At present, infections with bovine viral diarrhea virus (BVDV) type 2 occur nearly as frequently as those with BVDV type 1, so development of vaccines that protect cattle from both type 1 and type 2 BVDV has become critical. In this study, we compared various DNA prime–protein boost vaccination strategies to protect cattle from challenge with BVDV-2 using the major protective antigen of BVDV, glycoprotein E2. Calves were immunized with a plasmid encoding either type 1 E2 (E2.1) or type 2 E2 (E2.2) or with both plasmids (E2.1 + E2.2). This was followed by a heterologous boost with E2.1, E2.2 or E2.1 and E2.2 protein formulated with Emulsigen and a CpG oligodeoxynucleotide. Subsequently, the calves were challenged with BVDV-2 strain 1373. All vaccinated calves developed both humoral and cell-mediated immune responses, including virus-neutralizing antibodies and IFN-\(\gamma\)-secreting cells in the peripheral blood. Depletion studies showed that CD4\(^+\) T cells were responsible for IFN-\(\gamma\) production. Furthermore, the calves vaccinated with either the E2.2 or the E2.1 + E2.2 vaccines were very well protected from challenge with BVDV-2, having little leukopenia and showing no weight loss or temperature response. In addition, the animals vaccinated with the E2.1 vaccine were partially protected, so there was a certain level of cross-protection. These data demonstrate that a vaccination strategy consisting of priming with E2.2 or E2.1 + E2.2 DNA and boosting with E2.2 or E2.1 + E2.2 protein fully protects cattle from BVDV-2 challenge.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a widespread problem for beef and dairy herds and has been reported throughout the world. Acute BVDV infections caused by type 1 strains result in mild disease of short duration, characterized by fever, increased respiratory rate, diarrhoea and a reduction in white blood cells. Although animals generally recover, the effect of BVDV on the immune cells reduces the host’s resistance to disease. Type 2 strains can cause acute infections in herds, which are characterized by high fever, haemorrhaging, diarrhoea, reduction of white blood cells and platelets, and death. BVDV infections in pregnant cows can also result in abortions, malformations, poor-doers (weak, stunted calves) and persistently infected calves. These persistently infected animals may not only succumb to the lethal mucosal disease, which has a substantial economic impact, but also shed BVDV throughout their lifetime, so fetal infection is a major source of virus spread (Baker, 1995; Houe, 1995).

BVDV is a single-stranded RNA virus of positive polarity with a non-segmented genome of ~12.5 kb, which encodes a single polyprotein precursor that is co- and post-translationally processed by host and viral proteases to produce mature structural and non-structural proteins of the virus. The viral proteins are sequentially designated N\textsuperscript{PPA}-C-Ern-NS1-E1-E2-P7-NS2-NS4a-NS4b-NS5a-NS5b (Collett et al., 1988, 1991). Based on sequence comparison of the 5’-untranslated region (UTR) of the genome, BVDV could be classified into two genotypes by phylogenetic analysis, genotypes 1 and 2 (Pellerin et al., 1994; Ridpath et al., 1994). Genotype 1 BVDV isolates collected from around the world have been further clustered into 11 phylogenetic groups (BVDV-1a–BVDV-1k) (Vilcek et al., 2005). A highly virulent BVDV genotype 2 was identified in severe outbreaks of acute haemorrhagic disease in Canada and the USA (Bolin & Ridpath, 1992; Pellerin et al., 1994). Analysis of BVDV field isolates indicated that BVDV-2 has also spread across Europe, for instance into Germany (Wolfmeyer et al., 1997), Italy (Pratelli et al., 2001), France (Vilcek et al., 2001), Belgium (Couvreur et al., 2002), Austria (Vilcek et al., 2003) and the UK (Wakeley et al., 2004), as well as South America (Canal et al., 1998) and Asia (Kim et al., 2006; Shimazaki et al., 1998). However, BVDV-2 is mainly a problem in North America, where type 2 BVDV is currently isolated nearly as...
frequently as BVDV-1 (Bolin & Ridpath, 1998; Evermann & Ridpath, 2002).

Most modified-live virus (MLV) and killed virus (KV) BVDV vaccines licensed by the United States Department of Agriculture (USDA) and marketed commercially contain type 1a strains with different antigenic properties compared with other type 1 and type 2 phylogenetic groups. However, there is evidence that type 1a vaccines fail to protect calves from BVDV type 2 infection, although there appears to be some cross-protection (Fulton & Burge, 2000). Furthermore, antigenic differences observed between BVDV-1 and BVDV-2 have led to the conclusion that protection may be improved by inclusion of both type 1 and type 2 strains in BVDV vaccines (Ridpath, 2005).

Glycoprotein E2 is a major protective antigen of BVDV. In our previous studies, we compared different type 1 E2 DNA vaccines (Liang et al., 2005) and determined that a DNA prime–protein boost is an optimal vaccination strategy for induction of protective immunity against BVDV-1 in cattle (Liang et al., 2006). The purpose of this study was to evaluate the potential for a similar vaccination strategy to induce protection from BVDV-2 challenge in calves. Since an effective BVDV vaccine needs to protect from BVDV-1 as well, we also evaluated the efficacy of a mixture of type 1 and type 2 E2 vaccines. We were particularly interested in evaluating whether mixing type 1 and type 2 E2 vaccines would have any effect on the immunogenicity of the individual components. To determine whether a DNA prime–protein boost would protect animals from challenge with BVDV type 2, calves were vaccinated with individual plasmids encoding type 1 E2 (E2.1) or type 2 E2 (E2.2) or a mixture of these plasmids, followed by boosting with E2.1 and/or E2.2 proteins formulated with 10% Emulsigen (Em), a mineral oil in water emulsion, and CpG oligodeoxynucleotide (ODN). The calves were challenged with BVDV-2 strain 1373. The results demonstrate that a vaccination strategy consisting of priming with plasmid encoding E2.2 or E2.1 + E2.2 followed by boosting with E2.2 or E2.1 + E2.2 protein fully protected animals from a BVDV-2 challenge.

**METHODS**

**Cells and virus.** COS-7 cells were grown in Dulbecco’s minimum essential medium (DMEM; Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen) at 37°C in a CO₂ atmosphere. Madin Darby bovine kidney (MDBK) cells were purchased from the American Type Culture Collection and grown in Eagle’s minimum essential medium with 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM HEPES, 50 μg gentamicin ml⁻¹ and 10% FBS at 37°C in a CO₂ atmosphere. FBS was confirmed to be BVDV-free by virus neutralization assay and BVDV isolation after three passages (performed by Prairie Diagnostic Services). BVDV type 1a strain NADL was grown in MDBK cells and used for virus neutralization assays, and BVDV type 2 strain 1373 [USDA Animal and Plant Health Inspection Service (APHIS)] was grown in MDBK cells and used for viral challenge and virus neutralization assays.

**Construction, purification and expression of plasmids.** The construction of a plasmid that encodes a truncated secreted version of type 1 E2 with a tissue plasminogen activator signal sequence (tPAs), designated pMASIA-tPAs-AE2.1, has been described previously (Liang et al., 2005). A plasmid encoding a truncated secreted version of type 2 E2, designated pMASIA-tPAs-AE2.2, was constructed by cloning a truncated version of the E2 gene from BVDV strain Q140 (Pellerin et al., 1994) into pMASIA. Briefly, the full-length E2.2 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 to create pUC19-E2.2. The E2.2 gene without a membrane anchor (AE2.2) was amplified by PCR from pUC19-E2.2 using a pair of primers, 5’-CTACTGAGGATG-TTTCCCGTGAATGCAAGGAG-3’ and 5’-GGCGAAGATCTCAGAT-GAACCTCTGAGAAGTAG-3’. The PCR product was digested with NheI and BglII, cloned into pSLLA-tPAs and then subcloned into pMASIA to create pMASIA-tPAs-AE2.2.

In order to construct plasmids for production of E2.1 and E2.2 proteins, the tPAs-AE2.1 and tPAs-AE2.2 genes were amplified by PCR from pMASIA-tPAs-AE2.1 and pMASIA-tPAs-AE2.2 and cloned into pCDNA6/HA-His(B) (kindly provided by Dr Z. Chang, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, China) to obtain pCDNA-tPAs-AE2.1-His and pCDNA-tPAs-AE2.2-His.

All constructs were confirmed by restriction enzyme digestion and agarose gel electrophoresis and sequenced for cloning accuracy. The plasmids were grown in Escherichia coli DH5α cells and purified with Endofree Plasmid Giga kits (Qiagen). Expression of the E2 proteins was confirmed by transient transfection of COS-7 cells and Western blotting as described previously (Liang et al., 2005, 2006).

**Purification of recombinant E2 protein from transfected COS-7 cells.** COS-7 cells were transiently transfected with pCDNA-tPAs-AE2.1-His or pCDNA-tPAs-AE2.2-His by using a Bio-Rad Gene Pulser (Bio-Rad Laboratories). After addition of DMEM with 10% FBS immediately after transfection, the transfected COS-7 cells were incubated overnight in a CO₂ incubator at 37°C. Subsequently, the cell layer was washed twice with PBS (140 mM NaCl, 10 mM NaHPO₄/Na₂HPO₄, 2.7 mM KCl, pH 7.2) and then OPTI-MEM (Gibco/Invitrogen) was added. After 48 h, the media were collected, cleared by centrifugation and concentrated. The E2–His proteins were purified from the concentrated supernatants under native conditions using ProBond nickel-chelating resin (Gibco/Invitrogen). The E2 yields were determined by using a Bio-Rad protein assay, and the E2 purity was assessed by SDS-PAGE, followed by densitometry.

**Immunizations.** Hereford and Angus crossbred calves (8–9 months old) were screened with the HerdChek BVDV antigen/serum test kit and the HerdChek BVDV antibody test kit (IDEXX Laboratories). Twenty-four BVDV antigen-negative and BVDV antibody-negative calves were selected and randomly allocated to four groups of six animals each and immunized with 285 pmol (~1 mg) pMASIA (placebo), pMASIA-tPAs-AE2.1 (E2.1), pMASIA-tPAs-AE2.2 (E2.2), or a mixture of pMASIA-tPAs-AE2.1 and pMASIA-tPAs-AE2.2 (E2.1 + E2.2). The plasmids were delivered transdermally by needle-free injection with a Biojector (Bioject Medical Technologies). Since one of the calves in the E2.1 group had seroconverted to BVDV between screening and the first vaccination, this animal was eliminated from the trial. Three weeks after the second DNA vaccination, all calves received a subcutaneous vaccination with 50 μg E2.1 or E2.2, or 50 μg each of E2.1 and E2.2 proteins formulated with 10% Em (MVP Laboratories) and 1 mg CpG ODN 2007 (5’-TCCGCGTTTGGTGTTTGTGTTT-3’) in 2 ml. The diluent for the antigens and CpG ODN was PBS. The CpG ODN was provided by Merial. The calves in the placebo group received 10%
Em and 1 mg CpG ODN 2007. The animals received the DNA vaccinations on days 0 and 21 and the protein boost on day 42, and they were challenged with BVDV-2 on day 58.

**Challenge and clinical evaluation.** Two weeks after the protein immunization, BVDV strain 1373 [6 × 10^5.2 50% tissue culture infective dose (TCID₅₀)] in 4 ml PBS) was administered to each calf (2 ml into each nostril) using an intranasal cannula. Body temperatures and weights were measured, and clinical signs including fever, depression, anorexia, cough and diarrhoea were monitored on the day of challenge and for 13 days afterwards by a veterinarian who was unaware of the vaccination status of the animals. Blood for haematological assays and nasal swabs for virus isolation were collected on the day of challenge and for 13 days afterwards. Sera were collected prior to each immunization, on the challenge day and on day 12 post-challenge. All procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care.

**ELISA.** Bovine IgG titres were determined as described previously (Liang et al., 2005). Briefly, 96-well Immulon 2 High Binding U-bottom polystyrene microtitre plates (Thermo Electron Corporation) were coated overnight with E2.1 or E2.2 protein at 4 ng per well and incubated for 1.5 h at room temperature with fourfold diluted bovine sera. Alkaline phosphatase (AP)-conjugated goat anti-bovine IgG (Kirkgaard & Perry Laboratories) was used to detect bound IgG. The reaction was visualized with p-nitophenyl phosphate (Sigma-Aldrich). ELISA titres were calculated as the highest dilution, resulting in a reading of two SD above the value of a negative control serum.

**Virus neutralization (VN) assay.** Sera were heat-inactivated at 56 °C for 30 min. Two hundred TCID₅₀ of BVDV strain NADL or 1373 were pre-incubated with fourfold serum dilutions for 1.5 h at 37 °C. Fifty microlitres of these mixtures were added to duplicate microtitre plates containing 80–90% confluent MDBK cells for 1.5 h at 37 °C. One hundred and fifty microlitres of MEM with 2% FBS were added to each well. The plates were incubated in a CO₂ incubator at 37 °C for 4 days for the NADL assay and for 6 days for the 1373 assay. The reciprocal of the highest dilution that completely inhibited viral cytopathic effect in the two test wells was reported as the VN titre.

**Viral sampling and virus isolation.** Nasal secretions were collected 2 days prior to challenge and daily from day 1 until day 13 post-challenge with cotton swabs in 1 ml MEM supplemented with antibiotic–antimycotic solution (Gibco/Invitrogen) and stored at −80 °C. White blood cells (WBCs) were isolated from blood by lysis with ammonium chloride lysis buffer (0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 7.2) to lyse the erythrocytes, followed by two washes with PBS. The pellet was resuspended in 1 ml Eagle’s MEM (Gibco-BRL) and stored at −80 °C. To detect virus shedding, twofold diluted nasal secretions or WBCs were added to MDBK cells in duplicate in 96-well microtitre plates. Six days after infection, the cells were fixed with 80% acetone and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Subsequently, E2.2-specific rabbit antibody at a dilution of 1:500 was added to the cells. This antibody was generated in-house to purified E2.2 protein. After incubation for 1–2 h at room temperature, AP-conjugated goat anti-rabbit IgG (Kirkgaard & Perry Laboratories) was added. Finally, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich) was used for detection. Virus staining was observed with an Olympus CKX31 microscope. The reciprocal of the highest dilution still showing virus in both wells was reported as the virus titre.

**Lymphocyte proliferation and IFN-γ ELISPOT assay.** Peripheral blood was collected immediately prior to the protein boost, 2 days prior to challenge, and 12 days after challenge. Peripheral blood mononuclear cells (PBMCs) were isolated, and E2.1- and E2.2-specific lymphocyte proliferation and IFN-γ ELISPOT assays were performed as described previously (Liang et al., 2006). Briefly, for the lymphocyte proliferation assays, 3.5 × 10⁶ PBMCs per well were added to triplicate wells and restimulated in vitro for 72 h in the absence or presence of 0.5 μg E2.1 or 0.75 μg E2.2 protein per well. After 3 days, the cells were pulsed with 0.4 μCi (14.8 KBq) [methyl-3H] thymidine (Amersham Biosciences) per well. Cells were collected 18 h later, and thymidine uptake was measured by scintillation counting. Proliferation results were calculated as the means of triplicate wells and expressed as a stimulation index (SI) where SI = counts min⁻¹ in the presence of antigen counts min⁻¹ in the absence of antigen. For the IFN-γ ELISPOT assays, nitrocellulose plates (Millipore) were coated overnight with a bovine IFN-γ-specific monoclonal antibody (2-2-1 (Raggo et al., 2000)). PBMCs were dispensed at 5 × 10⁵ cells per well in triplicate wells in medium or in medium with 1 μg E2.1 or E2.2 protein, or 10 μg BVDV strain 1373-infected MDBK cell lysate per well and incubated at 37 °C for 24 h. IFN-γ-secreting cells were detected with a rabbit anti-bovine IFN-γ antibody 92-131 (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 numbers of IFN-γ-secreting cells were expressed as the difference between the number of spots per 10⁶ cells in E2.1–His or E2.2–His-stimulated cultures and the number of spots in control cultures.

**Magnetic cell sorting.** Peripheral blood was collected from one animal from each of the E2.1+E2.2, E2.2 and placebo groups immediately prior to challenge as well as 14 and 60 days post-challenge. PBMCs were isolated as described previously (Liang et al., 2005), counted and resuspended at a concentration 1 × 10⁶ cells ml⁻¹. The cell suspensions were incubated on ice with mAb (VMRD) specific for bovine CD4 (IL-A11, IgG2a isotype; VMRD) or CD8 (CACT80C, IgG1 isotype; VMRD) at a concentration of 1 μg per 10⁶ PBMCs for 20 min. Cells were washed and then incubated with goat anti-mouse IgG-coated microbeads (Milenyi Biotech) at a bead–target cell ratio of 5:1. Bead-bound cells were removed on MACS separation 25LS columns (Milenyi Biotech). The CD4-depleted cells (CD4⁻) and CD8-depleted cells (CD8⁺) were isolated. The cell suspensions were resuspended at 1 × 10⁷ cells ml⁻¹ in FAC buffer (0.01 M PBS with 0.2% gelatin and 0.03% sodium azide pH 7.2) for flow cytometry or in culture medium for ELISPOT assays.

**Flow cytometry.** PBMCs and CD4⁻ and CD8⁺ T-lymphocyte subsets were resuspended at a concentration of 1 × 10⁶ cells ml⁻¹ in FAC buffer. Cell suspension (50 μl) was added to the wells of 96-well polystyrene U-bottom microtitre plates (Costar) and stained with CD4-specific (IL-A11) or CD8-specific (CALT80C) mAb. The level of specific mAb staining was defined by setting the threshold with an irrelevant isotype (IgG2a-FITC for CD4 or IgG1-FITC for CD8) and concentration-matched mAb. After three washes in FAC buffer, cells were resuspended in a 50 μl volume and incubated with FITC-conjugated goat anti-mouse IgG2a or FITC-conjugated goat anti-mouse IgG1 (Gibco/Invitrogen) to detect cell-bound antibodies. After three washes, cells were fixed with 2% formaldehyde. Samples were analysed with a FACScan (BD Biosciences) using CellQuest software (BD Biosciences).

**Haematological analysis.** Blood samples obtained immediately prior to challenge and daily from days 1 to 13 after challenge were sent to Prairie Diagnostic Services to quantify total WBC counts and differential leukocyte counts including lymphocytes, monocytes and segregates as described previously (Liang et al., 2006).

**Statistical analysis.** All data were analysed with the aid of GraphPad Prism 4.0 (GraphPad Prism Software) and STATISTIX 7.0 software.
(Analytical Software). In general, the antibody data were not normally distributed and, therefore, median values and ranges are reported. The differences in immune responses among the vaccine groups were examined using the Kruskal–Wallis test. If the result of an ANOVA proved significant, then multiple post-test comparisons between medians were performed using Dunn’s test. The clinical data were normally distributed (means reported), while the haematological data were not normally distributed (medians reported). Dependent upon their distribution, differences in haematological and clinical data among vaccine groups were initially assessed by parametric or non-parametric ANOVA with repeated measures over time. Means of normally distributed or rank-transformed data were then compared using Tukey’s test. Where appropriate, differences between the vaccine and placebo groups were compared at individual time points using two-sample t-tests or the Wilcoxon rank sum test. Results were considered significant when P<0.05.

RESULTS

Construction of plasmids and production of E2 proteins

Plasmids encoding secreted forms of type 1 and type 2 E2 defined as pMASIA-tPAs-ΔE2.1 (Fig. 1a) and pMASIA-tPAs-ΔE2.2 (Fig. 1b) were constructed and confirmed to express secreted versions of E2.1 and E2.2 (data not shown). In order to produce histidine-tagged recombinant E2.1 and E2.2 proteins, COS-7 cells were transfected with pCDNA6-tPAs-ΔE2.1-His or pCDNA6-tPAs-ΔE2.2-His. The E2.1–His and E2.2–His proteins were purified from the culture supernatants on nickel resin. The purified proteins were analysed by SDS-PAGE followed by Coomassie brilliant blue staining or Western blotting (Fig. 1c and d), which demonstrated that the ΔE2.1–His and ΔE2.2–His proteins had apparent molecular masses as expected. The apparent difference in size between ΔE2.1–His (50 kDa) and ΔE2.2–His (57 kDa) is probably due to differences in glycosylation, since ΔE2.2–His has two more N-linked glycosylation sites. The purity of the His-tagged E2 proteins was ≥90%.

Humoral immune responses

After immunization with plasmid encoding E2.1, with or without plasmid encoding E2.2, calves developed significantly higher E2.1-specific IgG titres (Fig. 2a) (P<0.01 for both groups) and NADL (type 1)-specific VN titres (Fig. 3a) (P<0.05 for both groups) compared with those in the placebo group. Calves immunized with plasmid encoding E2.2, with and without plasmid encoding E2.1, developed higher E2.2-specific IgG titres (Fig. 2b) (P>0.05 and P<0.001, respectively) and strain 1373 (type 2)-specific VN titres (Fig. 3b) (P<0.05 and P<0.01, respectively) compared with those in the placebo group. Furthermore, after the protein boost, the vaccinated calves developed 10–13-fold higher E2.1-specific and E2.2-specific IgG titres (Fig. 2c and d) and 10–30-fold higher type 1-specific and type 2-specific VN titres (Fig. 3c and d) compared with the titres prior to the protein boost. In both assays, there was some cross-reactivity between type 1 and type 2. There were no differences before or after the protein boost.
boost in IgG or VN titres to the respective homologous viruses between the calves immunized with E2.1 + E2.2 and the animals immunized with E2.1 or E2.2 individually. The fact that the placebo group appeared to have very low titres is likely to be due to non-specific background as there were no virus-neutralizing antibody titres in this group.

Fig. 2. Antibody responses of calves after vaccination and after challenge with BVDV-2. Four groups of six calves were immunized twice with a 3 week interval with: (group 1) pMASIA (placebo); (group 2) pMASIA-tPAs-ΔE2.1 (E2.1); (group 3) pMASIA-tPAs-ΔE2.1 + pMASIA-tPAs-ΔE2.2 (E2.1 + E2.2); or (group 4) pMASIA-tPAs-ΔE2.2 (E2.2). Three weeks later the calves received a vaccination with: (group 1) PBS; (group 2) E2.1–His protein; (group 3) E2.1–His + E2.2–His proteins; or (group 4) E2.2–His protein. Each plasmid was administered at 285 pmol per dose by needle-free injection with a Biojector. The proteins were formulated with 10% Em and 1 mg CpG ODN and administered subcutaneously at 50 µg protein per dose. PBS was also formulated with 10% Em and 1 mg CpG ODN. Two weeks after protein vaccination, all calves were challenged with 6 × 10^6.2 TCID_{50} BVDV strain 1373 by nasal installation. E2.1-specific serum IgG titres (a, c and e) and E2.2-specific serum IgG titres (b, d and f) were determined on day 42, 3 weeks after the second DNA immunization (a and b), on day 58, 2 weeks after the protein boost (c and d) and on day 70, 12 days after challenge (e and f). Each data point represents an individual animal and median values are indicated by horizontal lines. *, P<0.05; **, P<0.01; ***, P<0.001.
To evaluate anamnestic responses after BVDV-2 challenge, sera were collected on day 12 after challenge. Both IgG (Fig. 2e and f) and VN (Fig. 3e and f) assays showed that there was a strong E2.2-specific anamnestic response in the three E2-vaccinated groups. However, there were no significant differences between animals immunized with E2.1 + E2.2 and those immunized with E2.1 or E2.2 individually. These results suggest that there was no interference or competition between the E2.1 and E2.2 vaccine.

**Cell-mediated immune responses**

The cell-mediated immune responses were evaluated based on lymphocyte proliferation and number of IFN-γ-secreting cells in PBMCs. Low or no cell-mediated...
responses were detected before the protein boost on day 42 (data not shown). However, after the protein boost, calves immunized with E2.1 alone developed significantly higher lymphocyte proliferative responses ($P<0.01$) in comparison to the placebo group (Fig. 4a). The low SI in the placebo group is probably due to non-specific stimulation, which sometimes occurs in an outbred population. Prior to challenge no proliferative responses were detected for E2.2 (Fig. 4b). Furthermore, after immunization with E2.1, E2.2, or both E2.1 and E2.2 proteins, the calves developed increased numbers of IFN-γ-secreting cells compared with the placebo-treated animals ($P<0.05$, for E2.1-induced IFN-γ in the E2.1 and E2.1+E2.2 groups and $P<0.01$ for E-2.2-induced IFN-γ in the E2.2 group, respectively) (Fig. 5a and b). After challenge with BVDV strain 1373, the lymphoproliferative responses increased significantly in the E2.1 and E2.2 groups ($P<0.01$ and $P<0.05$, respectively) (Fig. 4c and d). Similarly, the numbers of IFN-γ-secreting cells in the E2.1, E2.2 and E2.1+E2.2 groups increased ($P<0.01$ and $P<0.05$ for E2.1-induced IFN-γ in the E2.1 and E2.1+E2.2 groups and $P<0.01$ for E-2.2-induced IFN-γ in the E2.2 group, respectively) (Fig. 5c and d). There were no significant differences in cell-mediated immune responses between animals immunized with E2.1+E2.2 and those immunized with E2.1 or E2.2 individually before or after challenge. This further confirms that the E2.1 and E2.2 vaccines are compatible.

**Phenotype of T-cell subpopulations secreting IFN-γ in response to E2 protein and live BVDV**

In order to identify which T-lymphocyte subpopulation was responsible for IFN-γ secretion, CD4$^+$, CD4$^-$, CD8$^+$ and CD8$^-$ T-cell populations of the PBMCs were isolated. There were 15–20% CD4$^+$ cells and 7–15% CD8$^+$ T cells in the PBMCs (Fig. 6a and d). The purity of the CD4$^+$ and CD8$^-$ cells was >99% (Fig. 6b and e) and that of the CD4$^+$ and CD8$^+$ T cells was >92% (Fig. 6c and f). Representative ELISPOT results are shown for one calf from the E2.2 group (Fig. 6g and h) and one calf from the E2.1+E2.2 group (Fig. 6i and j). CD4 T-cell-depleted PBMCs did not secrete IFN-γ in response to E2.1 or E2.2, and there was a dose-dependent increase when 5, 10, 15 or 20% CD4$^+$ T cells were added to the CD4-depleted PBMCs (Fig. 6g and i). In contrast, depletion of CD8$^+$ lymphocytes did not affect the number of IFN-γ-secreting cells in the PBMCs, and there was no increase when 2.5, 5, 10 or 15% CD8$^+$ T cells were added to the CD8$^+$-depleted PBMCs (Fig. 6h and j), regardless of whether the depleted PBMCs were restimulated in vitro with E2 protein or live

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**Fig. 4.** Lymphocyte proliferation responses after vaccination and after challenge with BVDV-2. Calves were immunized and challenged as described in the legend to Fig. 2. PBMCs were collected on day 58, prior to challenge (a and b) and on day 70, 12 days after challenge (c and d). E2.1-specific (a and c) and E2.2-specific (b and d) lymphocyte proliferation was expressed as SI. Each data point represents an individual animal, and median values are indicated by horizontal lines. *, $P<0.05$; **, $P<0.01$. 

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BVDV. Consequently, the depletion studies showed that the cells responsible for IFN-γ production were CD4+ T cells.

Protection from challenge with BVDV type 2

After challenge with BVDV strain 1373, the calves were monitored for clinical signs and rectal temperatures, and the weights were measured. The placebo-treated calves had elevated temperatures between days 3 and 11, with the peak between days 8 and 11 (Fig. 7a). In contrast, the immunized calves had significantly lower temperatures than the placebo-treated group (P<0.001). There was no significant difference among the vaccine groups.

Weight measurements represent another objective parameter of disease severity. As shown in Fig. 7(b), the calves immunized with placebo lost weight between days 2 and 11, and those immunized with E2.1 lost weight between days 2 and 9 post-challenge, whereas the calves immunized with E2.2 or E2.1+E2.2 gained weight. There was a significant difference between the E2.2 or E2.1+E2.2 groups and the placebo group (P<0.01). However, there was no significant difference in weights among the vaccine groups.

In addition, the amount of virus shedding in the nasal fluids was determined. No virus was detected in any of the vaccinated groups (Fig. 7c). However, the calves in the placebo-treated groups shed virus from the nasal fluids between days 5 and 12, with a total of 29 days of virus shedding out of 66 days (Fig. 7c), whereas virus was recovered from the WBCs of one animal on day 6 and two other animals on days 6–8.

Haematological analyses including WBCs and differential leukocyte counts were performed daily from the day of challenge to day 13 (Fig. 8). The calves immunized with placebo had markedly decreased WBCs, monocytes, lymphocytes and segregated neutrophils in comparison to the vaccinated calves (P<0.01, 0.001, 0.01 and 0.01, respectively). The E2.1+E2.2 and E2.2 groups had significantly higher WBC (P=0.006) and neutrophil (P=0.004) counts than the placebo group, whereas the monocyte and lymphocyte counts for the E2.1+E2.2 group were significantly greater than those of the placebo group (P=0.001 and P=0.01, respectively). In contrast, there was no difference between the E2.1 group and the placebo group in WBCs, lymphocytes, monocytes or segregated neutrophils.

These results demonstrate that calves vaccinated with either the E2.2 vaccine or the E2.1+E2.2 vaccine were very well protected from challenge with BVDV-2, whereas the E2.1-vaccinated animals were partially protected.
DISCUSSION

The high prevalence of both BVDV-1 and BVDV-2 suggests that vaccines should contain both type 1 and type 2 strains to induce optimal protection. In the current study, the compatibility of plasmids encoding BVDV type 1 and type 2 E2, as well as type 1 and type 2 E2 protein formulations, was evaluated in a vaccination–protection trial. There were no differences in immune responses to the homologous viruses between calves immunized with both type 1 and type 2 E2 and animals immunized with type 1 or type 2 E2 individually. Similarly, there was no difference in protection between the type 2 E2 group and the group immunized with both type 1 and type 2 E2. Calves vaccinated with either E2.2 or E2.1 and E2.2 were very well protected from BVDV-2 challenge since these animals did not have increased temperatures or weight loss, and showed very little evidence of leukopenia. This suggests that there was no interference between the plasmids encoding BVDV type 1 and type 2 E2, or between the two E2 proteins. The fact that there was no enhancement of protection by adding E2.1 to the E2.2 vaccine suggests that either E2.2 alone elicited maximal levels of protection or that there was a low level of cross-reactivity between the E2.1 and E2.2 proteins, resulting in very low immune responses to heterologous virus. This would further confirm the need for type 1 and type 2 components in a BVDV vaccine.

Previous reports have suggested that BVDV-1 vaccines induce protection from BVDV-2 strains. For example, a type 1 MLV vaccine (strain WRL), provided protection from challenge with BVDV type 2 strain 890 (Dean & Leyh, 1999). Similarly, commercial inactivated BVDV-1 vaccines have been reported to afford cross-protection from BVDV-2 challenge (Ellis et al., 2001; Hamers et al., 2003). However, another study in which eight commercial MLV and KV vaccines were compared demonstrated that calves vaccinated with type 1 BVDV developed antibodies to a broad range of BVDV type 1 strains, but low or no titres to type 2 BVDV strains (Fulton & Burge, 2000). Similarly, a recent study with a BVDV type 1 vaccine demonstrated low cross-over titres to BVDV strain 890 (Reber et al., 2006). Furthermore, antigenic differences observed between BVDV-1 and BVDV-2 support the contention that both type 1 and type 2 BVDV should be included in vaccines to induce protection against both strains (Ridpath, 2005). Consequently, in recent vaccine trials generally both a BVDV-1 and a BVDV-2 vaccine strain is included (Ellsworth et al., 2006; Fairbanks et al., 2004; Ficken et al., 2006a, b; Fulton et al., 2006). Indeed, Ficken et al. (2006b) demonstrated that one dose of MLV vaccine containing both BVDV type 1 and type 2 reduced the incidence of persistent BVDV infection, whereas one or two doses of BVDV-1 did not induce protection. The results of the trial reported here support the need to include BVDV-2 components in a vaccine to be able to induce solid protection against type 2 BVDV challenge.

Based on previous reports it appears that mixing of plasmids in one vaccine formulation may result in interference, enhancement or no difference in the immune responses induced by individual plasmids. Multigene and multiclade DNA vaccines containing components from human immunodeficiency virus (HIV) A, B and C Env and Gag–Pol–Nef fusion protein, induced a broadened antiviral immune response without immune interference (Kong et al., 2003). Similarly, there was no evidence for interference between the components of a denguevirus tetravalent DNA vaccine consisting of plasmids expressing premembrane and envelope genes of each of the four serotypes of dengue virus. Indeed, higher antibody levels against denguevirus tetravalent DNA were shown compared with monovalent-vaccine-immunized mice (Konishi et al., 2006). In other instances interference between plasmids has been demonstrated. A nine-plasmid DNA vaccine encoding malaria antigens from the sporozoite, exoerythrocytic and erythrocytic stages of the parasite elicited dramatically reduced immune responses to the component antigens compared with the responses to the plasmids given singly (Sedegah et al., 2004). Furthermore, a plasmid encoding bovine herpesvirus-1 glycoprotein D interfered with plasmids encoding parainfluenzavirus-3 HN or influenza HA, when co-delivered in a mixture, whereas the HN- and HA-encoding plasmids did not cause interference (Braun et al., 1998). The bias of the immune response may also be altered, as shown for plasmids encoding measles virus HA and NP (Cardoso et al., 1998). Overall, this leads to the conclusion that without a rationale for making predictions, each new plasmid combination needs to be evaluated.

Heterologous prime–boost strategies have been used to increase immune responses to a number of DNA vaccines. Immunization regimens comprised of a DNA prime and a viral vector boost for instance for vaccinia virus (Dunachie et al., 2006; McConkey et al., 2003; Mwau et al., 2004), adenovirus (Shiver et al., 2002), fowlpox (Webster et al., 2006), and retrovirus (Anson, 2004), have been most frequently tested. Priming with DNA and boosting with protein is another promising approach. This regimen has been studied for HIV (Pal et al., 2006; Barnett et al., 1997), hepatitis C virus (Yu et al., 2004), anthrax (Galloway et al., 2004), tuberculosis (Li et al., 2006), Streptococcus pneumoniae (Moore et al., 2006) and BVDV (Liang et al., 2006). DNA vaccines and recombinant protein vaccines utilize different mechanisms to elicit antigen-specific responses. Due to the production of antigen in transfected cells of the host, a DNA vaccine induces robust T-cell responses, which are critical for the development of T-cell-dependent antibody responses (Liang et al., 2005; Martin et al., 2006). DNA immunization is also highly effective in priming antigen-specific memory B cells. In contrast, a recombinant protein vaccine generally is more effective at eliciting antibody responses than cell-mediated immune responses and may directly stimulate antigen-specific memory B cells to differentiate into antibody-secreting cells, resulting in production of high titre antigen-specific
antibodies (Lu, 2006). Therefore, a DNA prime plus protein boost is a complementary approach that overcomes each of their respective shortcomings. In order to make this viable as a vaccination strategy against BVDV in cattle it would be necessary to develop a slow- or pulsed-release delivery system, possibly based on microparticles (O’Hagan et al., 2006), which would mimic the DNA prime and protein boost. Another important criterion that needs to be addressed in the future is the ability of this strategy to protect against fetal infection with both types of BVDV. Modified live vaccines have been tested and shown to induce protection from fetal infection, in particular when given prior to breeding (Kovacs et al., 2003). However, MLV vaccines can also induce adverse effects including abortion, fetal infection, immunosuppression and respiratory signs (van Oirschot et al., 1999), so the development
of non-replicating vaccines that induce fetal protection might be desirable. Indeed, there is evidence that inactivated BVDV vaccines can provide adequate protection (Brownlie et al., 1995), which suggests that the DNA prime–protein boost strategy reported here might also be protective.

**Fig. 6.** Phenotypes of T-lymphocyte subpopulations of PBMCs-secreting IFN-γ. PBMCs were isolated from peripheral blood of one animal from each of the E2.1+E2.2, E2.2 and placebo groups prior to challenge and on days 14 and 60 after challenge. CD4-depleted T cells (CD4⁺) and CD8-depleted T cells (CD8⁻), as well as CD4⁺ and CD8⁺ T cells, were isolated by MACS. (a–f) The purity of the CD4⁺⁻ and CD8⁺⁻ T cells was determined by flow cytometric analysis with a FACScan using CellQuest software. The data are for the calf from the E2.2 group on day 14 post-challenge and are representative of all depletions. (a) PBMCs recognized by CD4 mAb. (b) CD4⁻ population recognized by CD4 mAb. (c) CD4⁺ population recognized by CD4 mAb. (d) PBMCs recognized by CD8 mAb. (e) CD8⁻ population recognized by CD8 mAb. (f) CD8⁺ population recognized by CD8 mAb. (g–j) T-lymphocyte subpopulations of PBMCs-secreting IFN-γ cells. (g) CD4-depleted PBMCs of one calf from the E2.2 group. (h) CD8-depleted PBMCs of one calf from the E2.2 group. (i) CD4-depleted PBMCs of one calf from E2.1+E2.2 group. (j) CD8-depleted PBMCs of one calf from the E2.1+E2.2 group. (g and h) E2.2-specific ELISPOT. (i and j) E2.1+E2.2-specific ELISPOT. The number of INFγ-secreting cells per 10⁶ PBMCs was expressed as the difference between the numbers of spots in the antigen-stimulated wells and the number of spots in the medium-control wells. FSC, Forward scatter.

**Fig. 7.** Clinical signs and virus shedding after challenge with BVDV-2. Calves were immunized and challenged as described in the legend to Fig. 2. Weights and rectal temperature were recorded daily and nasal swabs were collected daily. (a) Mean rectal temperatures of all three vaccine groups were significantly lower than that of the placebo group (P=0.0002). *, †, ‡=Days when E2.1, E2.1+E2.2 and E2.2 (respectively) varied significantly from the placebo group P<0.05. (b) Mean weight changes of vaccine groups E2.1+E2.1 and E2.2 were significantly greater than those of the placebo group (P=0.007). *=Days when both E2.1+E2.1 and E2.2 varied significantly from the placebo group (P<0.05). (c) Virus in nasal secretions.
It has been reported that in BVDV-seropositive animals, IFN-\(\gamma\) levels are significantly higher than in BVDV-seronegative animals (Waldvogel et al., 2000), and that there is a significant positive correlation between the IFN-\(\gamma\) levels and antibody titres. Similar results were obtained in the current study. Thus, IFN-\(\gamma\) may play an important role in protection from BVDV. In order to identify which T-lymphocyte subpopulation is responsible for secreting IFN-\(\gamma\), we performed CD4\(^+\) T-cell and CD8\(^+\) T-cell depletion ELISPOT assays using E2.1, E2.2 or live BVDV strain 1373 for in vitro stimulation. The CD4\(^+\) T-cell-depleted PBMCs did not secrete any IFN-\(\gamma\), whereas depletion of CD8\(^+\) lymphocytes did not affect the number of IFN-\(\gamma\)-secreting cells in the PBMCs, so we concluded that the cells responsible for IFN-\(\gamma\) production were CD4\(^+\) T cells and not CD8\(^+\) T cells. Since the cell-mediated and humoral immune responses showed a concurrent increase after protein boost and virus challenge in our study, we hypothesize that the CD4\(^+\) T-cell responses were critical for the development of T-cell-dependent antibody responses, CD4\(^+\) T-helper cells, as well as CD4\(^+\) memory T cells being involved in regulating B-cell functions.

In conclusion, our data demonstrate that a vaccination strategy consisting of priming with E2.2 DNA or E2.1+E2.2 DNA and boosting with E2.2 protein or E2.1+E2.2 protein fully protected calves from BVDV-2 challenge. Interestingly, vaccination with type 1 E2 induced some cross-protection from BVDV type 2 challenge. However, these results confirmed that type 2 E2 is needed for optimal protection from BVDV-2 infection. Furthermore, vaccination with both type 1 and type 2 E2 also induced effective protection from BVDV-2 and can be expected to provide protection from BVDV-1 as well, so a
Combination of E2.1 and E2.2 should protect cattle from both BVDV-1 and BVDV-2 strains. Since mixing of plasmids or proteins produced according to the same manufacturing protocols is a very simple approach, new vaccine strains could be added with relative ease, which would allow us to adapt BVDV vaccines according to the prevalent strains.

ACKNOWLEDGEMENTS

We thank the animal care staff, specifically Dr Don Wilson, Brock Evans and Sherry Tellet for care and handling of the calves. We are grateful to Sarah Mackenzie-Dyck, Laura Latimer and Marlene Snider for technical assistance. We acknowledge Dr Richard Stout (Bioject, Yuvalatin, OR, USA) for providing the Biojector device and Merial Limited for supplying CpG ODN 2007. Financial support was provided by the Beef Cattle Research Council (BCRC), British Columbia Cattle Industry Development Fund (BCIDF), Canadian Institutes of Health Research (CIHR), Natural Sciences and Engineering Research Council (NSERC), and Ontario Cattlemen’s Association (OCA). Published as VIDO journal series 467.

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