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Janice Jones Endsley
Iowa State University

Julia Ridpath
United States Department of Agriculture

John Neill
United States Department of Agriculture

Matthew Robert Sandbulte
Iowa State University

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Abstract
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Disciplines
Veterinary Infectious Diseases | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments
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Induction of T Lymphocytes Specific for Bovine Viral Diarrhea Virus in Calves with Maternal Antibody

JANICE J. ENDSLEY,1 JULIA F. RIDPATH,2 JOHN D. NEILL,2 MATTHEW R. SANDBULTE,1 and JAMES A. ROTH1

ABSTRACT

Passive antibody to bovine viral diarrhea virus (BVDV) acquired through colostrum intake may interfere with the development of a protective immune response by calves to this virus. The objective of this study was to determine if calves, with a high level of maternal antibody to bovine viral diarrhea virus (BVDV), develop CD4⁺, CD8⁺, or γδ T lymphocyte responses to BVDV in the absence of a measurable humoral immune response. Colostrum or milk replacer fed calves were challenged with virulent BVDV at 2–5 weeks of age and/or after maternal antibody had waned. Calves exposed to BVDV while passive antibody levels were high did not mount a measurable humoral immune response to BVDV. However, compared to nonexposed animals, these animals had CD4⁺, CD8⁺, and γδ T lymphocytes that were activated by BVDV after exposure to in vitro BVDV. The production of IFNγ by lymphocytes after in vitro BVDV exposure was also much greater in lymphocytes from calves exposed to BVDV in the presence of maternal antibody compared to the nonexposed calves. These data indicate that calves exposed to BVDV while maternal antibody levels are high can develop antigen specific CD4⁺, CD8⁺, and γδ T lymphocytes in the absence of an active antibody response. A manuscript presented separately demonstrates that the calves with T lymphocytes specific for BVDV in this study were also protected from virulent BVDV genotype 2 challenge after maternal antibody became undetectable.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a pestivirus responsible for major economic losses to the cattle industry due to respiratory, enteric, and reproductive disease (22). The disease manifestation varies with biotype, genotype, and strain of the BVD virus. BVDV isolates are designated as either cytopathic or noncytopathic based on the ability to cause cytolysis in cell culture (20). Both biotypes of BVDV can cross the placenta leading to abortion, mummification, or congenital defects (1). Fetal infection with the noncytopathic strains may lead to immunotolerant persistently infected animals (37), the most common source of...
transmission within a herd (22). Superinfection of a persistently infected animal with a homologous cytopathic strain may cause a fatal mucosal disease (5,11).

Two genotypes of BVDV have also been designated based on genomic differences in the 5′ untranslated region and differences in the monoclonal antibody binding pattern (30,33). Disease caused by genotype 1 BVDV (BVDV1) infection in immunocompetent animals is usually mild and transient, while disease caused by genotype 2 BVDV (BVDV2) can be severe and frequently fatal (13,14,32). Young calves are protected from clinical signs due to either genotype by passively acquired antibody derived from colostrum (6,23). Maternally derived antibody to BVDV has a half life of approximately 3 weeks and is usually catabolized by approximately 6 months of age (10,15,26,28). High titers of maternal antibody are capable of preventing the induction of an active antibody response to vaccination in calves (10). This has led to the conclusion that it is necessary to wait until maternal antibody has waned to effectively induce protective immunity.

Stimulating an immune response to BVDV in the presence of maternal antibody could generate a protective memory response to this virus at a much earlier age. Cell-mediated immune responses to some pathogens of domestic animals can occur in the presence of maternal antibody and in the absence of a detectable humoral response (18,27,38). Protective immune responses mediated by T lymphocytes may be overlooked because there is no simple serology based test to detect them. The objective of this trial was to determine if calves with a high level of maternal antibody to BVDV develop CD4\(^+\), CD8\(^+\), or γδ T lymphocyte responses to BVDV in the absence of a humoral immune response.

**MATERIALS AND METHODS**

**Experimental design.** Eighteen mixed breed male and female calves were used to determine if BVDV-specific T lymphocytes were generated when calves with circulating maternal antibody are challenged with BVDV. Pooled colostrum from cows immunized against BVDV during pregnancy, or milk replacer, was fed to BVDV-seronegative calves within 48 h of birth as described (34). The titer of neutralizing antibody against BVDV1 and BVDV2 in the colostrum and first milk was determined as previously described (8). The neutralizing antibody titer of the pooled colostrum to BVDV1b-NY-1 was 1/128,000 and titer to BVDV2-1373 was 1/4,096,000. The corresponding titers in the second milking after parturition were 1/1,448 against BVDV1 and 1/6,848 against BVDV2.

Calves receiving colostrum or milk replacer were assigned to one of four treatments. Groups A and B (n = 6 in each group) received colostrum with a high titer of antibody against BVDV, and groups C and D (n = 3 in each group) were fed milk replacer. Animals from groups A and C were inoculated intranasally with 5 mL (2.5 mL in each nostril) of freeze/thaw lysate from cultured bovine cells infected with BVDV2-1373 at 2–5 weeks of age. The titer of the lysates used for the inoculum was 10\(^6\) TCID\(_{50}\)/mL. The inoculated animals were housed separately from the non-inoculated animals for the remainder of the trial. Groups B and D were not challenged with BVDV at 2–5 weeks of age. Following inoculation, all surviving groups of animals were sampled approximately monthly for antibodies to BVDV, T lymphocyte subset activation by *in vitro* BVDV, and production of IFNγ by lymphocytes after *in vitro* exposure. Antibodies of maternal origin were monitored as described (34) until they were no longer detectable in the serum of calves in all treatment groups. At this time, all groups of animals were challenged with BVDV2-1373 as described above to determine if they had protective immunity.

Viral stocks of BVDV2-1373, BVDV2-890, BVDV1b-NY-1, and BVDV1b-TGAN were propagated as described previously (33). BVDV1-970 virus was grown in bovine turbinate (BT) cells in McCoy’s 5A medium supplemented with 10% fetal bovine serum that was free of BVDV virus and antibodies. Flasks of BT cells were seeded for 24 h and infected with 10\(^6\) TCID\(_{50}\) of BVDV. Virus was grown for 48 h and frozen at −80°C. Culture flasks were then thawed and cell culture media centrifuged at 2,000 \(\times\) g to remove cellular debris. Viral titers of BVDV were determined as described previously (7).

**Measurement of T cell activation.** Antigen-specific T cell subset activation was detected using a modification of a previously described procedure (31). Animals in all treatment groups were sampled for *in vitro* T cell responsiveness approximately monthly from 2 to 32 weeks following challenge with BVDV at
2–5 weeks of age. Peripheral blood mononuclear cells (PBMC) were isolated from approximately 40 mL of citrated blood, washed twice in Hanks balanced salt solution (HBSS), and resuspended at $4 \times 10^5$ cells in 200 $\mu$L of cRPMI media containing 2 mM L-glutamine, penicillin (60 $\mu$g/mL), 20 mM HEPES, and 7.5% fetal bovine serum (cRPMI). Triplicate wells of PBMCs from all animals were inoculated with a mock control of 25 $\mu$L of cRPMI or 25 $\mu$L of cRPMI containing BVDV2-890 (TCID$_{50}$ 10$^6$/mL), BVDV2-1373 (TCID$_{50}$ 4 $\times$ 10$^6$/mL), BVDV1-970 (TCID$_{50}$ 10$^7$/mL), BVDV1b-TGAN (TCID$_{50}$ 4 $\times$ 10$^6$/mL), or Concanavalin A for 5 days at 39°C in a 5% CO$_2$ atmosphere. The amount of viral antigen used in the CD25 expression assay was based on levels that gave an optimal response in animals previously vaccinated with modified live BVDV vaccine containing BVDV1.

Following the 5 days of culture, cells were washed twice with Hanks balanced salt solution (HBSS) and twice with a phosphate buffered saline (PBS) solution containing 0.5% bovine serum albumin and 0.1% NaN$_3$ (PBS$^+$). The washed cells were then stained with murine monoclonal antibodies (VMRD, Pullman, WA) specific for bovine CD4 (CACT138A), CD8 (CACT80C), and $\gamma\delta$ (GB21A) T cells and for bovine CD25 (CACT108A). The antibodies specific for CD4, CD8, and a matched isotype control were of the IgG1 isotype. Antibodies specific for $\gamma\delta$ TCR and a matched isotype control were of the IgG2b isotype. The antibody specific for CD25 was of the IgG2a isotype. Following a 30-min incubation at 4°C, cells were washed four times with PBS$^+\pm$ and then stained with phycoerythrin labeled anti-murine IgG2a and fluorescein isothiocyanate labeled anti-murine IgG2b ($\gamma\delta$ and isotype control) or anti-IgG1 (CD4, CD8, and isotype control) (Caltag, Burlingame, CA). After 30 min at 4°C, cells were washed five times in HBSS without phenol red and fixed in 1% paraformaldehyde. Triplicate wells were pooled prior to counting.

Measurement of in vitro IFN-γ production. Supernatants were collected from PBMC cultured with and without BVDV2-890 after 5 days of incubation and frozen at −20°C prior to analysis. Upon thawing, supernatant was pooled from six equivalent wells of a 96-well plate per sample. The Bovigam ELISA kit (Biocor Animal Health, Omaha, NE) was used to measure IFN-γ levels in the supernatants. The ELISA was performed according to the protocol supplied by the manufacturer, except that each sample was tested in triplicate rather than duplicate and the volume of sample per well was decreased from 50 to 25 $\mu$L so that optical density in most positive samples would not exceed the microplate reader’s photometric range. A standard curve for protein concentration was derived by regression of four ten-fold dilutions of recombinant bovine IFN-γ (Novartis Animal Health, Basel, Switzerland). Optical density readings at a wavelength of 450 nm were obtained with a V$_{max}$ Microplate Reader (Molecular Devices, Sunnyvale, CA).

Data analysis. Antigen-specific T cell responsiveness to in vitro antigen was determined by analyzing a total of 5,000 cells using a flow cytometer (Becton Dickinson FACScan, San Jose, CA). Data are reported as a CD25 expression index, which is a ratio of the lymphocyte responsiveness from each animal when stimulated with antigen versus when not stimulated with antigen. The Expression Index was calculated as follows: % stimulated T cells positive for CD25) * [mean fluorescent intensity of stimulated T cells positive for CD25])/([% unstimulated T cells positive for CD25) * [mean fluorescent intensity of unstimulated T cells positive for CD25]). Data are reported as treatment means plus the standard error of the mean. For clarity of presentation, the expression indices and IFNγ data are pooled by 10-week intervals following the first challenge with BVDV. A Wilcoxon rank sum test was used to determine the level of significance of the differences in CD25 expression indices and IFNγ production between treatment groups.

RESULTS

Calves fed colostrum with high titers of BVDV antibody did not show clinical signs of disease from BVDV after challenge at 2–5 weeks of age (34). The calves in group C that were fed milk replacer and challenged with BVDV at 2–5 weeks of age became acutely ill with BVDV and had to be euthanized. Colostrum fed calves had average serum neutralizing antibody titers of 2,211 against BVDV1 and 11,456 against BVDV2 at 1 week of age (34). Antibodies to BVDV1 and BVDV2 from colostrum fed calves declined at similar rates for the calves exposed to BVDV at 2–5 weeks of age compared to the nonexposed calves (34). The calves in groups A, B, and D were sampled for T lymphocyte subset responsiveness to four strains of BVDV in vitro, approximately monthly, until maternal antibodies were no longer detectable. 

T LYMPHOCYTES SPECIFIC FOR BVDV
Peripheral blood CD4^+, CD8^+, and γδ T cells from animals in all surviving treatment groups responded to *in vitro* stimulation with Concanavalin A (ConA) by increasing expression of CD25, indicating that all T cell subsets were capable of responding. Following exposure to ConA the average CD25 Expression Indices for CD4^+, CD8^+, and γδ T cells of calves challenged with BVDV at 2–5 weeks of age in the presence of colostral antibody were 42.8, 33.1, and 54.1, respectively. The average CD25 Expression Indices of CD4^+, CD8^+, and γδ T cells in response to ConA by calves that were not challenged with BVDV at 2–5 weeks of age in the presence of colostral antibody were 50.2, 41.5, and 36.2, respectively.

Antigen-specific CD4^+, CD8^+, and γδ T cell activation, in response to both BVDV1 and BVDV2 *in vitro*, was detected in calves challenged with BVDV2 while they had high titers of maternal antibody. Variation in the response of the three subsets of lymphocytes to the four strains of BVDV was observed (Figs. 1–4). The T lymphocyte responses of the calves fed milk replacer and not exposed to BVDV (group D) were not significantly (p < 0.05) different from the T lymphocyte responses of the calves fed colostrum and not exposed to BVDV (group B) (data not shown). Production of IFNγ by PBMC exposed *in vitro* to BVDV2 (strain 890) was significantly greater for calves exposed to BVDV at 2–5 weeks of age compared to colostrum fed calves that were not exposed to BVDV at 2–5 weeks of age (Fig. 5). Calves from all sur-

![Image](BVDV2-1373.png)

**FIG. 1.** CD25 Expression Index (mean ± SEM) for T cell subsets stimulated *in vitro* with BVDV2-1373 from calves fed colostrum and not challenged with BVDV (group B) and calves fed colostrum and challenged with BVDV2-1373 at 2–5 weeks of age (group A). Data is presented as weeks after challenge with BVDV at 2–5 weeks of age and is pooled from weeks 2–10 (n = 33), 11–20 (n = 37), and 21–32 (n = 31). There were no significant differences between treatment groups.
viving treatment groups were challenged with BVDV2-1373 when maternal antibodies were no longer detectable in peripheral blood. The calves in group A that had previously been exposed to BVDV when maternal antibody levels were high were protected from challenge compared to the animals from groups B and D that had not been previously exposed (34).

**DISCUSSION**

Previously, we reported that a protective immune response can be mounted by calves exposed to virulent BVDV in the face of passive antibody (34). In this report, we characterize the nature of that response. This is the first report to demonstrate that calves challenged with BVDV while maternal antibody levels are high can develop BVDV-specific T lymphocyte subset responses to *in vitro* BVDV antigen. The T lymphocyte response occurred in the absence of a detectable humoral immune response to BVDV in the challenged calves. As reported elsewhere (34), calves challenged with BVDV2-1373 after ingestion of colostrum with high titers of BVDV antibody did not develop a detectable serum neutralizing antibody response to BVDV1 or BVDV2. The amount of serum neutralizing antibody to BVDV declined steadily following

**FIG. 2.** CD25 Expression Index (mean ± SEM) for T cell subsets stimulated *in vitro* with BVDV2-890 from calves fed colostrum and not challenged with BVDV (group B) and calves fed colostrum and challenged with BVDV2-1373 at 2–5 weeks of age (group A). Data is presented as weeks after challenge with BVDV at 2–5 weeks of age and is pooled from weeks 2–10 (n = 33), 11–20 (n = 37), and 21–32 (n = 31). *p < 0.05, **p < 0.01, level of statistical significance between exposed and nonexposed values (mean ± SEM).
colostrum ingestion, indicating the antibodies were of maternal origin. In a similar experiment, calves challenged with BVDV while maternal antibody circulated were able to mount a moderate IgG2 response specific for BVDV (23). As discussed by Howard and colleagues, the level of serum neutralizing antibodies to BVDV in the calves mounting the IgG2 response did not decline at the expected rate for catabolism of maternally derived antibodies (23). The IgG2 response and prolonged serum neutralizing antibody titer in that trial may indicate that maternal antibody levels were not adequate to completely block a humoral response.

In the current trial, individual T cell subsets from colostrum fed, BVDV challenged calves were all activated to some degree in response to the homologous BVDV genotype. The T lymphocyte response by the animals exposed to BVDV while maternal antibody circulated persisted several months after exposure, indicating that a strong CMI response had occurred. When combined with surface phenotype markers, detection of CD25 expression indicates activation by individual T lymphocyte populations in response to specific antigen. The level of activation, however, does not provide information regarding the function of the responding T cells (29,31). The CD4+ and CD8+ T cells activated by BVDV are expected to produce cytokines and induce cytotoxic effects on infected cells, respectively. Purified CD4+ T cells from BVDV ex-

FIG. 3. CD25 Expression Index (mean ± SEM) for T cell subsets stimulated in vitro with BVDV1-970 from calves fed colostrum and not challenged with BVDV (group B) and calves fed colostrum and challenged with BVDV2-1373 at 2–5 weeks of age (group A). Data is presented as weeks after challenge with BVDV at 2–5 weeks of age and is pooled from weeks 2–10 (n = 33), 11–20 (n = 37), and 21–32 (n = 31). *p < 0.05, level of statistical significance between exposed and nonexposed values (mean ± SEM).
posed animals produce a predominant IL-4 cytokine response upon in vitro exposure to BVDV infected monocytes and CD8^+ T cells produce a predominant IFN and IL-2 response (35). Cytotoxic T lymphocytes (CTL) capable of MHC restricted killing of BVDV infected targets can be isolated from peripheral blood of animals immune to BVDV (3). The precise role of γδ T cells in cattle and other species is not well defined, making it difficult to explain the biological significance of antigen-specific γδ T cells following vaccination. Gamma delta T cells have been shown to act as antigen-presenting cells and cytotoxic T lymphocytes in cattle and as helper T cells in mice (4,12). In the current trial, γδ T cells were activated by both genotypes of BVDV in vitro. This result may reflect the less specific nature of the γδ T cell population in response to antigen (25) or the detection of epitopes conserved in both genotypes.

In the current trial, PBMC from calves exposed to BVDV while maternal antibody levels were high produced greater amounts of IFNγ than nonexposed counterparts upon re-stimulation with BVDV antigens. The relative contribution of individual T cell subsets to IFNγ production in the mixed lymphocyte pool was not determined. BVDV-specific production of IFNγ has been demonstrated in PBMC and isolated CD4^+ and CD8^+ populations from animals exposed to BVDV antigens (35,39). Isolated CD4^+ and CD8^+ T cells from immune animals proliferate in response to BVDV-infected monocytes and the primary source of IFNγ in supernatants from the proliferating T cells appears to be the CD8^+ T cells. Production of IFNγ by bovine γδ T cells in response to BVDV has not been reported previously, although IFNγ production by bovine γδ T cells in response to specific antigen has been demonstrated in a Theileria parva study (16).

**FIG. 4.** CD25 Expression Index (mean ± SEM) for T cell subsets stimulated in vitro with from calves fed colostrum and not challenged with BVDV (group B) and calves fed colostrum and challenged with BVDV2-1373 at 2–5 weeks of age (group A). Data is presented as weeks after challenge with BVDV at 2–5 weeks of age and is pooled from weeks 2–10 (n = 33), 11–20 (n = 37), and 21–32 (n = 31). There were no significant differences between treatment groups.
The detection of significant CD8\(^+\) and \(\gamma\delta\) T lymphocyte responses to a genotype of BVDV heterologous to the challenge strain in the current trial may indicate that cross protective epitopes are presented to T lymphocytes following BVDV exposure. Vaccination of cattle with modified live vaccine containing BVDV1 was shown to provide partial protection of cattle from BVDV2 challenge (17). It is interesting that the \textit{in vitro} T cell subset responses to the BVDV2-890 were more consistent than to the BVDV2-1373 challenge strain in the current trial. The challenge strain is highly virulent and may have a suppressive effect on antigen specific T cells \textit{in vitro} compared to BVDV2-890.

The development of T cell memory to pathogens in the absence of an antibody response has been described for other viruses in animals with circulating maternal antibody. Calves challenged with bovine herpesvirus 1 (BHV-1) while maternal antibody levels were high had T lymphocytes that proliferated and secreted IFN\(\gamma\) in response to \textit{in vitro} BHV-1 antigen in the absence of a measurable antibody response (18,27). Antigen-specific T cell proliferation or cytokine secretion in the absence of an antibody response has also been described in cattle vaccinated against bovine respiratory syncytial virus (18), neonatal mice vaccinated against measles (36), pigs vaccinated against pseudorabies (9), and human neonates exposed to various enteroviruses (24). Cell-mediated immune responses do not always occur in the face of maternal antibody, however. Maternal antibody inhibits the proliferative response of T cells to antigen in mice exposed to malaria (21) and dogs exposed to rabies or Sendai virus (40). In a direct comparison, maternal antibody in mice was shown to inhibit the generation of a cytotoxic T cell (CTL) response to respiratory syncytial virus, but not influenza virus (2). The ability of a young animal to generate a memory T cell response to antigen in the face of maternal antibody apparently varies among pathogens.

The route of inoculation may also affect the ability of an individual to develop a memory T cell response to a pathogen in the presence of maternal antibody. Pseudorabies virus (PRV) inoculation of pigs with maternal antibody resulted in a strong T cell proliferative response to \textit{in vitro} PRV when an intranasal inoculation protocol was used (9). The T cell response was absent when an intramuscular (i.m.) inoculation protocol was followed in the same study. Calves with high passive antibody titers to BHV-1 demonstrated a lymphoproliferative response to antigen following intramuscular (i.m.) vaccination and had a significant IFN\(\gamma\) secretion response following intranasal inoculation. The current study examined T cell responses following an intranasal inoculation with virulent BVDV. An intranasal inoculation best simulates the natural
route of infection; however, BVDV vaccines are administered i.m. or subcutaneously (s.c.). Seropositive calves vaccinated i.m. with a MLV BVDV2 vaccine at 10–14 days of age were not protected from virulent BVDV2 challenge at 4 months of age (19). In the same study, seronegative calves subjected to the same vaccination and challenge were protected from clinical signs of disease. The T lymphocyte responses specific to BVDV were not determined following vaccination. It is unclear if the disparity in results between the current study and those reported by Ellis and colleagues reflect differences in route of inoculation, differences in antigen persistence, or differences in the development of other immune parameters. The results of the current trial demonstrate that calves can be seronegative for BVDV and still have immune memory due to an exposure to the virus during the presence of maternal antibody. This conflicts with the current practice of identifying BVDV naive and susceptible animals based on the absence of acquired antibody. Currently, a non-persistently infected calf that is seronegative after maternal antibody has waned is considered to be immunologically naive to BVDV. The existence of seronegative calves that have T cell memory to BVDV could significantly impact interpretations of immune responses of calves to BVDV infection or vaccination.

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Address reprint requests to:
Dr. James A. Roth
2156 College of Veterinary Medicine
Iowa State University
Ames, IA 50011

E-mail: jaroth@iastate.edu

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