The Induction of a Cell-Mediated Immune Response to Bovine Viral Diarrhea Virus with an Adjuvanted Inactivated Vaccine*

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This study evaluated the induction of bovine viral diarrhea virus (BVDV) cell-mediated and humoral immune responses after vaccination with an adjuvanted inactivated product. In vaccinated animals, there was an overall treatment effect ($P < .05$), for an increased percentage of BVDV-specific CD8 T cells expressing interferon-$\gamma$ (IFN-$\gamma$). The percentages of IFN-$\gamma$ producing $\gamma\delta$-T cells in the vaccinated group were increased on days 7 ($P = .10$), 14 ($P = .09$), and 31 ($P = .12$). CD4 T cells expressing IFN-$\gamma$ were increased on day 42 ($P = .05$). Stimulated peripheral blood mononuclear cells of the vaccinated group had increased IFN-$\gamma$ production on days 14 and 35 ($P < .05$). Testing for BVDV types 1 and 2 titers began at day 14, with peak titers on days 42 and 35, respectively. In summary, the intracellular accumulation and release of IFN-$\gamma$, a T helper cell 1 cytokine, indicates that an adjuvanted inactivated BVDV vaccine is capable of invoking a cell-mediated response while delivering a targeted humoral response.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a major pathogen of cattle, causing respiratory and reproductive disease. BVDV has two genotypes, type 1 and type 2, and two biotypes, cytopathic (cp) and noncytopathic (ncp). A fetus

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that survives a noncytopathic BVDV infection between 30 and 125 days of gestation can become a persistently infected (PI) animal.\textsuperscript{1} Attempts to control BVDV involve herd vaccination, identification and elimination of PI cattle, and a biosecurity program.\textsuperscript{2} Vaccination can be a cost-effective tool for controlling BVDV, but the selection of an appropriate vaccine is crucial for an effective animal health program.

Most marketed vaccines for BVDV contain either modified-live or inactivated virus. One of the advantages of a modified-live vaccine (MLV) is that the attenuated virus replicates and stimulates humoral and cell-mediated immunity. Inactivated vaccines, which have no replicating virus, can be a safer alternative to MLV in certain classes of cattle, such as pregnant dams or high-risk, stressed calves. Inactivated vaccines are most often administered with an adjuvant, which usually results in strong humoral immunity but weaker T-cell responses.\textsuperscript{3} For optimal immunity, a robust helper T-cell response is necessary. There are two CD4\textsuperscript{+} helper T cell subpopulations, which are differentiated by the cytokines they secrete. T helper 1 (Th1) cells secrete interleukin (IL)-2 and interferon-\(\gamma\) (IFN-\(\gamma\)), which are important for generating long-term cell-mediated immunity to viruses. On the other hand, T helper 2 (Th2) cells secrete IL-4 and IL-10, and it is this Th2 response that generates large amounts of antibody.\textsuperscript{4,5} In addition to the Th1 and Th2 subpopulations, \(\gamma\delta\)-T cells, which have a prominent distribution in epithelium-rich areas, may also play an important role in long-term immunity. Unlike CD4 and CD8 T cells, \(\gamma\delta\)-T cells are not restricted by the major histo-

compatibility complex (MHC), allowing for diverse interaction with antigen-presenting cells.\textsuperscript{6}

In this study, an inactivated vaccine containing three strains of BVDV (ncp type 1, ncp type 2, and cp type 1), bovine herpesvirus 1 (BHV-1), parainfluenza 3 virus (PI3), and bovine respiratory syncytial virus (BRSV) was administered to seronegative Holstein calves. Before and after vaccination, both BVDV cellular immune responses and BVDV humoral antibody responses were measured over time.

\section*{MATERIALS AND METHODS}

\textbf{Animals and Experimental Design}

Protocols were reviewed and approved by the Rural Technologies (Brookings, SD) institutional animal care and use committee before study initiation. Twenty 8- to 10-month-old, BVDV- and BHV-1-seronegative, castrated male Holstein calves were randomly assigned to one of two treatment groups at enrollment using Microsoft Excel. Additionally, before enrollment, all calves were confirmed to have had no previous T-cell exposure to BVDV or BHV-1 by testing for a recall response following stimulation of T cells with either BVDV or BHV-1 and measuring IFN-\(\gamma\) production.\textsuperscript{7}

Calves in Group 1 (\(n = 10\)) received an SC sham vaccine (sterile saline solution) twice, 3 weeks apart, and served as the nonvaccinated (control) group. Calves in Group 2 (\(n = 10\)) received an SC inactivated, adjuvanted viral vaccine (ViraShield 6, Novartis Animal Health) containing three strains of BVDV (ncp type 1, ncp type 2, and cp type 1), BHV-1, PI3, and BRSV twice, 4 weeks apart, and served as the
vaccinated group. First vaccinations and sham vaccinations were administered on day 0.

**Bovine Viral Diarrhea Virus–specific Interferon-γ Production in Vitro**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with a modification in the protocol described by Reber et al.\(^8\) using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) rather than Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). The isolated PBMCs were separated from whole blood on day –7 (before vaccination) and days 7, 14, 21, 28, 31, 35, 38, 42, and 49 (after vaccination). Cells were counted and suspended in Roswell Park Memorial Institute (RPMI) complete medium (RPMI 1640 medium containing 10% certified BVDV-free fetal bovine serum [Atlanta Biologicals, Lawrenceville, GA]), 1% penicillin–streptomycin–amphotericin B solution (Atlanta Biologicals), and 25 mM HEPES (Sigma-Aldrich) at a concentration of \(2 \times 10^7\) cells/ml. Isolated PBMCs (\(5 \times 10^5\) cells/well) from each animal were incubated in duplicate wells of a 48-well microtiter tissue culture plate (Greiner, Monroe, NC). One well contained RPMI complete medium (nonstimulated control), and the other contained inactivated BVDV (TGAC).\(^9\) The plates were incubated for 5 days at 37°C and 5% carbon dioxide. After incubation, the cell culture supernatant was removed and the IFN-γ concentration was determined using the bovine IFN-γ Screening Set (Endogen, Rockford, IL) according to the manufacturer’s instructions. The absorbance of samples was measured at a wavelength of 450 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Winoski, VT). An IFN-γ standard curve was produced for each plate using Microsoft Excel XLStatistics, and the concentration of IFN-γ (pg/ml) was determined for each sample.

**Staining for Intracellular Cytokine Production**

PBMCs were isolated from whole blood and separated by Ficoll gradient centrifugation on day –7 (before vaccination) and days 7, 14, 21, 28, 31, 35, 38, 42, and 49 (after vaccination). One million cells of each PBMC suspension were added to each well of a 96-well, flat-bottom tissue culture plate (Greiner). RPMI complete medium was added to three wells, and Concanavalin A (0.05 µg/well) and 0.1 MOI (multiplicity of infection) BVDV (TGAC) were each added to duplicate wells. Plates were incubated for 4 days at 37°C and 5% carbon dioxide. After incubation, 100 µl of Brefeldin (2 µg; Sigma-Aldrich) was added to each well and plates were incubated for 4 hours at 37°C and 5% carbon dioxide.\(^7\) Plates underwent centrifugation at 350 \(\times\) g, and cells were washed three times with phosphate-buffered saline solution containing 1% bovine serum albumin (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). Plates underwent centrifugation again, and the primary monoclonal antibodies were added to the wells.

Primary monoclonal antibodies used for cell-surface staining included mouse antibovine CD4 isotype IgM (CACT83B, VMRD, Pullman, WA), mouse antibovine CD8 isotype IgG2a (MCA837G, Serotec, Raleigh, NC), and mouse antibovine \(\gamma\delta\) TCR1-N12 isotype IgM (CACT61A, VMRD). After incubating at 4°C for 30 minutes, plates were washed and centrifuged three times, and the secondary antibodies were added to the wells. Secondary antibodies consisted of goat antimouse IgM-fluorescein isothiocyanate (FITC) and goat antimouse IgG2a-allophycocyanin (APC). All secondary antibodies were purchased from Southern Biotech (Birmingham, AL). Plates were incubated at 4°C for 10 minutes, and then a wash step was performed. A 0.05% saponin solution (100 µl) was added to each well to per-
meabilize the cells, and plates were incubated at 4˚C for 20 minutes. Plates were washed, and mouse antiovine IFN-γ isotype IgG1 (MCA1964, Serotec) was added to the wells. After incubating at 4˚C for 30 minutes, the plates were washed and underwent centrifugation, and the secondary antibody goat antimouse IgG1-R-Phycoerythrin (R-PE) was added to the wells. The plates were incubated at 4˚C for 20 minutes, and then were washed and underwent centrifugation again.

Cells were suspended in a 1% solution of paraformaldehyde, and the percentage of cells expressing intracellular IFN-γ within the respective T-cell subset was evaluated by flow cytometry. Data were collected from at least 30,000 cells of each sample. Cells from each sample were counted using a dual laser (488 nm argon laser, 635 nm red diode laser) FACscalibur flow cytometer (Becton Dickinson, San Jose, CA), and cell data were analyzed using BD CellQuest Pro software (BD Biosciences, San Jose, CA). Three subsets of bovine T cells were identified: CD4⁺CD8⁻, CD4⁻CD8⁺, and γδTCR⁺. The percentages of each T-cell subset expressing IFN-γ were determined. The net increases in percent IFN-γ⁺ after in vitro stimulation were calculated by subtraction of antigen-stimulated cells from unstimulated cells.

**Serum Neutralizing Antibodies**

To determine the titers of serum-neutralizing (SN) antibodies, serum was collected on days –7, 0, 7, 14, 21, 28, 35, 42, and 56. The serum was heat inactivated and serially diluted in 96-well plates (Greiner) and incubated with BVDV type 1 (Singer) or BVDV type 2 (A125) at ≤500 TCID₅₀/ml. After incubation, BVDV-free bovine turbinate cells were added, and the plates were incubated at 37˚C and 5% carbon dioxide for 5 days before being evaluated for virus-induced cytopathic effect (CPE). The reciprocal of the last dilution that prevented CPE formation was designated the SN antibody titer. Geometric mean values were calculated by use of log₂ titer.

**Statistical Analysis**

The experimental unit was the individual animal. Statistical significance was established at the 5% level (P < .05). Each assay was evaluated using a repeated measures mixed linear model with a heterogeneous temporal covariance matrix. Linear functions of least squares means were used to calculate estimates of area under the curve (AUC) across all time points. Statistical analysis for each of the assays was also performed by each time point against the respective control group. Contrasts were created between treatment groups at each time point after detecting a significant overall treatment effect or overall treatment by time point interaction. For the statistical analysis of intracellular and supernatant IFN-γ, the net increase in IFN-γ production was derived by subtracting the IFN-γ production of non-antigen-stimulated cells from the IFN-γ production after stimulation with BVDV (TGAC). If the net IFN-γ production was less than zero, the value was changed to zero before statistical analysis.

**Overall, the vaccinated group had a higher percentage of CD8 T cells expressing IFN-γ compared with the control group.**
RESULTS

Overall, the vaccinated group had a higher \((P < .05)\) percentage of CD8 T cells expressing IFN-\(\gamma\) compared with the control group. Although an overall treatment effect for CD8 was observed, no significant differences were found between treatment groups within a day throughout the study. The vaccinated group had a twofold increase in the percentage of CD8 T cells expressing IFN-\(\gamma\) on days 21, 28, and 49 compared with control animals (Figure 1A). There was a tendency for increased percentages of \(\gamma\delta\)-T cells producing IFN-\(\gamma\) on postvaccination days 7 \((P = .10)\), 14 \((P = .09)\), and 31 \((P = 0.12)\) in the vaccinated group. The vaccinated group had 1.5- to fourfold higher levels of \(\gamma\delta\)-T cells expressing IFN-\(\gamma\) on days 7, 14, 31, 35, 38, and 49 compared with the control animals (Figure 1B). The CD4 subset was the most diminished in magnitude of the T-cell subsets evaluated until the last two sampling time points (days 42 and 49) in the vaccinated group. The percentage of CD4 T cells expressing IFN-\(\gamma\) was increased significantly on day 42 \((P = .05)\) in the vaccinated group. In the vaccinated group, there was a twofold increase in the percentage of CD4 T cells expressing IFN-\(\gamma\) on day 42 and a threefold increase on day 49 (Figure 1C).

The intracellular accumulation of IFN-\(\gamma\) also correlated with the release of IFN-\(\gamma\) in the culture medium after stimulation with BVDV. The mean IFN-\(\gamma\) production of isolated PBMCs was determined for vaccinated and control groups after in vitro stimulation with a heterologous strain of BVDV (TGAC). The increase in IFN-\(\gamma\) production in the cell supernatants of the vaccinated group was increased on day 14 and significantly increased on day 35 \((P < .05)\) (Figure 2). Day 35 after vaccination was 7 days after the booster vaccination.

Serum samples were tested for BVDV type 1 (Singer) and type 2 (A125) SN antibody titers by use of the constant virus–decreasing serum assay. BVDV type 1 titers began at day 14, with peak titers at day 42 (14 days after booster vaccination). BVDV type 2 titers also began at day 14, with peak titers achieved between days 35 and 42 (Figure 3).

DISCUSSION

This study demonstrated activation of several different cell subpopulations responsible for a cell-mediated response against BVDV. In the vaccinated group, there was a higher \((P < .05)\) percentage of CD8 T cells expressing IFN-\(\gamma\) compared with the control group. While assessing the immunity conferred by a different commercial inactivated BVDV vaccine, Platt and associates\(^9\) were able to detect a memory response in the CD8+\(\gamma\delta\) T cell subpopulation of vaccinated animals. Although we cannot make a direct comparison because the CD8+\(\gamma\delta\) T cell subpopulation was not identified here, the response detected may have been attributable to the CD8+\(\gamma\delta\) T cell subpopulation characterized by Platt and associates.\(^9\) Sandbulte and Roth\(^10\) evaluated T-cell priming in cattle after vaccination with BRSV with either an MLV or an inactivated vaccine. In that study, regardless of the vaccine formulation (MLV versus inactivated), the CD8 T cells had an increased level of CD25 expression, which suggested that the CD8 T cells were responsive to in vitro stimulation with BRSV. In the MLV group, the difference in CD25 expression between nonstimulated and BRSV-stimulated CD8 T cells peaked at 6 weeks after vaccination (22.5% difference between stimulated and nonstimulated cells) and then declined over the next 4 weeks (1% difference between stimulated and nonstimulated cells). Interestingly, in the inactivated vaccine group, the difference in CD25 expression between stimulated and nonstimulated cells remained steady; the difference was 27.5% at 6 weeks after vaccination and 25% at 10
weeks after vaccination. Both inactivated vaccines used by Platt and associates and Sandbulte and Roth were adjuvanted, albeit with different adjuvants. The inactivated BVDV vaccine used by Platt and associates had an adjuvant that contained Quil A, Amphigen, and cholesterol, whereas the inactivated BRSV vaccine used
by Sandbulte and Roth \cite{10} contained the proprietary adjuvant Xtend III.

\(\gamma\delta\)-T cells reside in epithelium-rich tissues and can expand significantly following infection.\cite{6} They also represent a large percentage of the circulating T cells in ruminant animals.\cite{11} Interestingly, we found that the \(\gamma\delta\)-T cells had the highest percentage of intracellular IFN-\(\gamma\) of the T-cell subsets evaluated. The tendency for intracellular IFN-\(\gamma\) responses to be increased in the vaccinated \(\gamma\delta\)-T cells after stimulation with BVDV was an important finding. Studies using an inactivated bacterial vaccine against \textit{Leptospira borgpetersenii} demonstrated that CD4 T cells and \(\gamma\delta\)-T cells from vaccinated animals divided and produced IFN-\(\gamma\) in an antigen recall response.\cite{12} Further characterization of the response showed that \(\gamma\delta\)-T cells bearing the WC1.1+ \(\gamma\delta\)-TcR were the most responsive.\cite{13} In a follow-up study, the magnitude and kinetics of the antigen recall response revealed that the WC1+ \(\gamma\delta\)-TcR cells were the major antigen-responding T-cell population in the first 4 weeks after vaccination and replicated more rapidly than CD4 T cells.\cite{14} Strikingly, the CD4 subset was the most diminished in magnitude of the T-cell subsets evaluated until the last two sampling time points (days 42 and 49).

After vaccination with an inactivated BVDV vaccine, we observed that BVDV type 1 titers began at day 14, with peak titers at day 42 (14 days after booster vaccination). BVDV type 2 titers also began at day 14, with peak titers achieved between days 35 and 42. (BVDV = bovine viral diarrhea virus; SN = serum neutralizing)
day 42 (14 days after booster vaccination). BVDV type 2 titers also began at day 14, with peak titers achieved between days 35 and 42 (Figure 3). Platt and associates\(^9\) demonstrated that modified-live BHV-1, PI3, and BRSV diluted in diluent containing inactivated BVDV type 1 (strain 5960) and BVDV type 2 (strain 53637) with a booster on day 21 could produce significant neutralizing titers of BVDV-specific antibodies to homologous BVDV type 1 (strain 5960) and a heterologous type 2 (strain 125C), with maximum titers reached on day 35 post-vaccination.\(^9\) On the other hand, Reber et al\(^8\) demonstrated that an inactivated BVDV type 1 (NADL) vaccine containing modified-live BHV-1 and PI3, with a booster on day 14, developed no significant neutralizing titers to a BVDV type 1 (NADL and Singer) and a type 2 (strain 890) above control animals at any time after vaccination. The pronounced humoral response observed to heterologous strains of BVDV is likely to have been potentiated by the diverse genotypes and biotypes of BVDV in combination with the adjuvant that was used in the vaccine formulation.

## CONCLUSION

The administration of an inactivated BVDV vaccine was able to induce cell-mediated and humoral responses. The intracellular accumulation and subsequent release of IFN-\(\gamma\), a Th1 cytokine, indicates that the adjuvant formulation of the inactivated BVDV vaccine is capable of invoking a cell-mediated response while delivering a targeted humoral response.

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## REFERENCES


