Two Doses of Bovine Viral Diarrhea Virus DNA Vaccine Delivered by Electroporation Induce Long-Term Protective Immune Responses

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Bovine viral diarrhea virus (BVDV) is a pathogen of major importance in cattle, so there is a need for new effective vaccines. DNA vaccines induce balanced immune responses and are relatively inexpensive and thus promising for both human and veterinary applications. In this study, newborn calves with maternal antibodies were vaccinated intramuscularly (i.m.) with a BVDV E2 DNA vaccine with the TriGrid Delivery System for i.m. delivery (TDS-IM). Two doses of this vaccine spaced 6 or 12 weeks apart were sufficient to induce significant virus-neutralizing antibody titers, numbers of activated T cells, and reduction in viral shedding and clinical presentations after BVDV-2 challenge. In contrast to the placebo-treated animals, the vaccinated calves did not lose any weight, which is an excellent indicator of the well-being of an animal and has a significant economic impact. Furthermore, the interval between the two vaccinations did not influence the magnitude of the immune responses or degree of clinical protection, and a third immunization was not necessary or beneficial. Since electroporation may enhance not only the magnitude but also the duration of immunity after DNA immunization, the interval between vaccination and challenge was extended in a second trial, which showed that two doses of this E2 DNA vaccine again significantly reduced clinical disease against BVDV for several months. These results are promising and support this technology for use against infectious diseases in cattle and large species, including humans, in general.

Bovine viral diarrhea virus (BVDV) is a Pestivirus in the family Flaviviridae and a pathogen of major importance in beef and dairy herds worldwide. BVDV strains have been classified into genotypes 1 and 2. BVDV-1 infections are common and result in fever, increased respiratory rate, diarrhea, and a reduction in white blood cells. Infections with type 2 BVDV strains may lead to high fever, hemorrhaging, diarrhea, reduction of white blood cells and platelets, and death, although only a minority of BVDV-2 strains are highly virulent. BVDV can also infect several types of immune cells, which results in functional impairment and down-regulation of the immune response not only to BVDV infection but also to secondary infections. As such, BVDV is a contributing factor to bovine respiratory disease (shipping fever). Each BVDV genotype contains cytopathic (CP) and noncytopathic (NCP) biotypes based on cell culture (1). NCP BVDV strains can cause persistent infections in fetuses of cows infected during early stages of gestation, while infection of cows after day ~120 leads to transient infection (2). Persistently infected calves shed virus for life and thus act as a reservoir of infection (3). If persistently infected calves become superinfected with a CP BVDV strain or the original virus mutates, they may develop mucosal disease. Although pregnant cows are vaccinated prior to breeding to prevent persistent infections (4), there still is a high incidence of BVDV in dairy and beef calves during the first 9 months of age. Consequently, it is important to be able to effectively vaccinate newborn calves against BVDV, even in the presence of maternal antibodies, which may be achieved by DNA immunization.

BVDV encodes a single polyprotein precursor, which, through co- and posttranslational processing by host and viral proteases, produces 12 structural and nonstructural proteins (5–8). BVDV virions are composed of the capsid protein C and the glycoproteins ENS, E1, and E2 (9). Since the envelope protein E2 plays a major role in viral attachment and penetration (10) and neutralizing antibodies are mostly E2 specific (11), the E2 protein is the most appropriate antigen in a subunit protein or DNA vaccine. Due to the endogenous production of proteins expressed in the host, DNA vaccines have the advantage of generally inducing balanced immune responses, as well as not being inactivated by maternal antibodies. Furthermore, DNA vaccines are stable, safe, and relatively inexpensive. One of the bottlenecks of DNA immunization was the relatively low protein expression in vaccinated large animals. In order to optimize the transfection efficiency in cattle, we previously adapted electroporation-mediated delivery for calves using the TriGrid Delivery System for intramuscular (i.m.) delivery (TDS-IM) (Ichor Medical Systems, San Diego, CA). Using the TDS-IM, we subsequently demonstrated significantly enhanced gene expression and improved immune responses to a plasmid encoding a model antigen (12) or BVDV E2 (13) after three immunizations.

In the current study, we first determined the frequency and interval of vaccinations required to induce protective immunity with the E2 DNA vaccine in newborn calves. This trial showed that two doses spaced 6 or 12 weeks apart are sufficient, while the third dose is not beneficial. Since electroporation enhances not only the magnitude but also the duration of immunity by DNA immunization (14, 15), we then performed a second trial in which the
interval between vaccination and challenge was extended. This demonstrated that two doses of the E2 DNA vaccine can induce protective immunity in newborn calves against BVDV challenge for several months.

MATERIALS AND METHODS

Cells and virus. Madin–Darby bovine kidney (MDBK) cells were grown in Eagle’s minimal essential medium (MEM) with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 50 µg/ml gentamicin, and 10% BVDV-free fetal bovine serum (FBS) at 37°C in a CO2 incubator. All cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA). The BVDV type 1a strain NADL and BVDV type 2 strain 1373 were grown in MDBK cells.

Construction, purification, and expression of plasmids. Plasmids pMASIA-tPAs-ΔE2.1 and pMASIA-tPAs-ΔE2.2, which encode truncated, secreted versions of BVDV type 1 (NADL) and type 2 (Q140) E2 (ΔE2.1 and ΔE2.2) were created as described previously (16). The codon bias of the E2 genes was optimized in favor of expression in bovine cells (http://www.kazusa.or.jp/codon/) (17, 18). The plasmids were grown in Escherichia coli DH5α on kanamycin-selective antibiotic plates, purified using Qiagen Endofree Plasmid Giga Kits (Qiagen, Mississauga, ON, Canada), and dissolved in calcium- and magnesium-free phosphate-buffered saline (PBS) (Gibco/Invitrogen). Expression of type 1 and type 2 E2 was confirmed by transient transfection of COS-7 cells and analysis by Western blotting (13).

Immunization and challenge of cattle. Two vaccination-challenge trials were performed. For both trials, ~6-week-old Angus cross calves born to cows that are routinely vaccinated with BVDV vaccine (Breed Back 9/Somnugen; Boehringer Ingelheim, Burlington, ON, Canada) were randomly allocated to treatment groups. The calves weighed approximately 60 kg and were housed at a local ranch during the first 6 weeks of the trial and at the research station of the Vaccine and Infectious Disease Organization during the remaining weeks. The trials were carried out according to the guidelines provided by the Canadian Council for Animal Care.

In the first trial, 28 calves were selected and randomly allocated to four groups of seven animals each. Calves in groups A, B, and C were injected i.m. in the right gluteus maximus muscle with TDS-IM (Ichor Medical Systems). The TriGrid is composed of four stainless steel electrodes positioned around a central injection port. The TriGrid array was adapted for use in cattle as described in detail previously (12). The electrodes were inserted, and the automatic injection device was activated to initiate IM injection of the plasmids. Subsequently, a 250-V/cm electrical field was locally applied for a total duration of 400 ms at a 10% duty cycle. The electroporation procedure was well tolerated, not requiring anesthesia.

Each calf received a DNA vaccine consisting of 1.5 mg of pMASIA-tPAs-ΔE2.1 and 1.5 mg pMASIA-tPAs-ΔE2.2 in 1 ml. Group D was injected with the diluent. Group A was vaccinated on days 0, 42, and 84, group B on days 0 and 84, and group C on days 42 and 84. Four weeks after the last vaccination, on day 112, the calves were challenged with BVDV strain 1373 (6 × 105.2 50% tissue culture infective dose [TCID50] in 4 ml PBS; 2 ml into each nostril) using an intranasal cannula (Pfizer Canada Inc.). On the day of challenge and for 14 days afterwards, the calves' temperatures were measured. Sera were collected at the start of the trial, as well as on days 28, 50, 70, 103, 137, 160, 171, 175, and 179. Peripheral blood was collected on days 70, 160, and 175. Nasal swabs and blood were collected 3 days prior to challenge, on the day of challenge, and on days 2, 4, 6, 8, 10, and 12 postchallenge. Temperatures and weights were recorded daily prior to and for 14 days after challenge.

In the second trial, 12 calves were selected and randomly allocated to two groups of 6 animals each. Group A was vaccinated with the same BVDV DNA vaccine (1.5 mg of pMASIA-tPAs-ΔE2.1 and 1.5 mg pMASIA-tPAs-ΔE2.2), and group B was injected with the diluent, both in 1 ml in the right glutaeus maximus muscle with the TDS-IM (Ichor Medical Systems). All calves were revaccinated after 58 days. The calves were challenged with BVDV strain 1373 on day 167 and monitored for 14 days afterwards as described for the previous trial. Sera were collected at the start of the trial, as well as on days 28, 58, 70, 103, 137, 160, 171, 175, and 179. Peripheral blood was collected on days 70, 160, and 175. Nasal swabs and blood were collected 3 days prior to challenge, on the day of challenge, and on days 2, 4, 6, 8, 10, and 12 postchallenge. Temperatures and weights were recorded daily prior to and for 14 days after challenge.

Enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) assay. BVDV E2-specific IgG titers were determined by ELISA as described in detail previously (16, 19). Immunol 2 HB U-bottom polystyrene microtiter plates (Dynatech Laboratories, Gaithersburg, MD) were coated overnight with culture supernatant containing ΔE2.1 or ΔE2.2 at 4 ng per well and incubated for 1.5 h at room temperature with serially diluted bovine sera. E2-specific antibodies were detected with alkaline phosphatase (AP)-conjugated goat anti-bovine IgG or anti-bovine IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and the reaction was visualized with p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich, Oakville, ON, Canada). Absorbance was read on a model Spectramax 340 PC® Microplate Spectrophotometer (Molecular Devices Corp.) at 405 nm, with a reference wavelength of 490 nm. ELISA titers were calculated as the highest dilution resulting in a reading of 2 standard deviations above the value of a negative-control serum.

Virus neutralization titers were determined as previously described (16, 19). Sera were inactivated for 30 min. at 56°C. Briefly, BVDV strain NADL or 1373 was incubated for 1.5 h at 37°C with serially diluted bovine sera. These samples were added to duplicate microtiter plates containing 80 to 90% confluent MDBK cells. The plates were incubated for 1.5 h at 37°C in a CO2 incubator, MEM with 2% FBS was added, and the plates were further incubated at 37°C for 4 days (NADL) or 6 days (1373). For strain 1373, the cells were fixed with 80% acetone in 0.85% saline at room temperature (RT), permeabilized with 0.5% Triton X-100 in PBS for 10 min, washed, and blocked with PBS containing 10% FBS. Subsequently, the cells were incubated with E2.2-specific rabbit antibody generated in-house, followed by AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories). BCIP/NBT (5-bromo-4-chloro-indolyolphosphate/nitroblue tetrazolium) was used for detection. The VN titer was reported as the reciprocal of the highest dilution that completely inhibited viral infection in the two replicate samples.

IFN-γ ELISPOT assay. Multiscreen-HA enzyme-linked immunosorbent spot assay (ELISPOT) plates (Millipore, Bedford, MA) were coated overnight with a bovine gamma interferon (IFN-γ)-specific monoclonal antibody (20) and blocked with 1% bovine serum albumin (BSA) in PBS. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (16, 19) and dispensed at 106 cells/well in triplicate wells in the absence or presence of 2 µg/ml of ΔE2.1 or ΔE2.2 protein. The plates were incubated at 37°C, and after 24 h bovine IFN-γ-specific rabbit serum (20) was added. AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) followed by BCP/NBT (Sigma-Aldrich Inc.) was used to visualize IFN-γ-secreting cells. The number of IFN-γ-secreting cells per 106 PBMCs was expressed as the difference between the number of spots in the BVDV E2-stimulated wells and the number of spots in the medium control wells.

Virus isolation. Nasal secretions were collected with cotton swabs in MEM supplemented with antibiotic-antimycotic (Gibco/Invitrogen), and white blood cells were isolated from blood with 0.83% ammonium chloride (Sigma-Aldrich), and resuspended in 1 ml Eagle’s MEM (Gibco/Invitrogen). Nasal swabs and WBCs were serially diluted and added to duplicate wells of a microtiter plate with MDBK cells. After 1.5 h of incubation at 37°C, MEM with 2% FBS was added, and the plates were further incubated. After 6 days, infected cells were fixed and identified by staining with a BVDV-2 E2-specific rabbit antibody as described for virus neutralization. The reciprocal of the highest dilution still showing virus in replicate wells was reported as the virus titer. The sera were considered positive if the titer was more than 1:10 and negative if less than 1:10.

Hematological analysis. Blood samples were analyzed on the day of challenge and on days 2, 4, 6, 8, 10, and 12 postchallenge by Prairie Diag-
nostic Services (Saskatoon, SK, Canada). Total WBC counts were quantified and expressed as counts per liter.

**Statistical analysis.** Data were analyzed with the aid of a software program (GraphPad Prism 5.0, San Diego, CA). As sample sizes were small, outcome variables were assumed not to be normally distributed. Therefore, in the first trial differences among all groups were examined using the Kruskal-Wallis test. If a significant difference was found among the groups, median ranks between pairs of groups were compared using the Mann-Whitney U test. In the second trial, differences between groups A and B were analyzed using the Mann-Whitney U test. Differences were considered significant if $P < 0.05$.

**RESULTS**

**Effects of frequency and interval between immunizations on the magnitude of the immune responses induced by a BVDV E2 DNA vaccine.** The goal of the first trial was to determine the effects of the number of the E2 DNA vaccine doses and of the interval between immunizations on the induction of protection against BVDV-2 challenge. To cover both BVDV genotypes, the calves were immunized with plasmids encoding E2.1 and E2.2. However, in view of the fact that BVDV-2 was used to evaluate the ability of the DNA vaccine to induce protective immunity, all immunological assays are reported for BVDV-2. To determine the influence of the different immunization regimens on the humoral immune responses, the BVDV-2-neutralizing antibody titers were determined. Prior to the first immunization, all calves had low levels of maternal antibodies, and there was no difference between the four experimental groups (Fig. 1A and B). Groups A and B were analyzed using the Mann-Whitney U test. Differences were considered significant if $P < 0.05$.

**FIG 1** BVDV-2-specific VN antibody titers of calves vaccinated with BVDV E2 DNA vaccine. Group A was vaccinated with BVDV E2 DNA vaccine on days 0, 42, and 84, group B on days 0 and 84, and group C on days 42 and 84. Group D was injected with the diluent. (A) Kinetics of BVDV-2-specific serum VN titers. (B to F) BVDV-2-specific serum VN titers immediately prior to vaccination (B), on day 42 (after the first immunization of groups A and B) (C), on day 70 (after the second immunization of group A and the first immunization of group C) (D), on day 112 (after the third immunization of group A and the second immunization of groups B and C) (E), and on day 124 (day 12 postchallenge) (F). In panel A, median values are shown. In panels B through F, each data point represents an individual animal and median values are indicated with the bars. The significance of differences is shown by asterisks: *, $P < 0.05$; **, $P < 0.01$. 

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after the second immunization as expected, while they remained at the same level after the first immunization of group C; in contrast, there was a decline in titers in group B, as well as a further decrease in group D (Fig. 1D). Interestingly, after the third immunization the titers in group A did not further increase, while groups B and C, which were immunized for the second time, showed an increase in VN titer such that there were no differences between the three vaccinated groups at the time of BVDV challenge or afterwards (Fig. 1E and F). The kinetics and levels of IgG production were very similar to those of the VN responses, confirming that the humoral responses induced by the three immunization regimens were equivalent in both magnitude and quality. The IgG and VN responses to BVDV-1 were also measured and found to be similar to those specific for BVDV-2 (data not shown).

The numbers of IFN-γ-secreting cells in the peripheral blood were also determined. The calves were not accessible until 6 weeks after the first immunization, at which time no increase in IFN-γ-secreting cells was observed in the two vaccinated groups (data not shown). This may be due to the fact that based on characteristic kinetics of activated T cells, this time point is not optimal for detection of BVDV-induced cell-mediated immune responses after primary immunization. However, significantly increased numbers of IFN-γ-secreting cells were observed after group A was reinmunized and group C was immunized for the first time, in comparison with groups B and D (Fig. 2). After the last immunization, groups A, B, and C all showed enhanced IFN-γ production compared to group D. Two weeks after BVDV-2 challenge, there were no differences between groups, although group D tended to have lower numbers of IFN-γ-secreting cells. However, due to the variable response in this group the difference was not significant. Again, the level of E2.1-induced T cell activation was very similar to that elicited by E2.2 (data not shown).

These data show that newborn calves with maternal BVDV-specific antibodies responded to vaccination with a BVDV E2 DNA vaccine. Furthermore, the interval between the two vaccinations did not influence the VN antibody titers or numbers of activated T cells achieved, and a third immunization administered 6 weeks after the second one was not necessary or beneficial.
blood cells was determined. While group D showed a significant decline in the number of WBCs from days 4 through 14, the three vaccinated groups (A, B, and C) had lower numbers only from days 4 through 6. The differences between groups A and B and group D were significant on days 8, 10, and 14 and between groups C and D on days 8 and 10 (Fig. 3C).

To determine the level of viremia, the amount of virus recovered from the nasal secretions and white blood cells was measured after BVDV challenge. Virus was detected in the nasal fluids from days 4 to 14 and in all animals in group D, while only two calves in group A and three calves in groups B and C shed virus for 1 to 2 days (Table 1). Virus was present in the WBCs from all animals in group D for 2 to 5 days after BVDV challenge, while only one animal in group A, two in group B, and three in group C had virus in the WBCs for 1 day only. Thus, in four or five of seven calves immunized with two or three doses, respectively, of E2 DNA vaccine, no virus was detected, while virus was present for only 1 to 2 days in the other calves in each group. Group A appeared to be slightly better protected than group B or C based on temperature response and virus replication, but this difference was not significant.

**Table 1** Virus in nasal fluids and white blood cells (trial 1)

| Sample and day | Group A | Group B | Group C | Group D |
|----------------|---------|---------|---------|---------|
| Nasal fluids   |         |         |         |         |
| 0              |         |         |         |         |
| 2              |         |         |         |         |
| 4              |         |         |         |         |
| 6              |         |         |         |         |
| 8              |         |         |         |         |
| 10             |         |         |         |         |
| 12             |         |         |         |         |
| 14             |         |         |         |         |
| WBCs           |         |         |         |         |
| 0              |         |         |         |         |
| 2              |         |         |         |         |
| 4              |         |         |         |         |
| 6              |         |         |         |         |
| 8              |         |         |         |         |
| 10             |         |         |         |         |
| 12             |         |         |         |         |
| 14             |         |         |         |         |

*Symbols: +, virus present; −, virus absent.*

After BVDV-2 challenge, the calves in group A had significantly higher IgG and VN titers than group B, showing an anamnestic antibody response, but the enhanced numbers of IFN-γ-secreting cells were similar in the two groups (Fig. 4). There was also a significant difference in the temperature response between the two experimental groups. While both groups showed slightly elevated temperatures between days 3 and 7, only group B continued to have increased temperatures, significantly higher than those in group A from day 8 through day 11 (Fig. 5A). Body weights were measured prior to and at the end of the trial and demonstrated that the calves in group A slightly increased in weight while the animals in group B had lost an average of ~6 kg (Fig. 5B). In addition, the calves in group B had significantly lower numbers of WBCs than those in group A between days 8 and 14 postchallenge (Fig. 5C). All animals in group B shed virus into the nasal fluids for 2 to 3 days, while in group A virus was detected in only three animals, for 1 day only each (Table 2). Similarly, virus was present in the WBCs from all calves in group B for 1 to 3 days, while virus was detected in two animals for 1 day in group A, demonstrating significant protection from BVDV challenge.

**DISCUSSION**

Despite the fact that DNA vaccines have several characteristics that make them promising as therapeutics or prophylactics, when used in large target species it is often necessary to administer more than two doses and/or use a heterologous prime-boost regimen. In this study, we demonstrated that two doses of a BVDV E2 DNA vaccine administered with the TDS-IM resulted in immune responses, reduction in viral shedding, and clinical disease after BVDV-2 challenge. After the first immunization, the antibody titers remained at the same level in groups A and B while they decreased 2- to 3-fold in groups C and D, suggesting that the vaccinated calves responded to the BVDV DNA vaccine. After the second immunization of group A, the VN titers were higher than those in the animals in groups B and C, who had only received one dose, which further supports that the calves were primed by the first immunization. This supports the ability of newborn calves

To determine the level of viremia, the amount of virus recovered from the nasal secretions and white blood cells was measured after BVDV challenge. Virus was detected in the nasal fluids from days 4 to 14 and in all animals in group D, while only two calves in group A and three calves in groups B and C shed virus for 1 to 2 days (Table 1). Virus was present in the WBCs from all animals in group D for 2 to 5 days after BVDV challenge, while only one animal in group A, two in group B, and three in group C had virus in the WBCs for 1 day only. Thus, in four or five of seven calves immunized with two or three doses, respectively, of E2 DNA vaccine, no virus was detected, while virus was present for only 1 to 2 days in the other calves in each group. Group A appeared to be slightly better protected than group B or C based on temperature response and virus replication, but this difference was not significant.

**Immunization of newborn calves with BVDV E2 DNA vaccine leads to long-term protective immunity against BVDV-2.** The goal of the second trial was to determine whether two immunizations with the BVDV DNA vaccine would provide protection for a longer period of time. Calves were immunized twice i.m. with electroporation. Again, prior to immunization both groups of calves had low IgG and VN titers (Fig. 4A and B). After two immunizations, group A developed increased IgG and VN titers, while the titers decreased in group B, which agrees with the previous trial. The vaccinated group showed a gradual decrease in the IgG titer to ~100 and in the VN titer to ~10 on the day of BVDV-2 challenge. Group A also had increased numbers of IFN-γ-secreting cells in the PBMCs upon in vitro restimulation in comparison with group B after the second immunization (Fig. 4C); despite some decline in group A over time, this difference was maintained until the day of BVDV-2 challenge.
with maternal antibodies to respond to vaccination. The interval between vaccinations did not influence the magnitude of the immune responses or protection, which suggests that calves could be vaccinated at branding and then again at entry into the feedlot. Interestingly, once groups B and C had received their second dose, both the VN titers and the numbers of IFN-γ-secreting T cells were equivalent to those of group A after three doses. No virus was detected from at least four of seven calves in each of the groups A, B, and C, while the other animals showed reduced durations of virus shedding, indicating significant protection from infection. Importantly, the vaccinated groups experienced only transient temperature responses and did not lose any weight, which is probably the best indicator of the well-being of an animal and has a significant economic impact. The fact that there was no significant difference in the level of protection between groups A, B, and C suggests that in the context of the immunization regimen tested, the third dose is not needed or beneficial. However, it is possible that, as reported for other species (D. Hannaman, unpublished data), a third dose spaced further apart from the second one might have been beneficial or aided in the duration of the immune response.

The protective efficacy of this DNA vaccine was confirmed in a second trial, in which again three of six animals did not have clinical signs, while the other three showed significantly less and shorter disease, even 3 months after the second vaccination. However, in this trial the animals recovered from leukopenia closer to the termination of the trial, although at that time their weights were stable and they did not show evidence of subsequent infec-

FIG 4 BVDV-2-specific immune responses of calves vaccinated with BVDV E2 DNA vaccine. Group A was vaccinated with BVDV E2 DNA vaccine, and group B was injected with the diluent. (A) Kinetics of E2.2-specific serum IgG titers. (B) Kinetics of BVDV-2-specific serum VN titers. (C) E2.2-induced T cell activation on day 70 (after the second immunization [Imm 2]), day 160 (7 days prior to challenge), and day 175 (day 11 postchallenge). The number of IFN-γ-secreting cells/10^6 PBMCs is expressed as the difference between the numbers of spots in the E2.2-stimulated and medium control wells. Bars represent median values and interquartile ranges. The significance of differences is shown by asterisks: *, P < 0.05; **, P < 0.01.

FIG 5 Changes in temperatures, weights, and white blood cell counts after challenge of calves with BVDV-2. Group A was vaccinated with BVDV E2 DNA vaccine, and group B was injected with the diluent. (A) Temperature change in °C; (B) weight change in kg; (C) change in WBC counts expressed as n x 10^9/liter. Data are shown as the medians for six calves. The significance of differences is shown by asterisks: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
TABLE 2 Virus in nasal fluids and white blood cells (trial 2)*

| Sample and day | Group A | Group B |
|----------------|---------|---------|
| Nasal fluids   |         |         |
| 0              | − − − − − − − − − − − − − − |
| 2              | − − − − − − − − − − − − − − |
| 4              | − − − − + − + + − + − + + + + |
| 6              | + + + − + − − + + + + + + + + + |
| 8              | − − − − + + + + + + + + + + + + |
| 10             | − − − − + + + + + + + + + + + + |
| 12             | − − − − + + + + + + + + + + + + |
| 14             | − − − − + + + + + + + + + + + + |

| WBCs           |         |         |
|----------------|---------|---------|
| 0              | − − − − − − − − − − − − − − |
| 2              | − − − − − − − − − − − − − − |
| 4              | − − − − + − + + − + − + + + + |
| 6              | + + + − + − − + + + + + + + + + |
| 8              | − − − − + + + + + + + + + + + + |
| 10             | − − − − + + + + + + + + + + + + |
| 12             | − − − − + + + + + + + + + + + + |
| 14             | − − − − + + + + + + + + + + + + |

*Symbols: +, virus present; −, virus absent.

mycobacterial antigens were found in cattle by using separate double-needle electrodes; however, humoral responses were not investigated (24). Expression of growth hormone-releasing hormone (GHRH) in the trapezius muscle of heifers was reported from a plasmid delivered by electroporation (25). We previously demonstrated enhanced gene expression, as well as improved immune responses, when plasmids encoding reporter antigens were administered with the TDS-IM (12). In addition, three doses of BVDV E2 DNA vaccine administered with the TDS-IM resulted in the induction of enhanced immune responses and protection from BVDV-2 challenge (13). The TDS-IM was also recently used in a heterologous prime-boost regimen with a foot-and-mouth disease virus (FMDV) DNA vaccine. In this study, electroporation also enhanced both humoral and cell-mediated immune responses, as well as clinical protection from FMDV challenge (26). However, these cattle received two doses of DNA vaccine followed by a protein boost, which is less favorable from a cost-benefit aspect.

To our knowledge, our present report represents one of the few demonstrating significant protective immunity with two doses of DNA vaccine in cattle. With the potential for development of battery-operated electroporation devices, this technology might be feasible for veterinary use in future. In addition, the fact that the E2 DNA vaccine in combination with electroporation is promising against BVDV supports the use of this technology against infectious disease in humans.

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