Primming with DNA encoding E2 and boosting with E2 protein formulated with CpG oligodeoxynucleotides induces strong immune responses and protection from *Bovine viral diarrhea virus* in cattle

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The objective of this study was to develop an optimal vaccination strategy for *Bovine viral diarrhea virus* (BVDV). The E2 protein of BVDV plays a major protective role against BVDV infection. In order to be able to compare DNA, protein and DNA prime–protein boost regimens, a plasmid was constructed encoding a secreted form of the NADL strain E2 protein (pMASIA-tPAaΔE2). Furthermore, a pure secreted recombinant ΔE2 (rΔE2) protein was produced. The rΔE2 protein was formulated with a combination of Emulsigen and CpG oligodeoxynucleotide. Groups of calves were immunized with pMASIA-tPAaΔE2 or with rΔE2, or first with pMASIA-tPAaΔE2 and then with rΔE2. To evaluate the protection against BVDV, calves were challenged with BVDV strain NY-1 after the last immunization. Although all immunized calves developed humoral and cellular immune responses, the antibody responses in the DNA prime–protein boost group were stronger than those elicited by either the DNA vaccine or the protein vaccine. In particular, E2-specific antibody titres were enhanced significantly after boosting the ΔE2 DNA-primed calves with rΔE2 protein. Moreover, protection against BVDV challenge was obtained in the calves treated with the DNA prime–protein boost vaccination regimen, as shown by a significant reduction in weight loss, viral excretion and lymphopenia, compared with the unvaccinated calves and the animals immunized with the DNA or protein only. These results demonstrate the advantage of a DNA prime–protein boost vaccination approach in an outbred species.

INTRODUCTION

Acute infections caused by *Bovine viral diarrhea virus* (BVDV) are very common in cattle and result in mild disease of short duration characterized by fever, increased respiratory rate, diarrhoea and a reduction in the number of white blood cells. Although animals generally recover, the effect of BVDV on the immune cells reduces the host’s resistance to disease, so BVDV may be an important pathogen in stressed cattle entering a feedlot and may play a role in shipping fever. The recently identified type 2 strains can cause acute infections in herds, characterized by high fever, haemorrhaging, diarrhoea, reduction in the number of white blood cells and platelets, and death. Infection of seronegative cattle with non-cytopathic BVDV during the first 120 days of pregnancy can result in the birth of immunotolerant calves that are persistently infected with BVDV (Brownlie, 1990). These BVDV carriers are the most important source of virus spread (Duffell & Harkness, 1985). BVDV is a member virus of the genus *Pestivirus*, which also contains *Classical swine fever virus* and ovine *Border disease virus*. Together with the genera *Flavivirus* and *Hepacivirus*, pestiviruses belong to the family *Flaviviridae*. The BVDV genome encodes a single polyprotein precursor that is co- and post-translationally processed by host and viral proteases to produce 11 or 12 mature structural and non-structural proteins (Bolin & Ridpath, 1990; Collett *et al.*, 1988, 1991). The capsid protein and the glycoproteins E<sub>RNS</sub>, E1 and E2 are structural components of the virion (Rümenapf *et al.*, 1993). The envelope protein E2 forms a heterodimer with E1 as well as homodimers (Weiland *et al.*, 1990) and may play a major role in virus attachment and entry (Donis, 1995). Neutralizing activity has been demonstrated predominantly for E2-specific antibodies (Bolin, 1993). The C terminus of E2 includes 30 hydrophobic amino acids, which could function as a transmembrane anchor for E2 and as a translocation signal for the downstream protein. Full-length E2 remains cell-associated in virus-infected cells (Rümenapf *et al.*, 1993).
Although DNA vaccines have been proposed as third-generation vaccines and examined for a variety of viral pathogens (Ulmer et al., 1993; van Drunen Littel-van den Hurk et al., 1998; Wang et al., 1993; Xiang et al., 1994) including BVDV (Harpin et al., 1997), BVDV DNA vaccines have been moderately effective in cattle (Harpin et al., 1997; Nobiron et al., 2003). Although other factors, such as antigen dose or the route of immunization and adjuvant, could affect the immune responses, strategies in which priming with DNA is followed by boosting with viral vectors could affect the immune responses, strategies in which CpG dinucleotides are unmethylated and present at an expected frequency in bacterial DNA, whereas they are underrepresented and selectively methylated in vertebrate genomes (Cardon et al., 1994). Thus, vertebrates recognize bacterial DNA as a pathogen-associated pattern (Krieg et al., 1998). By binding Toll-like receptors, CpG can activate dendritic cells and macrophages to trigger the production of interleukin-1 (IL-1), IL-6, IL-12 and tumour necrosis factor alpha, as well as natural killer cells and lymphocytes to produce gamma interferon (IFN-γ), IL-6, IL-10 and immunoglobulin, respectively (Ballas et al., 1996; Hartmann & Krieg, 2000; Hartmann et al., 2000). CpG DNA and CpG oligodeoxynucleotides (ODNs) stimulate Th1-type responses, characterized by IL-12 and IFN-γ secretion with little production of Th2 cytokines and a predominance of IgG2a over IgG1 in the mouse (Chu et al., 1997; Davis et al., 1998; Ioannou et al., 2002a; Jakob et al., 1998). The high efficacy of CpG ODN as an adjuvant has been demonstrated in a number of species, including mice and cattle (Davis, 2000; Ioannou et al., 2002a; b; Klinman, 2003; Krieg & Davis, 2001).

Previously, we evaluated plasmids encoding various truncated forms of BVDV E2 (ΔE2) either with the glycoprotein D signal or with the tissue plasminogen activator signal (tPAs) of Bovine herpes virus 1 (BHV-1). The plasmid pMASIA encoding ΔE2 with the tPAs (pMASIA-tPAsΔE2) elicited the strongest immune responses and could be a candidate DNA vaccine against BVDV (Liang et al., 2005). In the present study, we vaccinated calves with this ΔE2 DNA vaccine or with a ΔE2 protein vaccine, or we primed calves with pMASIA-tPAsΔE2 and boosted them with ΔE2 protein formulated with Emulsigen and CpG ODN to optimize further the vaccination strategy against BVDV.

**METHODS**

**Construction, purification and expression of plasmid.** All restriction enzymes were purchased from Amersham Pharmacia. The NADL strain E2 gene was resynthesized to optimize the codon bias in favour of expression in bovine cells (http://www.kazusa.or.jp/codon/) (Nakamura et al., 2000) and then inserted into pUC19. The ΔE2 gene, which lacks the C-terminal 30 aa constituting the membrane anchor, was amplified by PCR from pUC19-E2 using primers 5′-TCCCGGGGGGACACTTGAGTTGAAACT-3′ and 5′-GCGGAAAGATTCCTACAGAATGGACTGCGAA-3′. PCR products were digested with Smal and BglII and cloned into pSLIA-tPAs (Liang et al., 2005) to obtain pSLIA-tPAsΔE2 and then subcloned into pMASIA (Ponarollo et al., 2002) to obtain pMASIA-tPAsΔE2. The construct was confirmed by restriction enzyme digestion and agarose gel electrophoresis and sequenced for cloning accuracy. The plasmid used for immunization of cattle was grown in Escherichia coli DH5α cells and purified with an EndoFree Plasmid Giga kit (Qiagen). Expression of ΔE2 was confirmed by transient transfection of COS-7 cells and analysis by Western blotting.

**Purification of recombinant E2 protein from transformed Madin–Darby bovine kidney (MDBK) cells.** Truncated, secreted recombinant E2 (rΔE2) protein of the NADL strain was expressed in MDBK cells under the control of the hsp70 gene promoter as described previously for BHV-1 gD (Kowalski et al., 1993). Supernatant containing rΔE2 was collected and cellular debris was removed by centrifugation. The cleared rΔE2-containing supernatant was cyclic through an E2-specific monoclonal antibody affinity column. The column was washed with 10 mM Tris/HCl (pH 7-5), 500 mM NaCl, 0-1% NP-40, and rΔE2 protein was eluted with 50 mM diethylamine (pH 11-5), dialysed against PBS (pH 7-4) and concentrated 10-fold with an Amicon Ultra 10000 MWCO filter (Millipore). The concentration of purified rΔE2 was determined using a Bio-Rad protein assay and the purity was assessed by SDS-PAGE.

**Cells and virus.** COS-7 cells were grown in Dulbecco’s minimum essential medium (MEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) at 37°C in a CO2 atmosphere and used for transfections. MDBK cells were purchased from ATCC and confirmed to be BVDV-free by RT-PCR and immunofluorescence. Cells were grown in Eagle’s MEM with 1.0 mM sodium pyruvate, 0-1 mM non-essential amino acids, 10 mM HEPES, 50 μg gentamicin ml−1 and 10% horse serum (HS; Gibco-BRL) at 37°C in a CO2 atmosphere. MDBK cells were infected with BVDV type 1a (NADL strain) or type 1b (NY-1 strain) at an m.o.i. of 0-1 for 1 h at 37°C. After 96 h, cells were subjected to three freeze-thaw cycles and centrifuged at 1000 r.p.m. in a Sorvall Legend RT centrifuge (Kendro) for 5 min. The virus inocula were titrated and frozen at −70°C.

**Immunization and challenge.** Twenty BVDV-negative 6-month-old Hereford and Angus crossbred calves were purchased from a single source ranch in southern Saskatchewan. The calves were randomly allocated to four groups of five animals each and immunized as follows: (i) three times with saline; (ii) three times with 1 mg pMASIA-tPAsΔE2; (iii) twice with 1 mg pMASIA-tPAsΔE2, followed by 35 μg rΔE2 with 30% Emulsigen (MVP Laboratories) and 500 μg CpG ODN 2007; or (iv) three times with 35 μg rΔE2 with 30% Emulsigen and 500 μg CpG ODN 2007. ODN 2007 (5′-CGTGTTGTCCGTTCGTTGTT-3′) (CpG dinucleotides underlined) was synthesized by Qiagen and diluted in endotoxin-free PBS (Gibco-BRL). We have previously shown the combination of Emulsigen and CpG ODN to have excellent adjuvant activity (Ioannou et al., 2002a, b). The plasmid was delivered transdermally with a Biojector (Bioject) in the hip, whereas the protein and saline were delivered subcutaneously. Vaccinations took place on days 0, 25 and 50. Two weeks after the third immunization, calves were each exposed for 4 min to a 4 ml aerosol of 1×106 TCID50 BVDV strain NY-1, generated using a DeVilbiss Ultra-Neb 99 nebulizer. Body temperatures, weights and clinical status were determined daily for 12 days from the day of challenge. All procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care.

**Sampling.** Sera were collected prior to each immunization, on the challenge day and on days 4, 8 and 11 after challenge. Peripheral
blood was collected in EDTA-treated vacutainer tubes (Becton Dickinson) for lymphocyte proliferation and enzyme-linked immunospot (ELISPot) assays prior to each immunization, on the challenge day and on day 11 after challenge. Peripheral blood was collected daily in EDTA for white blood cell (WBC) counts and virus isolation, and nasal swabs were taken daily for virus isolation from the day of challenge until the end of the trial on day 12. Nasal secretions were collected 2 days prior to challenge and on days 4, 8 and 11 after challenge to measure local antibody production.

**Haematological profiles.** An automated haematological analyser (Cell-Dyn 3500R) was used to determine both total WBC counts and differential leucocyte counts. Lymphocytes, monocytes and segregates were quantified in the differential count. Results were expressed as counts × 10⁹ l⁻¹⁻¹.

**ELISAs.** To determine antibody titres, 96-well polystyrene microtitre plates (Immulon 2; Thermo Electron Corp.) were coated overnight with 4 ng rAE2 per well and incubated for 2 h at room temperature with fourfold-diluted bovine serum prepared in PBS with 0-05 % Tween 20 and 0-5 % gelatin. Affinity-purified alkaline phosphatase (AP)-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratories) was used to detect IgG and affinity-purified AP-conjugated rabbit anti-bovine IgA (Bethyl Laboratories) was used to detect IgA. AP-conjugated murine antibodies recognizing bovine IgG1 and IgG2 (Bethyl Laboratories) were used for Ig isotyping. The reactions were visualized with 0-01 M p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read on a model SpectraMax 340PC³⁸⁴ microplate spectrophotometer ( Molecular Devices) at 405 nm, with a reference wavelength of 490 nm. ELISA titres were calculated as the highest dilution resulting in a reading of two SD above the value of a negative control serum.

**Virus isolation.** Virus isolations from WBCs and nasal swabs were carried out in MDBG cells in 96-well tissue culture plates (Bruschke et al., 1999). WBCs were isolated from 5 ml EDTA/blood by adding 0-83 % ammonium chloride (Sigma-Aldrich) to lyse the erythrocytes, followed by two washes with PBS. The pellet was resuspended in 1 ml Eagle’s MEM and stored at −70 °C. WBCs and nasal swabs were serially diluted 10-fold starting at 1:10 and 100 μl was added to duplicate wells. After 1-5 h, 100 μl MEM with 2 % HS was added to each well. Four days later, cells were fixed with 80 % acetone at room temperature and permeabilized with 0-5 % Triton X-100 in PBS for 10 min. Subsequently, cells were washed and blocked with PBS containing 10 % HS, and bovine anti-BVDV antibodies diluted in PBS were added to the cells. After an incubation of 1-2 h at room temperature, AP-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratories) was added (50 μl per well) and the plates were further incubated for 1-2 h at room temperature. Finally, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich) were added for 45–60 min and the plates were washed three times with distilled H₂O. The reciprocal of the highest dilution still showing the presence of virus in both wells was reported as the virus titre. If the titre of a sample was more than 1:10, it was reported as positive (+) ; if less than 1:10, it was negative (−). To confirm very low BVDV titres, 200 μl of sample per well were incubated on a monolayer of MDBK cells in a 24-well tissue culture plate. After incubation for 1-5 h, the plates were washed, 0-5 ml MEM with 2 % HS was added and the plates were incubated for 4 days. After three passages, the plates were frozen and thawed twice and the samples were used for virus isolation.

**Virus neutralization (VN) assay.** VN assays were conducted using heat-inactivated bovine sera. One hundred microlitres containing 200 TCID₅₀ BVDV strain NADL or NY-1 were pre-incubated with 100 μl serum diluted twofold starting at 1:10 for 1-5 h at 37 °C. Fifty microlitres of this mixture was then added to duplicate wells of microtitre plates containing 80–90 % confluent MDBG cells at 37 °C for 1 h. MEM with 2 % HS (150 μl) was added to each well. The plates were incubated at 37 °C for 3 days. The NY-1-infected cells were fixed and stained as described for virus titration assays. The reciprocal of the highest dilution that completely inhibited viral cytopathic effect in the two test wells was reported as the VN titre.

**Lymphocyte proliferation assay.** Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood on Ficoll-Paque PLUS (Amersham Biosciences). PBMCs were dispensed at 1 × 10⁶ cells (ml culture medium⁻¹) consisting of MEM, 10 % FBS, 2 mM L-glutamine (Gibco-BRL), 50 μg gentamicin ml⁻¹, 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 1 ng dexamethasone (Sigma-Aldrich) ml⁻¹. Subsequently, PBMCs at a concentration of 3-5 × 10⁵ cells per well were added to triplicate wells and restimulated in vitro for 72 h in the absence or presence of 1 μg purified rAE2 protein per well. After 3 days in culture, cells were pulsed with 0-4 μCi [methyl-³¹H]mytidine (Amersham Biosciences) per well. Cells were collected 18 h later and thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the means of triplicate wells and expressed as the stimulation index (counts min⁻¹ in the presence of antigen/counts min⁻¹ in the absence of antigen).

**IFN-γ ELISpot assay.** Nitrocellulose plates (Millipore) were coated overnight with a bovine IFN-γ-specific monoclonal antibody (Raggo et al., 2000), washed and blocked for 1 h with Eagle’s MEM containing 10 % FBS, 2 mM L-glutamine, 50 μg gentamicin ml⁻¹, 50 μM 2-mercaptoethanol and 1 mg dexamethasone ml⁻¹. PBMCs in supplemented MEM were dispensed at 5 × 10⁶ cells per well in triplicate wells in medium or in medium with 2 μg rAE2 protein per well and incubated at 37 °C for 24 h. IFN-γ-secreting cells were detected with a rabbit serum specific for bovine IFN-γ (Raggo et al., 2000) followed by AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories). Spots representing IFN-γ-secreting cells were visualized with BCIP/NBT substrate. The number of IFN-γ-secreting cells per 5 × 10⁶ PBMCs was expressed as the difference between the number of spots in the BVDV-stimulated wells and the number of spots in the medium control wells.

**Statistical analysis.** All data were analysed with the aid of GraphPad Prism 3.0 software (San Diego, CA). The SEM was used as the error value and an unpaired t-test was used to measure the difference between selected groups.

**RESULTS**

**Construction of plasmid encoding ΔE2 and production of rAE2 protein**

A plasmid encoding a secreted form of E2 defined as pMASIA-tPAsAE2 (Fig. 1a) was constructed. To confirm that the recombinant plasmid expressed ΔE2 in vitro, COS-7 cells were transfected with pMASIA-tPAsAE2 or pMASIA and analysed by Western blotting. As shown in Fig. 1(b), the supernatant and cell lysate from pMASIA showed no reaction with E2-specific antibody. However, efficient secretion of ΔE2 with an apparent molecular mass of 53 kDa was observed in the supernatant of pMASIA-tPAsAE2-transfected cells. In the cell lysate, several species with molecular masses of between 45 and 53 kDa were found, which probably represent different degrees of glycosylation of E2. In addition, rAE2 produced in transformed MDBG cells and purified from the
culture medium was analysed by SDS-PAGE (Fig. 1c) and was confirmed to have an apparent molecular mass of 53 kDa.

**Immune responses pre-challenge**

On day 50, approximately 3-5 weeks after the second immunization, the DNA prime–protein boost ($P<0.05$) and protein ($P<0.01$) groups had higher IgG titres than the saline group. The difference between the DNA group and the saline group was not significant due to the slightly higher variation among animals. Furthermore, the rΔE2-immunized calves produced more IgG than the animals immunized with pMASIA-tPAsΔE2 ($P<0.001$) (Fig. 2a). VN titres were higher in all vaccinated groups than in the saline group ($P<0.05$) and the group immunized with rΔE2 had higher titres than the other three groups ($P<0.05$) (Fig. 2b). On day 63, 2 weeks after the third immunization, all vaccinated calves continued to have higher IgG and VN titres than the saline-treated animals ($P<0.001$). Furthermore, the DNA prime–protein boost group had the highest IgG (Fig. 2a) and VN (Fig. 2b) levels in comparison with the other groups. There was a significant difference in IgG titres between the DNA prime–protein boost and all other groups ($P<0.001$) and in VN titres between the DNA prime–protein boost and the DNA and saline groups ($P<0.001$).

To assess antigen-specific cell-mediated immune responses, lymphocyte proliferation assays were performed with PBMCs restimulated in vitro with rΔE2. However, there were no differences between the stimulation indices of any of the groups (Fig. 2c). To evaluate T-cell activation further, IFN-γ ELISPOT assays were performed after in vitro restimulation with rΔE2. Although immunization with pMASIA-tPAsΔE2 ($P<0.05$), pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.001$) or rΔE2 ($P<0.05$) resulted in higher numbers of IFN-γ-secreting cells than treatment with saline, there were no differences in the numbers of IFN-γ-secreting cells between the three vaccinated groups (Fig. 2d).

**Humoral immune responses post-challenge**

To evaluate anamnestic responses after challenge, antibody titres were measured in the serum and nasal fluids. Calves immunized with pMASIA-tPAsΔE2 ($P<0.05$), pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.001$) or rΔE2 ($P<0.01$) continued to have higher IgG titres than the saline-treated animals during the period after challenge (Fig. 3a). Furthermore, the DNA prime–protein boost group had higher IgG titres than the other groups on days 4 ($P<0.01$) and 8 ($P<0.001$). All vaccinated groups developed an anamnestic response, as was evident from the increased ΔE2-specific IgG titres on day 11 compared with day 0 ($P<0.05$), but there were no differences between the three vaccine groups.

To evaluate the type of antibody responses elicited, isotypes were determined prior to and after challenge (Fig. 3b). Before challenge, the groups immunized with pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.01$) or with rΔE2 ($P<0.05$) had higher IgG1 titres than the groups immunized with pMASIA-tPAsΔE2 or treated with saline, whereas the DNA-vaccinated group had higher IgG1 titres than the saline group ($P<0.05$). By day 11, the ΔE2-specific IgG1 titres had increased significantly in the groups immunized with pMASIA-tPAsΔE2 ($P<0.01$) or with pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.05$). Furthermore, the DNA prime–protein boost group had higher IgG1

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**Fig. 1.** *In vitro* analysis of pMASIA-tPAsΔE2 encoding ΔE2 and of purified rΔE2 protein. (a) Schematic diagram of pMASIA-tPAsΔE2. (b) COS-7 cells were transfected with pMASIA or pMASIA-tPAsΔE2 and the presence of ΔE2 in the transfected cells was detected at 48 h post-transfection by Western blotting with a rabbit anti-E2 antibody. S, Culture supernatant; L, cell lysate. (c) Analysis of affinity-purified rΔE2 protein by SDS-PAGE. Molecular mass markers (kDa) are shown on the left.
titres than the groups immunized with pMASIA-tPAsΔE2 or rΔE2 ($P<0.01$). All calves, whether immunized with pMASIA-tPAsΔE2, pMASIA-tPAsΔE2 followed by rΔE2 or rΔE2, developed higher IgG2 levels by day 11 after challenge than calves treated with saline ($P<0.05$).

To determine the biological significance of the antibody response, sera were tested for BVDV-neutralizing antibodies against the NADL and NY-1 strains (Fig. 3c). Prior to challenge, NADL-specific neutralizing antibody titres were higher ($P<0.05$) in the DNA prime–protein boost and protein-injected groups than in the saline-treated and DNA-immunized groups. However, as expected, these antibodies did not neutralize the NY-1 strain as effectively as the NADL strain ($P<0.05$). Eleven days after challenge, the NADL-specific VN antibody titres of calves in the pMASIA-tPAsΔE2 group were significantly enhanced compared with those on day 0 ($P<0.001$). Furthermore, the VN titres specific for NY-1 increased in all groups, regardless of whether they were immunized with pMASIA-tPAsΔE2 ($P<0.005$), pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.005$) or rΔE2 ($P<0.001$) or treated with saline ($P<0.001$). There was a significant difference between the NY-1-specific VN titres of the DNA prime–protein boost and DNA or protein groups ($P<0.05$). Interestingly, the groups immunized with pMASIA-tPAsΔE2 or rΔE2 or treated with saline tended to have higher VN titres against NY-1 than against NADL 11 days after challenge, indicating virus replication, whereas the group immunized with the DNA prime–protein boost regimen still had higher NADL-specific VN titres, suggestive of less virus replication and stronger anamnestic responses.

Prior to challenge, calves immunized with pMASIA-tPAsΔE2 ($P<0.005$), pMASIA-tPAsΔE2 followed by rΔE2 ($P<0.001$) or rΔE2 ($P<0.001$) had higher nasal IgG titles than the saline-treated animals. These titles decreased on day 4 after challenge, whereas there was a significant increase

**Fig. 2.** Immune responses of calves before challenge. Groups of 6-month-old Hereford and Angus crossbred calves were injected as follows: (i) three times with saline (Saline); (ii) three times with 1 mg pMASIA-tPAsΔE2 (DNA); (iii) twice with 1 mg pMASIA-tPAsΔE2 followed by 35 μg rΔE2 with 500 μg CpG ODN 2007 and 30% Emulsigen (DNA–protein); and (iv) three times with 35 μg rΔE2, 500 μg CpG ODN 2007 and 30% Emulsigen (Protein). The vaccinations took place on days 0, 25 and 50. (a) Serum IgG titres. (b) Strain NADL-specific serum VN titres. (c) Lymphocyte proliferation expressed as the stimulation index. The geometric mean background level of thymidine incorporation was 851 c.p.m. (d) Number of IFN-γ-secreting cells per $5 \times 10^5$ PBMCs. Data are shown as the means ± SEM of five calves.
between days 8 and 11 ($P<0.001$) (Fig. 4a). Similarly, the VN titres in the nasal fluids of all vaccinated groups were higher than those in the saline-treated group before challenge ($P<0.05$), whereas they were reduced on day 4 and then again significantly increased on day 11 ($P<0.05$) (Fig. 4b). Furthermore, on day 11 the nasal VN titres were significantly higher in the DNA prime–protein boost group than in the DNA group ($P<0.05$). As we immunized the calves parenterally, the nasal IgG was probably a transudate from the serum. No IgA was detected prior to or after challenge, which agreed with the parenteral route of vaccination.

**Cellular immune responses post-challenge**

After challenge, the stimulation indices of the three vaccinated groups increased strongly and became significantly higher than those of the saline-treated group on day 11 ($P<0.01$) (Fig. 5a). However, there were no differences among the three vaccinated groups. The number of IFN-γ-secreting cells increased, in particular in the DNA prime–protein boost group (Fig. 5b). Consequently, more IFN-γ-secreting cells were observed in the group immunized with pMASIA-tPAsΔE2 followed by rΔE2 than in the saline-treated or rΔE2-injected groups ($P<0.001$) 11 days after challenge. Although there also appeared to be a difference between the DNA prime–protein boost group and the DNA group, this was not significant.

**Clinical observations after challenge**

Two of the five calves in the control group had an occasional cough from day 5 to 11 and were slightly depressed from day 10 to 11. The calves in the three vaccinated groups had no signs of disease throughout the challenge period. There was a decrease in weight in the groups immunized with pMASIA-tPAsΔE2 or rΔE2, or treated with saline. A comparison of weight changes demonstrated a significant difference between the DNA prime–protein boost group and the DNA and saline groups ($P<0.05$) (Fig. 6a). There was some evidence for a biphasic febrile response in all groups, particularly in the control group and the DNA group, where the mean body temperature was moderately elevated on days 2–5, followed by a peak of 40.6 ± 0.54 and 40.3 ± 0.52 °C, respectively, on day 7. The groups immunized with either pMASIA-tPAsΔE2 followed by rΔE2 or with rΔE2 appeared to have elevated temperatures 1 day later than the other two groups. The saline group tended to have the highest body temperature overall, although there were no significant differences between any experimental groups.

**Haematological profiles**

Haematological analyses including both total WBCs and differential leukocyte counts were performed daily from the day of challenge to day 12. In comparison with calves immunized with pMASIA-tPAsΔE2 followed by rΔE2, animals treated with saline or immunized with pMASIA-
tPAs or rE2 had markedly decreased WBC ($P<0.01$) and lymphocyte ($P<0.001$) counts from 2 days after challenge. In contrast, the mean WBC and lymphocyte counts in the DNA prime–protein boost group did not decrease (Fig. 6c, d). There were no remarkable changes in monocytes or neutrophils (data not shown).

**Virus isolation**

Virus isolations from WBCs and nasal swabs were performed daily after challenge. The frequencies of virus isolation from WBCs and nasal swabs are shown in Table 1. BVDV was isolated from WBCs from all control animals between days 4 and 10. In contrast, in the DNA prime–protein boost group, virus was isolated from three of five calves on 1 day only, on day 6 or 7, whereas four of five calves in the DNA group showed detectable viraemia for up to 4 days and all calves in the rE2 protein group shed virus for 1–5 days. Virus shedding in the nasal swabs was similar to that in the WBCs. Two of five calves in the DNA prime–protein boost group shed virus only for 1 day, whereas all calves in the DNA and protein groups shed virus for 2–7 days and all animals in the control group were viraemic for up to 8 days. Since the DNA prime–protein boost group had the highest nasal VN titres and the lowest level of virus shedding overall, we analysed whether there was a correlation between nasal VN titres and the duration of virus shedding. We found that calves with higher nasal VN titres tended to shed for a longer duration.
shorter period of time in the nasal fluids ($r = -0.62$) and WBCs ($r = -0.75$) than animals with lower VN titres (Fig. 7).

**DISCUSSION**

A number of commercial modified-live virus (MLV) and inactivated BVDV vaccines are available. Most of these are combination vaccines, containing BVDV and other viral and bacterial agents. The MLV vaccines usually contain a single strain of attenuated cytopathic BVDV, for instance NADL, Singer or C24V, whereas several inactivated vaccines are bivalent, containing both BVDV type 1 and type 2 strains. The current MLV and inactivated vaccines play a role in the control of BVDV infections (Kelling et al., 2005). However, there are several disadvantages associated with MLV, including failure of immunization by improper storage or handling, safety issues in or around pregnant cattle, immunosuppression and potential genetic recombination (Bolin, 1995; Grooms et al., 1998; Roeder & Harkness, 1986). In contrast, inactivated vaccines are safe for use in pregnant cattle, but have other disadvantages such as lower efficacy, higher production costs and localized inflammatory reactions. Both types of vaccines are subject to interference by maternal antibodies. Furthermore, Kelling (2004) concluded in a recent review that there is no commercial BVDV vaccine that can induce complete fetal protection, although some recent trials are promising in protecting the fetus (Dean et al., 2003; Fairbanks et al., 2004; Moennig et al., 2005).

These considerations suggest that, although there are commercial vaccines, there is a need for improved BVDV vaccines. DNA vaccines and CpG-formulated subunit vaccines have several advantages, in particular the induction of both cell-mediated and humoral immunity and efficacy in newborns. In order to optimize an experimental BVDV E2 DNA vaccine, we previously assessed several variables that...
might influence DNA vaccine efficacy and demonstrated that the C-terminally truncated E2 gene fused to the tPAs constructed in the pMASIA vector elicited humoral and cellular responses to BVDV after intradermal immunization (Liang et al., 2005). Nevertheless, based on previously reported and our own results, we expected that the immune responses generated by this DNA vaccine in calves might not be optimal and that further improvements might be needed.

In several instances, a combination of a DNA vaccine and a different boost elicited better immune responses than either vaccine alone (Dégano et al., 1999; Estcourt et al., 2002; Robinson et al., 1999). Priming with DNA and boosting with protein improved binding and neutralizing antibody titres (Barnett et al., 1997; Mossman et al., 1999). Song et al. (2000) tested different prime–boost regimes and demonstrated that if the protein boost was administered after two consecutive DNA injections instead of one injection, antibody titres were dramatically increased in mice. These data indicated that sufficient DNA priming is essential for the enhancement of DNA-encoded antigen-specific immunity by a booster immunization with recombinant protein.

In the present study, we used a DNA prime–protein boost vaccination strategy for BVDV in cattle and, indeed, induced stronger humoral immune responses and better protection compared with immunization with DNA or protein only, which confirms the efficacy of this strategy in a large-animal model. The IgG subclasses induced by ΔE2 DNA were balanced with equivalent levels of IgG1 and IgG2 and, although there was some predominance of IgG1, both isotypes were induced by the DNA prime–protein boost and the rΔE protein, which confirms our previous studies (Ioannou et al., 2002b; van Drunen Littel-van den Hurk et al., 1998). In cattle, both IgG1 and IgG2 can have neutralizing activity and, ultimately, one of the most important correlates of protection appeared to be the higher total IgG and VN titres in the DNA prime–protein boost group in comparison with the DNA and protein groups, both in the serum and in the nasal secretions. This contention is supported by the observation that animals with higher VN titres tended to shed virus for a shorter period of time.

A few BVDV recombinant vaccines and novel formulations for inactivated whole BVDV virus vaccines have been

**Table 1. Virus isolation post-challenge**

<table>
<thead>
<tr>
<th>Day</th>
<th>Saline</th>
<th>DNA</th>
<th>DNA + protein</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells</td>
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<tr>
<td>12</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

| Nasal swabs |
| 0   | -      | -   | -            | -       |
| 1   | -      | -   | -            | -       |
| 2   | +      | -   | +            | -       |
| 3   | +      | -   | +            | +       |
| 4   | +      | -   | +            | +       |
| 5   | +      | -   | +            | +       |
| 6   | +      | -   | +            | +       |
| 7   | +      | -   | +            | +       |
| 8   | +      | -   | +            | +       |
| 9   | +      | -   | +            | +       |
| 10  | +      | -   | +            | +       |
| 11  | +      | -   | +            | +       |
| 12  | +      | -   | +            | +       |

**Fig. 7.** Correlation between VN titres and days of virus shedding. (a) Virus shedding in nasal fluids. (b) Virus shedding in WBCs. Values on the x axis represent the number of days of virus shedding. Values on the y axis represent nasal VN titres for individual animals that shed virus. Each data point represents one animal. An inverse correlation was observed between the virus titre and the number of days that an animal shed virus ($r = -0.62$ for nasal fluids and $r = -0.75$ for WBCs).
evaluated in cattle. An inactivated BVDV vaccine formulated in immunostimulating complexes induced neutralizing antibodies but was not evaluated for protective capacity in cattle (Kamstrup et al., 1999). Vaccination with a C-terminally truncated E2 subunit produced in recombinant baculovirus-infected insect cells induced neutralizing antibody titres and limited protection after challenge in cattle (Bolin & Ridpath, 1996). Microparticle-mediated in vitro-transcribed BVDV RNA immunization was demonstrated to induce neutralizing antibodies in two calves, but was not evaluated further for protection (Vassilev et al., 2001). Vaccination of cattle with a DNA vaccine encoding the full-length E2 or a C-terminally truncated E2 elicited moderate immune responses and partial protection from BVDV challenge (Harpin et al., 1999; Nobiron et al., 2003). Harpin et al. (1999) provided a clinical score based on a number of parameters including temperature and demonstrated that one animal was protected from BVDV-induced disease, whilst the other two animals in the vaccinated group showed reduced disease. Nobiron et al. (2003) also reported a disease-sparing effect but no significant differences in haematological parameters, and there was a less-pronounced decrease in virus shedding. Recently, an NS3 DNA vaccine was tested in cattle. Although NS3-specific antibodies were observed in one of five animals, fever and virus production was prevented in two calves (Young et al., 2005). Although not measured in this trial, cell-mediated immunity may have played a role, as the virus was cleared more rapidly in all vaccines. As NS3 is a conserved protein, the use of NS3 in a BVDV vaccine would have the advantage of generating cross-protection among strains and could be promising when used in combination with E2.

In conclusion, when compared with these reports, the DNA prime–protein boost strategy reported here induced excellent protection from BVDV challenge, as shown by a significant reduction in weight loss, viral excretion and leukopenia.

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