmid vaccines

Plasmids were constructed by cloning the BoHV-1 gene encoding the truncated form of gD into pVAX (Invitrogen, San Diego, CA) under the control of enhancer/early promoter of cytomegalovirus and with a bovine growth hormone polyadenylation signal (BGH polyA). The truncated form of glycoprotein D (tgD) was constructed by terminating the protein at aminoacid 360, immediately upstream the membrane anchor [8]. The tgD sequence was generated by polymerase chain reaction (PCR) using the following primers:

- sense 5′-CCGGAAGCTTTGCTGCGAGCGGGCGAACATGCAA -3′;
- antisense 5′-CCGGTCTAGAGGCGTCGGGGGCCGCGGGCGT -3′.

PCR product was digested with HindIII and XbaI and cloned into the multiple cloning site of pVAX to form pVAX-tgD (Fig. 1).

Fig. 1. Plasmid pVAX-tgD.

Fig. 2. Plasmid pVAX-UbiLacI-tgD-L.

Plasmid pVAX-UbiLacI-tgD-L (Fig. 2) was constructed by cloning the BoHV-1 gene, encoding tgD with an additional deletion of 18 amino acids at the N-terminal, and fused with the chimeric protein UbiLacI. Eighteen amino acids were deleted in order to eliminate the secretion signal of gD. The sequence for tgD-L was generated by PCR using the following primer:

- sense 5′-TGCGAATCCTTGCCTACACCCGCGCCGCGG-3′,

and cloning into pVAX EcoRI and XbaI restriction sites. The sequence UbiLacI, which encodes for ubiquitin in fusion with 45 amino acid "destabilizing" residues from LacI gene [9] was generated by PCR as previously reported [10]. It was cloned into HindIII and EcoRI restriction sites in frame with tgD-L sequence. The primers used for this PCR reaction were:

- sense 5′-CCAAGCTTCCGGAGCCGCAGCCGCCACCATGCA-GATCTTCGTGAAGACCCTGACTGGTAAGACC-3′;
- antisense 5′-GCCCGAATTCTCGGGAAACCTGTGGTGCCA-GCTGCATTAA-3′.

The sense oligonucleotide was designed to include a Kozak sequence, which allows efficient transcription in mammalian cells [11].

Plasmid pVAX-48CpG (Fig. 3) was constructed by introduction of CpG motifs based on the bovine immunostimulatory sequence from ODN 2135 [11]. Briefly, two complementary oligodeoxynucleotides (BstEII forw: 5′-AATTCGGTTACCTCTAGACAAACCAACCAAT-3′; BstEII rev: 5′ CTAGATTGGTTGGTTGGTCTAGAGGTAACCG 3′) were annealed to form a duplex containing the BstEII restriction site, and then cloned between EcoRI and XbaI sites in the vector pcDNA3.1 (Invitrogen, San Diego, CA). Other two complementary oligodeoxynucleotides were annealed to form a duplex containing 12 CpG motifs with protruding ends complementary to the restriction site BstEII.

- CpGBstEII sense: 5′-GTTACGTCGTTTGTCGTTTTGTCGT-TTCGTCGTTTGTCGTTTTGTCGTTTCGTCGTTTGTCGTTTTG-TCGTTG-3′;

Fig. 3. Plasmid pVAX-48CpG.

- CpGBstEII antisense: 5′-GTAACCAACGACAAAACGACAA-ACGACGAAACGACAAAACGACAAACGACGAAACGACAAA-ACGACAAACGAC-3′.

Since the oligo CpG BstEII sense has a C in lieu of G was possible to clone in succession four CpG annealed product into BstEII locus. Finally, the whole sequence was modified by PCR using the primers:

- sense 5′-GTGTGGTGGAATTGGGTTACGT-3′;
- antisense 5′-GTGCGGGCCCACTAGAGGAAACCAACG-3′, and blunt-cloned into the Eco721 site of pVAX.

Plasmid DNA for immunization was purified from *Escherichia coli* strain DH5α using Qiagen Plasmid-Giga kits (Qiagen Chatsworth, CA), resuspended at 1 mg/ml in sterile endotoxin-free water (Gibco BRI) and stored at −20 °C.

2.3. Experimental design

Fifteen calves of three months of age, devoid of BoHV-1 neutralizing antibodies, were used. The calves were housed in isolation units, and fed twice a day with a diet of hay concentrate and water *ad libitum*. The maintenance and experimental protocols were established according to the animal care guidelines of International Guiding Principles

for Biomedical Research Involving Animals and the European Agency for the Evaluation of Medicinal Products (CVMP/IWP/07/98). The experimental design was performed after the approval of the local ethical committee.

Calves were divided into five groups of three each (Table 1). The calves in the first four groups were injected with the vaccine by intramuscular (i.m.) route in the retroauricolar region; calves of the remaining group (group 5) served as control and were injected with the plasmid vector. Group 1 received 1 mg of pVAX-tgD plasmid in 1 ml of 0.1 M phosphate buffer saline (PBS) (Vaccine A); group 2 was co-immunised with 1 mg of pVAX-tgD in 1 ml of PBS and 500 μg of pVAX-48CpG in 500 μl PBS (Vaccine B); group 3 received 1 mg of pVAX-UbiLacI-tgD-L in 1 ml of PBS (Vaccine C); group 4 was co-immunised with 1 mg of pVAX-UbiLacI-tgD-L in 1 ml PBS and with 500 μg of pVAX-48CpG in 500 μl PBS (Vaccine D). Calves of group 5 were inoculated with 1.5 mg of pVAX (plasmid vector) in 1.5 ml PBS and used as unvaccinated and challenge infection controls. All animals were immunised three times, at 28-day intervals.

Ninety days following the first immunisation all animals were challenged with a virulent BoHV-1 [7]. The virus were given by the intranasal route (i.n.) at a dose of $5\,ml \times 10^{9.50}\,TCID_{50}/ml$ for each animal. The calves were observed for 30 days and temperatures were taken daily. Nasal swabs in transport fluid minimum essential medium (MEM) were obtained from each calf on post-challenge day (PCD) 0, 2, 4, 7, 10 and used for viral isolation and titration assays. Serum samples were taken from each calf on the day of challenge (PCD 0) and on PCD 13, 21, 28. At the end of the study animals were killed and the target tissues (trachea, lung, mediastinic lymph-node, tonsils, spleen, thymus and trigeminal ganglia) were collected for histological observation.

2.4. Virus isolation

Serial dilutions ranging from 10^{-1} to 10^{-9} of the supernatants from each nasal swabbing, were inoculated at volume of 0.1 ml into three wells of a 24-well plastic plate (Corning Inc., NY, USA) containing monolayers of MDBK (Madin, Darby Bovine Kidney) cells line grown in MEM (GibcoBRL, Life Technologies Inc., Grand Island, NY). After 60 min incubation at 37 °C in 5% CO_2 atmosphere,

Table 1
Bovine herpesvirus-1 (BoHV-1) DNA vaccines used in the experiment.

Group[1]	Number of calves	Vaccine identification[2]	Type and composition	Concentration (1000 μg/1000 μl)	Number of inoculation	Inoculation route[3]
1	3	A	pVAX-tgD	1000 μg	3	i.m.
2	3	B	pVAX-tgD + pVAX-48CpG	1000 μg 500 μg	3	i.m.
3	3	C	pVAX-UbiLacI-tgD-L	1000 μg	3	i.m.
4	3	D	pVAX-UbiLacI-tgD-L + pVAX-48CpG	1000 μg 500 μg	3	i.m.
5	3	Plasmid vector	pVAX	1500 μg	3	i.m.

[1]All groups of calves were housed together.
[2]Vaccine or plasmid only were administered three times, i.e. 90, 62 and 34 days before challenge infection.
[3]The vaccine or plasmid were inoculated by intramuscular route in the retroauricolar region.

1 ml of MEM enriched with 2% FCS (BioWhittaker Inc., MD, USA) was added to each well. A positive control was prepared from MDBK cell cultures infected with Cooper strain 90/180 TN of BoHV-1 (subtype 1). MDBK cell cultures free of BoHV-1 were used as a negative control. The plates were incubated for 7 days at 37 °C in 5% CO_2 atmosphere and observed daily for appearance of cytopathic effect (CPE). Virus titre was determined according to Reed and Muench [12], and expressed as $TCID_{50}$/ml.

2.5. Neutralization test

Twenty-five μl of undiluted serum samples and twofold dilutions of each were mixed with 25 μl of 100 $TCID_{50}$ of BoHV-1 in 96-well microtitre plates (Corning Inc., Corning, NY). The plates were held at room temperature (22 °C) for 90 min and then 20,000 MDBK cells at 50 μl volumes were added to each well. Positive serum control was previously prepared from calves subjected to challenge infections with Cooper strain 90/180 TN of BoHV-1 (subtype 1) and negative serum control was prepared from calves free of BoHV-1 infections (data not published). Neutralization titres were expressed as $\log_2$ of the highest dilution inhibiting cytopathology.

2.6. Enzyme-linked immunosorbent assay (ELISA) test

Immuno 96 MicroWell plates (Nunc International, Rochester, NY) were coated with tgD diluted to 0.85 μg/ml in 0.05 M carbonate buffer, pH 9.6. After incubation overnight at 4 °C, the plates were rinsed in PBS containing 0.05% Tween 20. Twofold dilutions (from 1:100 to 1:12,800) of serum samples were tested in duplicate [13]. All plates were washed three times with PBS, incubated at 37 °C for 1 h, and after addition of anti-bovine IgG horseradish peroxidase diluted 1:500 and prepared at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, were incubated for 1 h at 37 °C. The substrate solution was prepared by adding 60 mg of o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO), and 50 μl of 30% H_2O_2 (Sigma–Aldrich, Milano, Italy) to 10 ml of 0.05 M citric acid (Sigma–Aldrich, Milano, Italy) buffer, pH 4.5. The reaction was blocked after 10–15 min by adding 2N H_2SO_4 (Sigma–Aldrich, Milano, Italy) to each well. Readings were performed using a spectrophotometer with a 492 nm filter. Positive serum control was previously prepared from calves subjected to challenge infections with Cooper strain 90/180 TN of BoHV-1 (subtype 1) and negative serum control was prepared from calves free of BoHV-1 infections (data not published). For the end-point titre, a positive value was scored for any sample with an OD 492 nm greater than three standard deviations above the background (calculated using 12 wells free of primary antibodies) and expressed as $\log_2$. All reagents were added in 100 μl volumes. Tests were repeated three times.

2.7. IFN-γ Enzyme-Linked ImmunoSpot (ELISpot) assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by density gradient centrifugation at $400 \times g$ for 30 min at room temperature (22 °C) using Histopaque 1077 (Sigma–Aldrich, Milano, Italy). Buffy coats were collected, resuspended in RPMI-1640 (BioWhittaker Inc., MD, USA) supplemented with 10% inactivated FCS (BioWhittaker Inc., MD, USA), 50,000 IU/l penicillin, 50 mg/ml streptomycin and 2 μg/ml amphotericin B (BioWhittaker Inc., MD, USA). Nitrocellulose 96-well plates (Millipore Multiscreen-PVDF-Pierce, Rockford, IL) coated with anti-bovine IFN-γ antibody (Pierce, Rockford, IL) were used. PBMC were added to triplicate wells and incubated at a concentration of 5×10^6 cells/ml at volumes of 50 μl/well for 24 h at 37 °C. The PBMCs were stimulated by addition of 0.85 μg/ml of tgD [13] or by 10 μg/ml of the T-cell mitogen phytohaemaglutinin (PHA) (Sigma, Milano, Italy) as a positive control. Unstimulated PBMC were used to assess background levels of INF-γ secretion. After 24 h of incubation at 37 °C in 5% CO_2, cells were removed and 50 μl/well of bovine biotin-conjugated antibody (Pierce, Rockford, IL) were added to each well and incubated 1 h at 37 °C. Finally, plates were incubated with a 1:1000 dilution of AP-conjugated streptavidin (Pierce, Rockford, IL) for 1 h at room temperature. The plates were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma–Aldrich, Milano, Italy). The number of IFN-γ secreting cells (SCs) was expressed as the difference between the mean number of spots per 10^6 cells/ml in tgD-stimulated wells and in unstimulated wells. Test were repeated three times. The data were generated by ELI.Scan software version 4.0, considered 1 spot ≥ 500 μmm^2.

2.8. Gross pathology and histology

Necropsies were performed on all target tissues collected at slaughter and immediately fixed in calcium-buffered formalin solution 10%, pH 7.4, and then paraffin embedded (56–58 °C, Bio-Optica, Italy). Microtome sections, 5 μm thick, were stained with H&E, Van Gieson and Schiff's reaction (PAS).

Slides were viewed with a Nikon Eclipse E800 microscope using a Nikon PLAN APO lens (Nikon, Tokyo, Japan) and Bio Mount medium (Bio-Optica, Milan, Italy). Images were acquired using a DS Camera Control Unit DS-L2 (Nikon) and were processed by Adobe Photoshop software version 7.0 (Adobe Systems, San Jose, CA).

2.9. Statistical analysis

Proportions of animals showing clinical signs in each group were compared by Pearson's chi-square test on cumulated data from PCD 0 to PCD8, i.e. the day in which all animals recovered. Means of virus titres after challenge infection were compared by Wilcoxon Rank-Sum Test. P values <0.05 were considered significant. Analysis was performed with SPSS v. 12.0 (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Clinical response of calves

The test vaccines did not induce any clinical signs in the immunised calves prior to challenge at day 90. The rectal

Table 2
Clinical response of calves immunised with experimental BoHV-1 DNA vaccines and challenge infected with virulent BoHV-1.

Group	Vaccine type and composition[1,2]	Clinical signs after challenge infection[3]				
		Fever $\geq 40\,°C$	Nasal discharge	Congiuntival lesions	Dyspnoea	Cough
1	A[c]	2/4	2/3[a]	2/3[abc]	1/3[ab]	1/2[ab]
2	B	1/3[a]	1/3[b]	1/3[def]	1/1[acde]	1/1[cd]
3	C	2/5	2/5	2/6[ad]	2/4[cf]	2/4[ce]
4	D	2/5	2/5	2/6[be]	2/5[dg]	2/5[ad]
5	Plasmid vector	2/6[a]	2/6[ab]	3/6[cf]	2/7[befg]	2/6[be]

[1]See Table 1 for the vaccine identification.
[2]Vaccine or plasmid only were administered three times, i.e. 90, 62 and 34 days before challenge infection.
[3]Day of onset after challenge infection/length of period during which clinical signs were detectable. Length periods followed by the same letter within columns are significantly different according to Pearson's chi-square test at $P \leq 0.05$.

Table 3
Bovine herpesvirus-1 (BoHV-1) isolation from calves, immunised with experimental BoHV-1 DNA vaccines, and challenge infected with virulent BoHV-1.

Group	Vaccine type and composition[1,2]	Viral isolation and titration after challenge infection on days[3]				
		0	2	4	7	10
1	A	–	9.50 (3)[4]	9.50 (3)	4.24 (3)	N.I.
2	B	–	9.50 (3)	9.50 (3)	3.74 (3)	N.I.
3	C	–	9.50 (3)	9.50 (3)	6.99 (3)[ab]	N.I.
4	D	–	9.50 (3)	9.50 (3)	7.49 (3)[ac]	N.I.
5	Plasmid vector	–	9.50 (3)	9.50 (3)	6.50 (3)[bc]	N.I.

N.I. = not isolated.
[1]See Table 1 for the vaccine identification.
[2]Vaccine or plasmid only were administered to the calves three times, i.e. 90, 62 and 34 days before challenge infection.
[3]Reciprocal value of the negative log of the $TCID_{50}$/ml (group mean value); means followed by the same letter within columns are not significantly different according to Wilcoxon Rank-Sum Test at $P \leq 0.05$.
[4]Between brackets number of calves from which virus was recovered.

temperatures were within the normal values and similar to the control values (range 39.0–39.5 °C). After challenge, all immunised calves presented clinical signs which were similar to those observed in the controls (Table 2). They had high fever (41.0–42.0 °C) from PCD 1 (group 2) and PCD 2 (other groups), which lasted for 2–4 days. In animals of group 2, fever lasted for significantly less time ($P < 0.05$) than controls. Nasal mucus discharge, lesions of the nasal mucosa consisting of pseudomembranes associated with mucopurulent exudate, dyspnoea and cough were detected from PCD 1 (groups 1 and 2), PCD 2 and 3 (other groups) from 1 till 5 days. All animals had recovered after 3 weeks following challenge.

3.2. Virus shedding

After challenge infection, all vaccinated calves shed virus on PCD 2, 4 and 7. The mean titres of the virus recovered from all groups on PCD 2 and 4 were $10^{9.50}$ $TCID_{50}$/ml. By PCD 7 the titres generally dropped varying from little more than 3 log units (Vaccines C and D) to 5.26–5.76 log units (Vaccines A and B). On PCD7, virus titres of groups 1 and 2 were significantly lower than titres of groups 3, 4, and 5 (control). No virus was isolated from any of the calves on PCD 10. Control calves shed virus up to PCD 7. The virus titres were $10^{9.50}$ $TCID_{50}$/ml in all nasal swabs taken on PCD 2 and 4. On PCD 7 the titre decreased to $10^{6.50}$ $TCID_{50}$/ml (Table 3).

3.3. Neutralizing antibody

No increase in antibody titre to BoHV-1 was detected in the vaccinated calves except for animals treated with Vaccine B which presented a mean neutralization titre of 1.00 $\log_2$ on post-vaccination day (PVD) 56, which increased to 1.30 $\log_2$ on PVD 90. The neutralizing antibody titre of calves immunised with Vaccine B increased after challenge infection, reaching values of 4.11 $\log_2$ on PCD 13

Table 4
Serum neutralizing antibody response of calves immunised with experimental BoHV-1 DNA vaccines and challenge infected with virulent BoHV-1.

Group	Vaccine type and composition[1,2]	Neutralizing antibody titres to BoHV-1 after challenge on day[3]			
		0	13	21	28
1	A	<1.00	5.73	4.73	5.22
2	B	1.30	5.41	4.73	4.73
3	C	<1.00	3.21	3.40	3.73
4	D	<1.00	4.41	3.73	4.21
5	Plasmid vector	<1.00	2.41	2.41	2.77

[1]See Table 1 for the vaccine identification.
[2]Vaccine or plasmid only were administered three times, i.e. 90, 62 and 34 days before challenge infection.
[3]Expressed as $\log_2$ of the reciprocal of the highest dilution inhibiting cytopathogenic effects (mean value).

Table 5
Antibody titres, measured by ELISA, against truncated glycoprotein D (tgD) of BoHV-1 in calves immunised with experimental bovine herpesvirus-1 (BoHV-1) DNA vaccines and challenge infected with virulent BoHV-1.

Group	Vaccine type and composition[1,2]	Antibody titres to tgD of BoHV-1[3] after challenge infection	
		PCD 0	PCD 13
1	A	<1.00	3.06
2	B	2.60	3.72
3	C	<1.00	2.82
4	D	<1.00	3.87
5	Plasmid vector	<1.00	3.07

PCD = post-challenge day.
[1]See Table 1 for vaccine identification.
[2]Vaccine or plasmid only were administered three times, i.e. 90, 62 and 34 days before challenge infection.
[3]Expressed as $\log_2$ of the reciprocal of the highest serum dilution positive by ELISA (mean value).

and 4.73 $\log_2$ on PCD 21, and persisted at the same mean value until PCD 28. In the other groups which received Vaccines A, C, D or plasmid vector, neutralizing antibodies were detected only on PCD 13, with mean titres of 5.73, 3.21, 4.41 $\log_2$ for calves vaccinated with Vaccine A, C and D, respectively. These titres did not vary significantly on PCD 28. No seroconversion was detected in the control group inoculated with the plasmid vector. After challenge infection neutralizing antibodies evaluated on PCD 13 and 21 had a titre of 2.41 $\log_2$ which increased of 0.36 $\log_2$ on PCD 28 (Table 4).

3.4. ELISA test

Antibodies against tgD evaluated by ELISA test were first detected on PVD 90 in calves inoculated with Vaccine B, with a mean titre of 2.60 $\log_2$ and that increased to 3.72 $\log_2$ on PCD 13. No antibodies were detected in the other vaccinated groups as well as in the controls PVD 90. In these animals as well as in the control group, antibodies were detected only following BoHV-1 experimental infection with mean titres of 3.06, 2.82, 3.87, and 3.07 $\log_2$ for calves vaccinated with products A, C, D, and plasmid vector, respectively PCD 13 (Table 5).

3.5. ELISpot test

IFN-γ producing cells could be detected only in the groups of calves inoculated with Vaccines A and B, with mean number of spots of 75 and 115/10^6 cells, respectively. A further increase was detected in these animals on PCD 21 when the mean number of spots was 389 (Vaccine A) and 179 (Vaccine B)/10^6 cells. In the other groups either immunised with Vaccines C and D or in the controls a rise in the IFN-γ producing cells was detected only after challenge, with a mean number of spots ranging from 120 to 150/10^6 cells (Fig. 4).

3.6. Gross pathology and histology

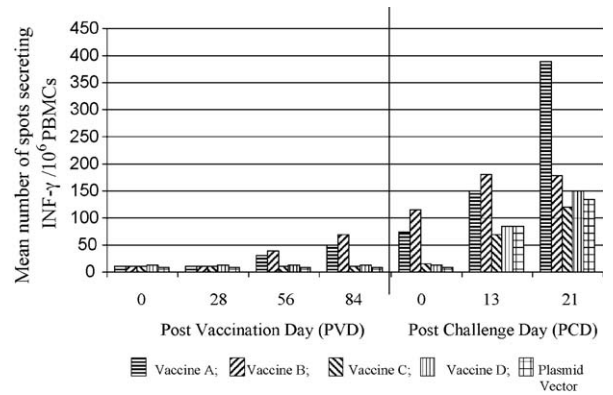Necropsy was conducted 30 days after challenge. During the examination, macroscopic lesions relating to BoHV-1

Fig. 4. Number of BoHV-1 IFN-γ secreting cells in calves vaccinated with BoHV-1 DNA vaccines and infected with the virulent strain of BoHV-1 measured by ELISpot assay. The results are reported as the average number of spots.

infection were not observed in the vaccinated or control calves. Histopathology identified lymphocytic hyperplasia in the lymphoid organs of all infected calves (Figs. 5 and 6). There was a slightly lymphocyte depletion of the thymus associated with both vaccinated and control calves. Moreover, mononuclear infiltrations were constantly observed in the submucosa of the trachea from all vaccinated groups but not the controls suggesting that these histological lesions were related to the vaccine response and were not the result of side-effect. No lesions were observed in lung parenchyma of calves in the vaccinated and control group. No histological lesions were detected in any of the target tissues analyzed from all five groups.

4. Discussion

Viral surface glycoproteins of BoHV-1 have been identified as the main targets for protective humoral or cell-mediated immune responses and they have been selected as candidate antigens in novel vaccine strategies such as DNA immunization. Glycoproteins B, C and D of BoHV-1 have been tested to evaluate their safety and
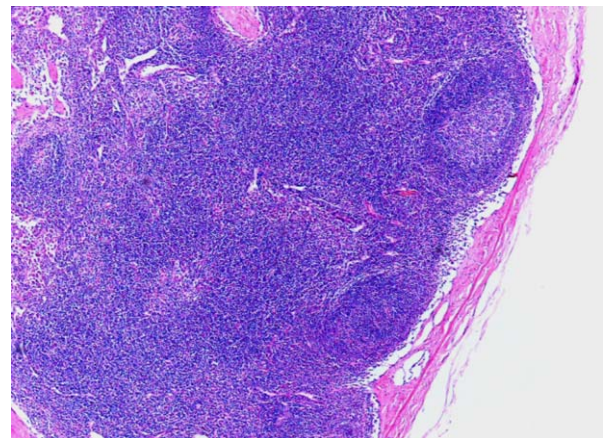
Fig. 5. Mediastinic lymph-node: mild follicular hyperplasia. Several reactive follicles and surrounding mantle zone are visible, sinus histiocytosis is also present (H&E – optical microscopy 4×).

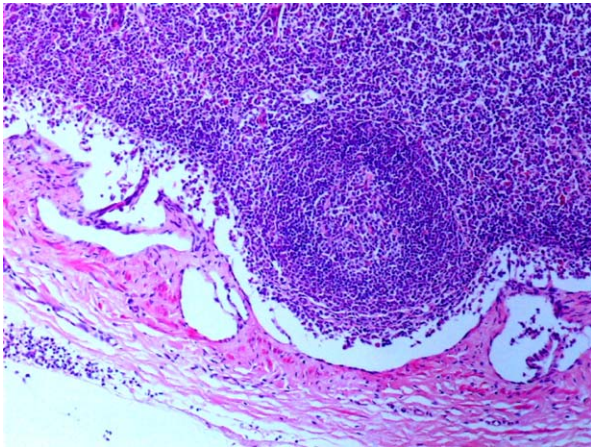**Fig. 6.** Mediastinic lymph-node: higher magnification of previous image (H&E – optical microscopy 10×).

efficacy with disappointing results in large animals. In order to increase the immunogenicity of the DNA vaccines, novel adjuvant approaches like the incorporation of CpG oligodeoxynucleotides (ODN) have been tested for their ability to enhance immune responses against viral antigens [14]. More specifically, the cytokine profile induced by CpG motifs is generally preferential for a Th1-type immune response [15]. It has been demonstrated [16] that the level of stimulation of a cellular immune response to BoHV-1 glycoprotein D induced by vaccination with CpG-enhanced plasmid vectors was related to the number of CpG motifs. Results from a previous study [6] have shown that a DNA vaccine encoding the truncated form of BoHV-1 glycoprotein D (tgD) was able to stimulate low levels of virus neutralizing antibody, but provided no protection against subsequent BoHV-1 challenge [17].

To improve DNA vaccine efficacy a number of improvements have been suggested. These include increasing the number of vaccine injections, increasing the concentration of the expressed protein, the addition of adjuvant molecules such as ODN CpG and the UbiLacI chimeric proteins. In this study a plasmid (pVAX-48CpG) was constructed containing 12 copies of the CpG hexamer (GTCGTT), organized in the same way as the ODN 2135 [16]. The pVAX-48CpG was co-immunised with the tgD plasmid because addition of the CpG motifs directly to the backbone of the plasmid encoding the glycoprotein D of BoHV-1 may cause a possible bias in the experiment [18]. We have also included an additional approach to enhance the cellular response to tgD. This involved conjugation of tgD with a proteasome-dependent degradation signal, as it has been reported that the ubiquitin–proteosome pathway is responsible for intracellular protein degradation and the production of peptides for antigen presentation via MHC class I. Hence, increased antigen turnover should increase the number and variety peptides available for MHC-I binding, which may enhance the cell-mediated immune response to the vaccine antigens [18].

Nevertheless, previous studies suggest that an enhanced cellular immunity to BoHV-1 does not correlate with protection unless a strong humoral response is induced as well [18].

Based on the results of this study, the BoHV-1 DNA vaccines did not protect the calves against the infection with virulent BoHV-1. The low or absence of protection observed under the experimental conditions might also be due to the high virus titre ($10^{9.50}$ TCID$_{50}$/ml) of the virulent BoHV-1 used, a concentration that is not usually found in the field. However, calves of group B and, to a less extent, those in group A developed milder clinical signs compared to the controls. This feature was also displayed by DNA vaccines expressing glycoprotein A [18] or D [17] of BoHV-1 as a result of their inability to stimulate a Th-1 type response. Moreover, in calves of groups A and B, earlier clearance of challenge virus was observed as indicated by the virus titres which were significantly lower then those detected in the other vaccinated groups as well as in the controls. This finding seems to be caused by the inhibition of the replication in target tissues by the antibodies to tgD.

The delay in inhibiting virus replication could be the result of the absence of a complete immune response directed to the virus due to the expression only of the tgD in the vaccine. This hypothesis has been confirmed by the humoral and cell-mediated immune response in calves of group B after vaccination and by their increasing following challenge exposure, which indicates a priming, but an incomplete protection and by the level of neutralizing antibody titres in group 5 which remained at lower level than those of the groups exposed to the vaccines expressing tgD glycoprotein.

Beside the importance of clinical protection and humoral immune response, the evaluation of the cell-mediated immunity is now assuming a role as fundamental parameter of immune efficiency during vaccination protocols. It is well known that the Th-1 cell-mediated response and the T cytotoxic lymphocytes activation play a major role in the early reduction in virus replication and disease prevention. Recently, more information has been obtained about the specific T-cell-mediated response during a viral infection, thanks to qualitative and quantitative determination of IFN-γ cytokine by an ELISpot assay, an antigen-specific and high sensitive technique employed for the determination of cells secreting immune cytokines implied in cell-mediated immunity [19].

In order to evaluate the role of IFN-γ in this study, the synthesis of this cytokines was analyzed by ELISpot assay using PBMC from the calves above mentioned.

We demonstrated that the mean number of spots secreting IFN-γ 5×10^6 cells/ml PBMC responding *in vitro* to tgD of BoHV-1 mainly in the calves vaccinated with the pVAX-tgD (Vaccine A) and pVAX-tgD combined with pVAX-48CpG. This suggests that BoHV-1-specific memory cells increase after exposure to BoHV-1 virulent strain. IFN-γ increased in this group up to 21 post-challenge days. Interestingly IFN-γ (unstimulated) was detected in the same period and might be associated with the presentation *in vivo* of viral antigen by infected circulating PBMC. In other works [20,21], in BoHV-1 experimentally infected calves cell-associated viremia was detected within the first week after challenge infection and lasted approximately 1–2 weeks depending on immune status [22,23]. The first peak of IFN-γ secretion observed in this work is presently after 21 days post-challenge. The kinetics of IFN-γ

synthesis described in this study resembled a CD8$^+$ T-cell response to viral infection namely a first expansion phase (days 0–20), cumulating in the generation of BoHV-1-specific T-cells secreting IFN-γ and coincided with the decline of cell-associated viremia.

In contrast, the ubiquitin sequences included in Vaccines C and D together with tgD expression, were not able to stimulate an immune response.

The truncated gD protein expressed in fusion with the ubiquitin in two vaccines proposed herein may not contain immunodominant CD4$^+$ epitopes able to help the activation of an effective CTL response against the virus. Notably our data are consistent with previous observations demonstrating that increased antigen degradation produces decreased antibody induction *in vivo*, presumably due to insufficient amounts of antigen remaining to interact with B-cells [24–26]. Nevertheless previous studies suggest that an enhanced cellular immunity to BoHV-1 does not correlate with protection unless a strong humoral response is induced as well [16].

To conclude, the results of this study indicate that vaccination of calves with a DNA vaccines expressing tgD of BoHV-1 combined with GpG motifs has been able to prime the immune system.

However, this response was able to protect only partially animals from virulent BoHV-1 challenge infection as shown by the less severity of clinical signs and by the lower amount of virus shed.

All DNA vaccines used in this experiment did not show any residual pathogenicity for respiratory apparatus as shown by macroscopic investigations and histology. These findings could be caused by the long interval period between challenge infection and necropsy. These results indicated that BoHV-1 DNA vaccines expressing viral surface glycoproteins, combined with CpG olygodeoxynucleotides (ODN) in the plasmid backbone, could be used safely for priming the immune system against BoHV-1 infection.

Conflict of interest

Acknowledgements

References

[1] Castrucci G, Frigeri F, Salvatori D, Ferrari M, Sardonini Q, Cassai E, et al. Vaccination of calves against bovine herpesvirus-1: assessment of the protective value of eight vaccines. Comp Immunol Microbiol Infect Dis 2002;25:29–41.

[2] Castrucci G, Frigeri F, Salvatori D, Ferrari M, Lo Dico M, Rotola A, et al. A study on latency in calves by five vaccines against bovine herpes-virus-1 infection. Comp Immunol Microbiol Infect Dis 2002;25:205–15.

[3] Babiuk LA, Lewis J, van den Hurk S, Braun R. DNA immunization: present and future. Adv Vet Med 1999;41:163–79.

[4] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1999;247:1465–8.

[5] Caselli E, Boni M, Di Luca D, Salvatori D, Vita A, Cassai E. A combined bovine herpesvirus 1 gB-gD DNA vaccine induces immune response in mice. Comp Immunol Microbiol Infect Dis 2005;28:155–66.

[6] Castrucci G, Ferrari M, Marchini C, Salvatori D, Provinciali M, Tosini A, et al. Immunization against bovine herpesvirus-1 infection. Preliminary tests in calves with a DNA vaccine. Comp Immunol Microbiol Infect Dis 2004;27:171–9.

[7] Castrucci G, Ferrari M, Traldi V, Tartaglione E. Effects in calves of mixed infections with bovine viral diarrhoea virus and several other bovine viruses. Comp Immunol Microbiol Infect Dis 1992;15:261–70.

[8] Tikoo SK, Zamb TJ, Babiuk LA. Analysis of bovine herpesvirus 1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia viruses. J Virol 1993;67:2103–9.

[9] Bachmair A, Varshavsky A. The degradation signal in a short-lived protein. Cell 1989;56:1019–32.

[10] Bachmair A, Finley D, Varshavsky A. In vivo half-life of a protein as a function of its amino-terminal residue. Science 1986;234:179–86.

[11] Kozak M. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J Mol Biol 1987;196:947–50.

[12] Reed LJ, Muench HA. A simple method of estimating 50% end points. Am J Hyg 1933;27:493–7.

[13] Petrini S, Ferrari M, Vincenzetti S, Vita A, Amici A, Ramadori G. An immunoenzyme linked assay (ELISA) for the detection of antibody to truncated glycoprotein D (tgD) of bovine herpesvirus-1. Vet Res Commun 2006;30:257–9.

[14] Mutwiri G, Benjamin P, Soita H, Babiuk LA. Co-administration of polyphosphazenes with CpG oligonucleotides strongly enhances immune responses in mice immunized with Hepatitis B virus surface antigen. Vaccine 2008;23:2680–8.

[15] Babiuk LA, Pontarollo R, Babiuk S, Loehr B, Van Drunen Little-van den Hurk S. Induction of immune responses by vaccines in large animals. Vaccine 2003;21:649–58.

[16] Pontarollo RA, Babiuk LA, Hecker R, Van Drunen Little-van den Hurk S. Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors. J Gen Virol 2002;83:2973–81.

[17] Cox GJM, Zamb TJ, Babiuk LA. Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. J Virol 1993;67:5664–7.

[18] Gupta PK, Saini M, Gupta LK, Rao VDP, Bandyopadhyay SK, Butchaiah G, et al. Induction of immune responses in cattle with a DNA vaccine encoding glycoprotein C of bovine herpesvirus-1. Vet Microbiol 2001;78:293–305.

[19] Corradi A, Ferrari L, Borghetti P. Parameters for evaluating the cell-mediated immune response during viral infection: diagnostic and prognostic applications. Vet Res Commun 2007;31(Suppl 1):103–7.

[20] Huang Y, Babiuk LA, Van Drunen Little-van den Hurk S. Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic t-lymphocyte responses in mice and cattle. J Gen Virol 2005;86:887–98.

[21] Gupta PK, Saini M, Gupta LK, Rao VDP, Bandyopadhyay SK, Butchaiaiah G, et al. Induction of immune response in cattle with a DNA vaccine encoding glycoprotein C of bovine herpesvirus-1. Vet Microbiol 2001;78:293–305.

[22] Babiuk LA, Rouse BT. Herpesvirus vaccines. Adv Drugs Deliv Rev 1996;21(1):63–76.

[23] Babiuk LA, Lewis J, Van Drunen Little-van den Hurk S, Braun R. Adv Vet Med 1999;41:201–7.

[24] Day C, Lauer GM, Robbins GK, McGovern B, Wurcel AG, Gandhy RT, et al. Broad specificity of virus-specific CD4$^+$ T-helper-cell responses in resolved hepatitis C virus infection. J Virol 2002;76:12584–95.

[25] Godkin A, Thomas H, Openshaw P. Evolution of epitope specific memory CD4 T cells after clearance of hepatitis C virus. J Immunol 2002;169:2210–4.

[26] Ahmadzadeh M, Farber DL. Functional plasticity of an antigen-specific memory CD4 T cell population. Proc Natl Acad Sci USA 2002;99:11802–7.