Adaptive immunity in the colostrum-deprived calf: Response to early vaccination with *Mycobacterium bovis* strain bacille Calmette Guerin and ovalbumin¹

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ABSTRACT

Responses of the newborn calf to vaccination are frequently characterized by marginal antibody (Ab) responses. The present study evaluated effects of colostrum ingestion on the adaptive immune response of the preruminant calf to early vaccination. Colostrum-fed (CF) and colostrum-deprived (CD) calves were vaccinated at 2 d of age with *Mycobacterium bovis*, Pasteur strain of bacille Calmette Guerin (BCG), and ovalbumin (OVA) to track development of the adaptive immune response during the first 8 wk of life. Dams were also vaccinated with BCG prepartum. At wk 0, serum IgG₁, IgG₂, IgA, and IgM were elevated in CF calves, with IgG₁ predominating. In these calves, IgG₂, IgA, and IgM concentrations decreased with age. The CD calves, in contrast, had very low or undetectable serum immunoglobulin concentrations at wk 0 followed by an age-related increase in IgG₁, IgG₂, and IgM concentrations, suggesting endogenous production of these immunoglobulin classes. Immunoblot and ELISA analyses of Ab response to BCG vaccination indicated that colostrum ingestion was associated with measurable serum anti-mycobacterial Ab in CF calves during the first month postpartum, with substantially lower levels at 7 wk of age. Although mycobacteria-specific Ab was undetectable in CD calves at wk 0, it was present at 4 and 7 wk of age, suggesting that these calves, unlike CF calves, were capable of generating an Ab response to BCG vaccination. Antibody responses of CF and CD calves to vaccination with OVA, an antigen not present in the natural environment of dairy cattle,

were of comparable magnitude and characterized by a progressive increase in Ab levels from birth (wk 0) to 7 wk of age. The disparate Ab responses of CF calves to BCG and OVA suggest that maternal antigenic experience or exposure influences Ab responses of the colostrum-fed preruminant calf to early vaccination. Ex vivo, antigen [OVA and *M. bovis*-derived purified protein derivative (PPDb)]-induced IFN- γ and nitric oxide responses of blood mononuclear cells (PBMC) from CF and CD calves were comparable at wk 0 and wk 7. As expected, responses were very low or nonexistent at wk 0. Responses for all calves were greater at wk 7 than at wk 0, suggesting a colostrum-independent maturation of the cell-mediated immune response capacity of the preruminant calf. The consistently greater proliferative responses of antigen-stimulated T-cell subsets at wk 7 versus wk 0 indicate the development of antigen-specific lymphocyte responses to early vaccination. Total numbers of blood leukocytes as well as numbers of lymphocytes and monocytes were unaffected by colostrum feeding; however, granulocyte numbers were higher in CD than in CF calves at wk 0. Granulocyte numbers decreased and monocyte numbers increased with age in all calves. Within the lymphocyte population, only natural killer (NK⁺) cell percentages were affected by colostrum ingestion, with higher percentages of NK⁺ cells in CD calves at wk 0 and wk 7. Antigen-induced proliferation of lymphocyte subsets including IgM⁺ cells was unaffected by colostrum ingestion. In conclusion, ingestion of colostrum within hours after birth inhibited the capacity of the calf to produce antigen-specific immunoglobulin (i.e., antibody) in response to vaccination, with little or no effect on cell-mediated immune responses. Although colostrum appeared to block endogenous antibody production, certain B-cell functions were retained. These findings will aid in development of new vaccination strategies for improving health of the preruminant calf.

Key words: preruminant calf, neonatal vaccination, colostrum deprived, bacille Calmette Guerin vaccination

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INTRODUCTION

High morbidity and mortality rates of neonatal calves attributable to infectious diseases cause substantial economic losses to producers. Recent USDA-APHIS-National Animal Health Monitoring System (NAHMS) data indicated that in 2006 the mortality rate for preweaned dairy heifers was approximately 7.8%. Diarrhea or other digestive problems contributed to 56.5% of preweaned heifer calf deaths, with respiratory disease being the second largest cause of mortality (22.5%; USDA-APHIS NAHMS, 2010). Susceptibility of the calf to infectious disease can be reduced by feeding high-quality colostrum within the first hours after birth, providing adequate nutrition during the preruminant phase of development, minimizing environmental stress during the first weeks of life, and by administering vaccines against pathogens associated with respiratory and enteric diseases. Effects of early vaccination on the adaptive immune response of the neonatal calf have not been well described. Protection afforded by early vaccination is critical given the diversity and number of potential pathogens in the calf's environment. It is generally assumed that the developmental immaturity of the immune system of the neonate, including the newborn calf, compromises the efficacy of vaccination protocols and exacerbates the calf's susceptibility to infectious disease (Siegrist, 2001; Chase et al., 2008).

The calf is born agammaglobulinemic and relies on ingestion of colostrum to ensure adequate passive immunity. The significance of colostrum in conferring protective immunity to the calf was reported almost 90 yr ago (Smith and Little, 1922). Colostrum is also a critical source of viable leukocytes, growth factors, and essential nutrients for the newborn calf. Interestingly, almost 20% of preweaned heifers demonstrate a failure of passive immunity based on serum IgG testing (USDA-APHIS NAHMS, 2010). Although the importance of colostrum for passive transfer of maternal immunoglobulins is well established, it has been suggested that colostral immunoglobulin, representing maternal antigenic experience, compromises the antibody (Ab) response of the preruminant calf to early vaccination (Husband and Lascelles, 1975; Aldridge et al., 1998). For example, neonatal calves fail to develop immunoglobulin responses to *Brucella abortus* if Ag-specific maternal immunoglobulin is present at the time of vaccination (Husband and Lascelles, 1975). Colostrum-deprived calves, however, generate robust Ab responses to *B. abortus*. In addition, neonatal calves sensitized to an antigen to which there was no maternal immunoglobulin mount humoral responses at birth similar to that of older cattle, regardless of colostral status (Husband and Lascelles, 1975). Likewise, neona-

tal calves vaccinated with *Mycobacterium bovis* strain bacille Calmette Guérin (BCG) failed to mount antigen (Ag)-specific humoral responses, whereas BCG-vaccinated adult cattle did, suggesting that maternal Ab exported via colostrum may be contributory (Nonnecke et al., 2005). In contrast, functional capacities of T cells from BCG-vaccinated calves were similar to those of BCG-vaccinated adults and the magnitude of their cutaneous delayed-type hypersensitivity responses to intradermal administration of purified protein derivative b (PPDb) was comparable to those of vaccinated adults (Nonnecke et al., 2005). Overall, these results suggest that maternal antigenic experience does not inhibit all aspects of the adaptive response of the calf to vaccination. Other research indicates that calves given a bovine viral diarrhea (BVD) virus vaccine develop Ag-specific CD4⁺, CD8⁺, and $\gamma\delta$ T-cell receptor ($\gamma\delta$ TCR⁺) cell responses and generate memory T and B cells, despite the absence of primary Ab responses (Endsley et al., 2003, 2004). Taken together, these results suggest that although maternal immunoglobulin blocks endogenous immunoglobulin production, B cells retain other functional capacities (i.e., differentiation into memory B cells).

Foote et al. (2007) characterized the composition and function of B-cell populations from neonatal calves vaccinated at an early age with ovalbumin (OVA) and BCG. Vaccination with OVA elicited measurable OVA-specific Ab responses that were amplified by revaccination at 5 wk of age. Vaccination with BCG, in contrast, was not associated with increased levels of mycobacteria-specific Ab. Interestingly, IgM⁺ lymph node-derived cells stimulated with PPDb or OVA showed increased expression of CD25 (i.e., a protein present on activated B and T cells that serves as receptor for IL-2) and CD80 (i.e., a protein found on activated B cells and monocytes that provides co-stimulatory signal for T-cell activation), suggesting the generation of Ag-specific memory B cells in response to vaccination with both antigens.

The objective of the present study was to evaluate the effects of colostrum ingestion on the development of the adaptive immune response in the preruminant calf to early vaccination. Colostrum-fed (CF) and colostrum-deprived (CD) calves were vaccinated at less than 2 d of age with 2 antigens, *Mycobacterium bovis* strain BCG and OVA, to track the development of the adaptive immune response during the first 8 wk of life.

MATERIALS AND METHODS

Calf Management and Treatments

The National Animal Disease Center Animal Care and Use Committee preapproved all procedures involv-

ing animals. Holstein bull calves ($n = 16$) born to dams housed at the National Animal Disease Center were caught at birth to prevent them from suckling their dams. Calves weighing >36.3 kg (80 lb) and without developmental problems were selected for the study. Navels were dipped in 7% iodine daily from birth to 72 h postpartum. Colostrum-fed calves were fed 3.8 L of colostrum from their dams within 4 h after birth.

Colostrum-deprived ($n = 9$) and CF calves ($n = 7$) were fed Similac infant formula (2 cans in 1.5 bottles of warm water at each feeding, Abbott Laboratories, Abbott Park, IL) twice daily for the first 48 h and then transitioned to a milk replacer diet. Calves were housed individually in elevated calf crates (1.6 m long $\times$ 0.9 m wide $\times$ 0.9 m high) in a temperature-controlled (18°C) barn and fed a medicated 20% CP and 20% fat milk replacer (Land O'Lakes-Purina Feed, Shoreview, MN) twice daily (0700 and 1800 h) following the manufacturer's recommendations. Calves were weighed weekly and the amount of milk replacer fed was adjusted at these times to allow for changes in live weight. Clean water was available between feedings. Starter grain was not fed during the study.

Preparation and Administration of Vaccines

Mycobacterium bovis BCG (Pasteur strain) and OVA vaccines were prepared as described by Foote et al. (2007). At less than 2 d of age, all calves were vaccinated subcutaneously in the right mid-cervical region with 1×10^7 cfu of BCG and in the left mid-cervical region with 4 mL of OVA/incomplete Freund's adjuvant emulsion as described previously (Nonnecke et al., 2009). Calves were revaccinated with OVA/incomplete Freund's adjuvant 4 wk later. All dams were vaccinated with BCG approximately 60 d prepartum.

Quantification of Serum Immunoglobulins

Total immunoglobulins (IgG₁, IgG₂, IgM, and IgA) in weekly serum samples were quantified by capture ELISA. Assays were established in ELISA-specific 96-well plates (Immunolon 2HB plates, VWR, West Chester, PA). Coating antibodies were affinity-purified sheep anti-bovine IgG₁, IgG₂, IgM, and IgA (Bethyl Laboratories, Montgomery, TX) diluted 1:100 in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6; Sigma, St Louis, MO). Calibrators consisted of bovine reference sera with defined amounts of each immunoglobulin isotype (Bethyl Laboratories) serially diluted from 15.6 to 10,000 ng/mL. Standards and test sera were diluted in PBS with Tween-20 (PBST; 0.014 M NaCl, 50 mM Trizma, 0.05% Tween 20, pH 8.0). Detection Ab were sheep anti-bovine IgG₁, IgG₂, IgM,

or IgA conjugated to horseradish peroxidase (Bethyl Laboratories). Dilutions of each were optimized before the evaluation of test sera. SureBlue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) was used as the chromogenic substrate. Plate preparation was initiated by addition of coating Ab (100 μL) to each well followed by 1 h at 22°C . After washing 3 times with PBST, 200 μL of blocking solution (PBS with 1% vol/vol gelatin) were added. Plates were washed 3 times and then 100 μL of diluted test sera, reagent diluent only (blank), and standards were added to the appropriate wells. Plates were incubated for 1 h at 22°C and then washed 5 times with PBST. After addition of horseradish peroxidase-conjugated Ab (100 μL), plates were incubated 1 h at 22°C and then washed 3 times. Chromogenic substrate (100 μL) was added and plates were incubated for 10 min at 22°C . The enzyme-substrate reaction was stopped by addition of TMP stop solution (100 mL, KPL) to each well. Absorbance at 450 nm was measured using an automated plate reader (Flex Station 3, Molecular Devices, Sunnyvale, CA). Concentrations of each immunoglobulin class were determined by comparing absorbance of test samples with that of serially diluted standards within a linear curve fit. Data were corrected for dilution factor to arrive at immunoglobulin concentrations (mg/mL) in test samples.

Quantification of Serum Haptoglobin

Haptoglobin concentrations were determined as described previously (Nonnecke et al., 2009) using a bovine-specific haptoglobin ELISA (Immunology Consultants Laboratory Inc., Newberg, OR) following the manufacturer's instructions. Haptoglobin concentrations in test sera, evaluated in duplicate, were determined by comparing the absorbance of test samples with those of serially diluted standards within a linear curve fit. Data were corrected for dilution factor to arrive at haptoglobin concentrations ($\mu\text{g/mL}$) in test samples.

Evaluation of Serum Ab Responses

Antibody responses were evaluated using both ELISA and immunoblot analysis. Sera collected at 0 (prevaccination), 4, and 7 wk were analyzed. For ELISA, a lipoarabinomannan-enriched fraction of proteinase-digested, whole-cell sonicate of *M. bovis* strain 95-1315 (WCS1315), referred to as WCS1315-LAM, was prepared as described by Waters et al. (2002). Anti-OVA and anti-WCS1315-LAM specific IgG₁ levels in serum samples collected at 0, 4, and 7 wk after vaccination were determined by capture ELISA as described pre-

viously (Foote et al., 2007) with modifications. Both assays used 96-well Immulon 2HB plates (VWR). For each sample analyzed, duplicate wells without Ag (coating buffer consisting of 0.05 M carbonate-bicarbonate coating buffer, pH 9.6, 100 mL total volume, Sigma) and duplicate wells with Ag (coating buffer with WCS1315-LAM at 20 µg/mL or OVA at 1.56 µg/mL) were prepared and incubated overnight at 4°C in zip-loc bags. Plates were washed 3 times with PBST (200 µL/well) and blocked with a commercial milk diluent blocking solution (KPL) diluted 1:20 in double deionized water and added at 200 µL/well. After 1 h at 37°C, plates were washed 9 times in PBST and test sera were added to wells (100 µL/well). Test sera for both assays were diluted 1:50 in PBS with 0.1% gelatin. Optimal dilutions of test sera were determined by evaluating the reactivity of undiluted sera and sera diluted from 1:10 to 1:100. After 20 h at 4°C, plates were washed 9 times with PBST. Plates were incubated an additional 1 h at 37°C after addition of 100 µL of horseradish peroxidase-sheep anti-bovine IgG₁ conjugates (diluted 1:10,000 PBS/gelatin, 100 mL/well; Bethyl Laboratories Inc.). Plates were subsequently washed 9 times with PBST before addition of chromogenic substrate (SureBlue TMB Microwell Peroxidase Substrate, 100 µL/well; KPL). Plates were read kinetically every minute for 15 min at 650 nm using an automated plate reader (Flex Station 3, Molecular Devices). Data were analyzed using Softmax Pro software (v 5.2, Molecular Devices). Raw maximum initial rate of reaction ($V_{\max}$) was calculated by subtracting the mean $V_{\max}$ (milli-units/min, $n = 2$ wells) of the no-Ag wells from the mean $V_{\max}$ of Ag wells ($n = 2$). Raw $V_{\max}$ values for the positive and negative control wells also were determined. The sample-to-positive ratios (**S/P**) for test sera were calculated using the following formula: $[V_{\max}(\text{test sample}) - V_{\max}(\text{negative control})] / [V_{\max}(\text{positive control}) - V_{\max}(\text{negative control})]$.

Immunoblot analysis of Ab responses to BCG vaccination was performed as described by Bannantine and Stabel (2000) with the following modifications. Antigens included *M. bovis*-derived WCS1315 and OVA (2 mg/mL, Sigma). Antigens were electrophoresed through preparative 12% (wt/vol) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose was accomplished with a Bio-Rad Trans Blot cell (Bio-Rad Laboratories, Mississauga, ON, Canada) using PBS (25 mM, pH 7.8) at 0.8 A for 90 min. After transfer, membranes were blocked with PBST with 2% (wt/vol) BSA (**PBST-BSA**). Membranes were placed into a 20-slot mini-protean multi-screen device (Bio-Rad), and serum from each calf was diluted 1:200 in PBST-BSA and added to independent slots. After 2 h at 22°C with gentle rocking, membranes were washed

3 times with PBST and incubated with peroxidase-conjugated Protein G (Sigma) diluted 1:20,000 in PBST-BSA for 1.5 h. Membranes were washed again with PBST and developed for chemiluminescence using SuperSignal detection reagent (Pierce Chemical Co., Rockford, IL).

Isolation and Enrichment of PBMC

Peripheral blood was collected into 10% (vol/vol) 2 × acid-citrate-dextrose (77 µM sodium citrate, 38 µM citric acid, and 122 µM dextrose) tubes and also into Vacutainers with potassium EDTA (Becton Dickinson, Franklin Lakes, NJ). Blood was collected after an overnight fasting period and before the morning feeding. Blood mononuclear cells were isolated (Nonnecke et al., 2003) and resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) with 25 mM HEPES buffer, L-glutamine (2 mM, Sigma), antibiotics (100 U/mL of penicillin G and 100 µg/mL of streptomycin sulfate, Sigma), 2-mercaptoethanol (50 µM, Sigma), 1% nonessential AA (Sigma), and heat-inactivated fetal bovine serum (**FBS**, 10% vol/vol; Hyclone Laboratories Inc., Logan, UT).

In Vitro Production and Measurement of IFN-γ and Inducible Nitric Oxide

Secretion of IFN-γ and nitrite, the stable oxidation product of nitric oxide (NO), by nonstimulated and stimulated peripheral blood mononuclear cell (**PBMC**) cultures was evaluated in 96-well, flat-bottomed tissue culture plates (Immunolon). Wells were seeded with 4×10^5 PBMC in a culture volume of 200 µL (final concentration of 2×10^6 cells/mL in culture). Culture medium consisted of supplemented RPMI 1640 (Gibco Laboratories) as described above for PBMC isolation and enrichment. Cultures were nonstimulated (medium alone), stimulated with pokeweed mitogen (**PWM**, 2 µg/mL, Sigma), OVA (10 µg/mL) or PPD_b (10 µg/mL). Cultures, consisting of 6 wells for each culture condition, were incubated for 72 h in a humidified atmosphere at 39°C with 5% CO₂.

For NO-producing cultures, N^G-monomethyl-L-arginine, a competitive inhibitor of inducible NO synthase, was used as a negative control. The inhibitor was added to parallel nonstimulated or stimulated cultures to verify that nitrite production was due to inducible NO synthase. Nitrite (µM) in supernatants freshly harvested after centrifugation of plates (400 × g, 21°C, 5 min), was measured by the method of Green et al. (1982) incorporating modifications described previously (Rajaraman et al., 1998). Supernatants from cultures producing IFN-γ were harvested and stored at

Table 1. Primary antibodies used in the analysis of blood mononuclear cell populations from colostrum-fed and colostrums-deprived calves

Primary antibody specificity	Clone ¹	Isotype	Dilution, $\mu\text{g/mL}$
CD4 T cell	GC50A1	IgG _{2a}	15
CD8 T cell	CACT80C	IgM	7
$\gamma\delta$ TCR ⁺ T cell	GB21A	IgG _{2b}	14
Natural killer cell	AKS1	IgG ₁	7
IgM ⁺ (B cell)	PIG45A	IgG _{2b}	7
CD14 ⁺ (monocyte)	CAM36A	IgG ₁	7

¹Primary monoclonal antibodies were from VMRD (Pullman, WA); Serotec (Raleigh, NC); and M. Estes (Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX).

–80°C until analyses could be performed. Interferon- γ (ng/mL) in supernatants was measured by an IFN- γ capture ELISA (Ametaj et al., 1996). Responses were analyzed after IFN- γ and nitrite concentrations in non-stimulated cultures were subtracted from concentrations in stimulated cultures.

Composition and Proliferative Responses of Blood Leukocyte Populations

The composition of blood leukocyte populations at wk 0 and 7 was determined by flow cytometry using a modification of a previous method (Nonnecke et al., 2003). A 25- μL aliquot of anticoagulated blood from each animal was centrifuged ($1,000 \times g$, at room temperature for 20 min). Contaminating erythrocytes in recovered buffy coats were eliminated by hypotonic lysis. Recovered leukocytes were washed and resuspended in PBS containing 0.02% NaN₃ and 1% (vol/vol) FBS. Approximately 5×10^5 cells in PBS (100- μL aliquot) were added to each of 8 wells of a 96-well, round-bottomed microtiter plate (Sigma). Individual wells were preloaded with a 50- μL aliquot of a subset of the primary monoclonal antibodies (Table 1) diluted in PBS with 0.02% NaN₃ and 1% FBS. Plates were incubated for 15 min at 22°C in the dark and centrifuged ($400 \times g$ for 2 min at room temperature). The washed cells were resuspended in 50 μL of fluorochrome-conjugated secondary antibodies in PBS containing 0.02% NaN₃ and 1% FBS. Incubation and centrifugation steps were repeated as described above. Cells were resuspended in 200 μL of FACSlyse buffer (Becton Dickinson, San Jose, CA) and 10,000 cells exhibiting light-scattering properties consistent with bovine mononuclear and granulocytic leukocytes were analyzed. Markers were positioned for negative control samples to provide a background of approximately 2% and were maintained at this position for all samples. Data were acquired and analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson). Percentages of cells staining positive for a specific marker were recorded.

Percentage data in combination with total numbers of circulating leukocytes were used to estimate numbers of cells positive for each marker.

Lymphocyte subset proliferation assays were performed as described by Foote et al. (2005). Briefly, 4×10^7 PBMC were resuspended in diluent C (2 mL) provided in the PKH67 kit (Sigma). Diluted cells were added to 1 mL of diluted (4 μM) PKH67 green fluorescent dye, incubated for 5 min, and then incubated for 1 min with 4 mL of inactivated FBS to adsorb excess dye and stop dye uptake. Individual wells of 96-well tissue culture plates were seeded with 2×10^5 PKH67-stained PBMC in a total volume of 200 μL (6 replicates for each treatment). Cultures were nonstimulated (medium alone), stimulated with PWM (2 $\mu\text{g/mL}$, Sigma), stimulated with OVA (10 $\mu\text{g/mL}$, Sigma), or stimulated with PPD_b (10 $\mu\text{g/mL}$). Cultures were incubated for 7 d at 39°C in a humidified atmosphere with 5% CO₂. Approximately 2×10^5 pooled PKH67 stained cells in 150 μL of culture medium were added to individual wells of 96-well round-bottomed microtiter plates. Stained cells were labeled with 1 of 4 phenotype makers (CD4, CD8, $\gamma\delta\text{TCR}$, IgM⁺). Primary Ab used in the analyses are listed in Table 1. Antibodies (1 $\mu\text{g/well}$) diluted in PBS containing 0.02% NaN₃ and 1% (vol/vol) inactivated FBS were added to individual wells in 25- μL aliquots. Cells were incubated for 15 min at 22°C, centrifuged, and resuspended in 100 μL of secondary fluorochrome-conjugated, isotype-specific Ab diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were incubated for 15 min at 22°C in the dark, centrifuged as described above, resuspended in 200 μL of PBS, and evaluated the same day by flow cytometry.

Ten thousand cells exhibiting light-scattering properties consistent with bovine PBMC were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson). Lymphocyte subset proliferation data were acquired using CellQuest (Becton Dickinson) software and analyzed using Modfit LT (Verity Software House Inc., Topsham, ME) software. Proliferation profiles were determined for gated lymphocyte subsets within

the PBMC population. Lymphocyte proliferation was estimated by measuring the fluorescence intensity of PKH67-stained cells. Because fluorescence intensity decreases progressively with the number of cell divisions, cell generation can be mathematically determined by fluorescence intensity using Modfit LT software. Data are presented as the mean ($\pm$ SEM) percentages of specific T-cell subsets in the first through fifth generation beyond the parent population. The cell population was considered to have proliferated if it was not in the parent generation.

Cutaneous Delayed-Type Hypersensitivity Reaction

The comparative cervical skin test, performed according to the Bovine Tuberculosis Eradication Uniform Methods and Rules (USDA-APHIS, 2005), was used to gauge the magnitude of the delayed-type hypersensitivity response elicited by BCG vaccination. This test uses biologically balanced USDA bovine and avian PPDb tuberculin. All calves were injected intradermally with 100 μ L of PPDb and *Mycobacterium avium* PPD (PPDa) at separate sites in the mid-cervical region 7 wk after BCG vaccination. Skin-fold thickness was measured before tuberculin administration and 24 h and 72 h later. Responses were analyzed after subtracting pretest skin-fold thicknesses from skin-fold thicknesses at 24 h and 72 h after antigen administration.

Statistical Analyses

Data were analyzed as a completely randomized design using Statview software (version 5.0, SAS Institute Inc., Cary, NC). Calf served as the experimental unit in the analysis of all data. Composition and proliferative responses of PBMC populations, serum antibody, and haptoglobin, and in vitro IFN- γ and NO secretion were analyzed as a split-plot with repeated-measures ANOVA. The model included fixed effects of treatments (colostrum-fed vs. colostrum-deprived), time (week on experiment), and treatment $\times$ time interaction, and calf was included as the random effect. Fisher's protected LSD test was applied when effects detected by the model were significant ($P < 0.05$). Delayed-type

hypersensitivity data were analyzed as a split-plot with factorial ANOVA. Colostrum treatment and calf were included in the model as fixed and random effects, respectively. Fisher's protected LSD test was applied when effects detected by the model were significant ($P < 0.05$).

RESULTS

Serum Immunoglobulin and Haptoglobin Concentrations

Immunoglobulin concentrations in sera collected weekly from CF and CD calves are shown in Figure 1. Immunoglobulin G₁ (panel a), IgG₂ (panel b), IgA (panel c), and IgM (panel d) were not detectable in CD calves at wk 0; however, all isotypes were detectable in CF calves at wk 0, with IgG₁ predominating. At wk 0, IgG₁, IgG₂, IgA, and IgM concentrations in CF calves were 9.63, 1.12, 2.23, and 1.01 mg/mL, respectively. In CD calves, IgG₁ concentrations increased ($P < 0.05$) progressively during the study and were comparable to those in CF calves by wk 7. Concentrations of IgG₂ in CD calves also increased ($P < 0.05$) with age and were comparable to concentrations in CF calves from wk 4 to 7. Immunoglobulin A and IgM concentrations in CF calves decreased ($P < 0.05$) progressively from their maximal concentrations at wk 0 to ≤ 0.10 mg/mL from wk 4 to 7. In CD calves, IgA and IgM concentrations remained low throughout the 7-wk period, although IgM concentrations were marginally higher ($P < 0.05$) from wk 2 through wk 7 than at wk 0.

Effects of colostrum deprivation on serum haptoglobin concentrations are presented in Table 2. Colostrum-deprived calves had markedly higher haptoglobin concentrations than CF calves from wk 1 through wk 5; however, by wk 6 and 7, haptoglobin concentrations had decreased and were comparable to those in CF calves.

Antigen-Specific Responses to Early Vaccination with OVA and BCG

Antibody Responses to Early Vaccination. Antibody responses elicited by vaccination with BCG

Table 2. Mean ($\pm$ SEM) serum haptoglobin concentrations (μ g/dL) in colostrum-fed (n = 7) and colostrum-deprived (n = 9) calves

Treatment	Age, ¹ wk						
	0	1	2	3	5	6	7
Colostrum-fed	0.27 (0.05)	0.10 (0.03)	0.04 (0.02)	0.04 (0.01)	0.05 (0.01)	0.03 (0.01)	0.10 (0.04)
Colostrum-deprived	5.17 (4.28)	28.9 (10.2)***	13.19 (6.90)*	9.04 (4.55)*	6.77 (2.79)*	0.18 (0.06)	0.18 (0.07)

¹Average age at wk 0 sampling time was 1.9 d (± 0.19 d); average age at wk 7 sampling time was 51.1 d (± 0.2 d).

* $P < 0.05$; *** $P < 0.001$: treatment means that differed at a specific time.

and OVA at wk 0 were evaluated using ELISA (Figure 2) and immunoblot (Figures 3 and 4). Mycobacterial (WCS1315)-specific IgG₁ levels in serum from CF and CD calves are shown in Figure 2a. At the time of vaccination (wk 0), mycobacteria-specific IgG₁ was detected in CF calves (S/P ratio: 0.653) and was undetectable in CD calves. During the weeks following vaccination, mycobacteria-specific IgG₁ levels in CF calves decreased progressively and by wk 7 were lower ($P < 0.05$) than levels in sera collected at the time of vaccination. In contrast, mycobacteria-specific IgG₁ levels in CD calves at wk 4 and 7 increased ($P < 0.01$) relative to wk 0 values and at wk 7 were higher than at wk 0 and wk 4 and were comparable to levels in CF calves. At the time of calving, mycobacteria-specific IgG₁ concentrations in sera from the BCG-vaccinated dams averaged 0.626 (SEM $\pm$ 0.081).

Ovalbumin-specific IgG₁ levels in CF and CD calves are shown in Figure 2b. All calves had low but measurable serum OVA-specific IgG₁ at the time of vaccination (wk 0). Although levels in CD calves were numerically lower than levels in CF calves at this time, these values were not different. Ovalbumin-specific IgG₁ responses of CF and CD calves increased ($P < 0.0001$) between wk 0 and wk 4 and again between wk 4 and wk 7 ($P < 0.0001$). Responses of CF and CD calves were not different at wk 4 and 7. Ovalbumin-specific IgG₁ was undetectable in maternal sera at parturition (data not shown).

Immunoblot responses to WCS1315 (Figure 3) and OVA (Figure 4) by 3 representative calves demonstrate Ab responses to BCG and OVA vaccination. At parturition, all the dams responded to mycobacteria (Figure 3) in immunoblot assays (data not shown). Mycobacte-

Figure 1. Mean ($\pm$ SEM) IgG₁ (a), IgG₂ (b), IgA (c), and IgM (d) concentrations (mg/mL) in serum from colostrum-fed ($n = 7$) and colostrum-deprived ($n = 9$) preruminant calves. Calves were bled weekly from wk 0 (pre-vaccination) to wk 7. Individual immunoglobulin isotypes were quantified by capture ELISA using bovine-specific reagents. Treatment means that differed at a specific time are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 2. Mycobacteria (a) and ovalbumin (OVA, b) specific IgG₁ levels in serum collected at wk 0 (pre-vaccination), wk 4, and wk 7 from colostrum-fed ($n = 7$) and colostrum-deprived calves ($n = 9$) vaccinated with OVA and *Mycobacterium bovis* strain bacille Calmette Guerin (BCG). All calves were vaccinated with antigens shortly after birth (mean age: 1.9 ± 0.2 d) and again with OVA at 37 d (± 0.2 d) of age. Mean ($\pm$ SEM) anti-OVA and anti-mycobacterium [capture antigen: lipoarabinomannan-enriched, proteinase digest of *M. bovis* strain 1315 (WCS-1315-LAM)] IgG₁ concentrations were expressed as sample-to-positive ratios (S/P) as defined in the Materials and Methods. Anti-OVA and anti-WCS-1315-LAM IgG₁ levels in maternal sera at calving were $0.013 (\pm 0.006)$ and $0.626 (\pm 0.081)$, respectively.

rial antibody (Figure 3b) was absent in CD calves at the time vaccination (i.e., wk 0), whereas all of the CF calves (Figure 3a) had measureable responses. Responses of CF calves to mycobacteria waned from 0 to 4 to 7 wk, with a few exceptions (e.g., response to 42-kDa antigen(s) by calf 1; Figure 3a). In contrast, responses by CD calves to WCS1315 either increased (e.g., calf 16 and 18; 7 of 9 calves) or were undetectable (e.g., calf 4; 2 of 9 calves) from 0 to 4 to 7 wk (Figure 3b). None of the CD or CF calf sera reacted to OVA at wk 0; however, responses were detectable in all calves by wk 7 and in 1 calf (i.e., calf 3) at wk 4 (Figure 4). Ovalbumin-specific Ab was not detectable in sera from dams (data not shown).

Antigen-Induced IFN- γ and NO Secretion. Interferon- γ responses of PBMC from CF and CD calves to both antigens (panels a and b) and PWM (panel c) are shown in Figure 5. Responses of cells from CF and CD calves to both antigens and PWM were not different ($P > 0.05$) at wk 0 or wk 7. Ovalbumin-, mycobacteria (PPDb)-, and PWM-induced IFN- γ responses of all calves were greater at wk 7 than at wk 0

(OVA: $P < 0.0001$, mycobacteria: $P = 0.02$, PWM; $P < 0.0001$). At wk 7, IFN- γ responses of all calves to PWM exceeded ($P < 0.0001$) their responses to either Ag.

Nitric oxide responses by PBMC from CF and CD calves to both antigens (panels a and b) and PWM (panel c) are shown in Figure 6. Responses of cells from CF and CD calves to both antigens and PWM were not different at wk 0 or wk 7. Mitogen-induced responses by PBMC from all calves at wk 0 were comparable to responses at wk 7; however, OVA and mycobacteria responses were greater ($P < 0.01$) at wk 7 than responses at wk 0. At wk 0, PWM-induced responses of all calves exceeded their responses to either OVA ($P < 0.001$) or mycobacteria ($P = 0.002$). Responses of all calves to PWM and mycobacteria were not different at wk 7 ($P = 0.07$); however, OVA-elicited responses were weaker than responses to PWM ($P = 0.01$).

Antigen-Induced Lymphocyte Subset Proliferation. Because initial analyses of lymphocyte proliferation data indicated that treatment effects and treatment $\times$ time interactions were not significant for CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, and IgM⁺ lymphocyte subsets,

a. Colostrum-fed calves **b. Colostrum-deprived calves**

Figure 3. Immunoblots of a whole-cell sonicate of *Mycobacterium bovis* strain 1315 (WCS1315) probed with sera from 3 representative colostrum-fed (a) or colostrum-deprived (b) calves. Molecular mass (kDa) markers are indicated in the left margin and weeks relative to birth immediately above the blots. Responses by dams are provided immediately before the series of blots for their respective calves. Serum from dams was collected several weeks before calving (first lane) and at parturition (second lane).

proliferative responses of lymphocytes from all calves at wk 0 and at wk 7 were pooled before analysis. These data are shown in Figures 7 and 8.

Responses of CD4⁺ cells (Figure 7a) to PWM at wk 0 were not different from corresponding responses at

wk 7; however, percentages decreased ($P < 0.0001$) with increasing generation (i.e., number of cell divisions) at both wk 0 and wk 7. Responses of this subset to both antigens (Figure 7b and Figure 7c) differed markedly from responses to PWM, a possible conse-

a. Colostrum-fed calves **b. Colostrum-deprived calves**

Figure 4. Immunoblots of ovalbumin (OVA) probed with sera from 3 representative colostrum-fed (a) and colostrum-deprived (b) calves collected at wk 0 (prevaccination), wk 4, and wk 7. Calves were vaccinated with OVA and *Mycobacterium bovis* strain bacille Calmette Guérin (BCG) at 1.9 d (± 0.2 d) of age and again with OVA at 37 d (± 0.2 d) of age.

quence of the effects of early vaccination on subsequent antigen-specific reactivity of the $CD4^+$ cell population. Unlike the responses of PWM-stimulated cells, percentages of antigen-stimulated $CD4^+$ cells in generations 3 to 5 were very low at the time of vaccination (wk 0). Percentages of mycobacteria- and OVA-stimulated cells in generations 3, 4, and 5 were higher at wk 7 than percentages in the same generations at the time of vaccination (wk 0).

Responses of $CD8^+$ cells (Figure 7d) to PWM were higher at wk 7 than at wk 0. In particular, percentages of cells in generations 1 and 3 at wk 7 were greater than the corresponding percentages at wk 0. Antigen-induced responses of this population at wk 0 and wk 7 (Figure 7e and Figure 7f) followed a pattern similar to that observed for antigen-stimulated $CD4^+$ cells. Percentages of cells in generations 2 through 5 were negligible at the time of vaccination (wk 0) and by wk 7 were detectable and greater than at wk 0. Interestingly, percentages of antigen-induced $CD8^+$ cells in generations 3 through 5 at wk 7 were lower ($P < 0.05$) than the corresponding percentages of antigen-stimulated $CD4^+$ cells.

Responses of $\gamma\delta TCR^+$ cells (Figure 8a) to PWM were greater at wk 7 than at wk 0. This difference is attributable to the significantly higher percentage in

generation 1 at wk 7 than at wk 0. Of the lymphocyte subsets examined, the $\gamma\delta TCR^+$ cells had the weakest responses to antigenic stimulation (Figure 8b and Figure 8c). Although these responses were of low magnitude, the percentage of PPD_b-stimulated $\gamma\delta TCR^+$ cells in generation 3 at wk 7 was higher ($P < 0.05$) than at the time of vaccination (wk 0). For OVA-stimulated cells, percentages in generation 3 and 5 were higher ($P < 0.05$) at wk 7 than at wk 0.

Responses of IgM^+ cells to PWM (Figure 8d) at wk 0 were not different from corresponding responses at wk 7; however, percentages of cells in generations 2 through 5 decreased ($P < 0.01$) progressively at both sampling times with the highest percentages in generation 2. Responses to PPD_b and OVA are shown in Figures 8e and 8f. Percentages of antigen (OVA and mycobacteria)-stimulated cells in generations 2 through 5 were higher ($P < 0.05$) at wk 7 than the corresponding percentages at the time of vaccination (wk 0). For all lymphocyte subsets, responses to PWM appeared to be limited to generations 1 through 3. In contrast, responses to both antigens were minimal at the time of vaccination, especially in generations 3 through 5; however, by wk 7 responses were most pronounced in these generations.

Comparative Skin-Fold Test Results. Results from the comparative cervical skin-fold test performed 8 wk after BCG vaccination indicate that colostrum deprivation did not influence *in vivo*, antigen-specific recall responses (data not shown). Briefly, PPD_b-induced responses (i.e., skin-fold thickness (mm) as defined in Materials and Methods) of CF and CD calves at 24 h (5.3 vs. 5.6) and 72 h (8.4 vs. 9.3) were not different. Similarly, responses of CF and CD calves to PPD_a were not different at 24 or 72 h. Responses of all calves to PPD_a were of lower magnitude ($P < 0.05$) than their responses to PPD_b induced at 24 h (3.3 vs. 5.5) and 72 h (5.1 vs. 8.9).

Composition of Blood Leukocyte Populations

Total numbers of circulating leukocytes in CF and CD calves from wk 0 to wk 7 are shown in Figure 9a. Leukocyte numbers in CF and CD calves were not different at any of the weekly time points. Leukocyte numbers in CF and CD calves during the 8-wk period averaged $13.2 (\pm 0.59)$ and $15.1 (\pm 0.71) \times 10^6/\text{mL}$, respectively, and were not different. When all calves were considered, weekly changes in leukocyte numbers were not significant.

Absolute numbers of granulocytes, lymphocytes, and monocytes in the circulation of CF and CD calves are shown in Figure 9b. At wk 0, CD calves had substantially higher numbers of granulocytes than CF calves.

Figure 5. Mean ($\pm$ SEM) IFN- γ concentrations in supernatants from blood mononuclear cell cultures stimulated with ovalbumin (OVA, a), *Mycobacterium bovis* purified protein derivative (PPDb, b), and pokeweed mitogen (PWM, c). Cells were harvested at wk 0 (prevaccination) and at 7 wk postvaccination. Calves were vaccinated with OVA and *M. bovis* strain bacille Calmette Guérin (BCG) at 1.9 d ($\pm$ 0.2 d) of age and again with OVA at 37 d ($\pm$ 0.2 d) of age. Responses of nonstimulated cultures were subtracted from responses by parallel, stimulated cultures before data were analyzed. For each type of stimulation, responses of colostrum-fed and colostrum-deprived calves at wk 7 were greater ($P < 0.05$) than their responses at wk 0.

Figure 6. Mean ($\pm$ SEM) nitrite concentrations in supernatants from blood mononuclear cell cultures stimulated with ovalbumin (OVA, a), *Mycobacterium bovis* purified protein derivative (PPDb, b), and pokeweed mitogen (PWM, c). Cells were harvested at wk 0 (prevaccination) and at 7 wk postvaccination. Calves were vaccinated with OVA and *M. bovis* strain bacille Calmette Guérin (BCG) 1.9 d ($\pm$ 0.2 d) of age and again with OVA at 37 d ($\pm$ 0.2 d) of age. Responses of nonstimulated cultures were subtracted from responses by parallel, stimulated cultures before data were analyzed.

Figure 7. Mean ($\pm$ SEM) proliferative responses of CD4⁺ and CD8⁺ T cells from all calves ($n = 16$) to pokeweed mitogen (PWM), purified protein derivative (PPDb), and ovalbumin (OVA). Blood leukocytes were harvested before vaccination (wk 0) and at 7 wk of age. All calves were vaccinated with both antigens at 1.9 d (± 0.2 d) of age and again with OVA at 37 d (± 0.2 d) of age. Data represent percentages of CD4⁺ and CD8⁺ cells in generations 1 through 5 at the time of vaccination (wk 0) and at 7 wk of age. Before analysis, responses of non-stimulated cultures were subtracted from responses of parallel, stimulated cultures. For a specific subset, wk 0 and wk 7 percentages that differed are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Treatment effects and treatment $\times$ time interactions were not significant ($P > 0.05$, data not shown) for either cell type.

Granulocyte numbers at wk 7 and lymphocyte and monocytes numbers at wk 0 and wk 7 were unaffected by colostrum deprivation. For all calves, granulocyte numbers at wk 7 were lower ($P < 0.001$) than at wk 0. Numbers of lymphocytes and monocytes, in contrast, were higher ($P < 0.05$) at wk 7 than at wk 0.

Percentages of CD8⁺, CD4⁺, $\gamma\delta$ TCR⁺, NK⁺, and IgM⁺ cells in gated lymphocyte populations from CF

and CD calves are presented in Table 3. Of the lymphocyte subsets examined, only NK⁺ cell percentages were affected by colostrum deprivation. The CD calves had higher NK cell percentages than CF calves at wk 0 ($P = 0.04$) and at wk 7 their NK cell percentages tended ($P = 0.09$) to be higher than those in CF calves. Although NK cell percentages in CD calves were higher ($P < 0.05$) at wk 7 than at wk 0, those in CF calves at

Figure 8. Mean ($\pm$ SEM) proliferative responses of $\gamma\delta\text{TCR}^+$ and IgM^+ cells from all calves ($n = 16$) to pokeweed mitogen (PWM), purified protein derivative (PPDb), and ovalbumin (OVA). Blood leukocytes were harvested from all calves before vaccination (wk 0) and at 7 wk of age. Data represent percentages of $\gamma\delta\text{TCR}^+$ and B cells in generations 1 through 5 before vaccination (wk 0) and at 7 wk of age. All calves were vaccinated with both antigens 1.9 d (± 0.2 d) of age and again with OVA at 37 d (± 0.2 d) of age. Responses of nonstimulated cultures were subtracted from responses by parallel, stimulated cultures before data were analyzed. For a specific subset, wk 0 and wk 7 percentages that differed are indicated by * $P < 0.05$ and ** $P < 0.01$. Treatment effect and treatment $\times$ time interactions were not significant ($P > 0.05$, data not shown) for either cell type.

wk 0 and wk 7 were not different. Treatment effects, time effects, and treatment $\times$ time interactions were not significant for CD8^+ , CD4^+ , $\gamma\delta\text{TCR}^+$, and IgM^+ percentages.

DISCUSSION

Ingestion of colostrum within the first hours after birth provides the dairy calf with not only a complex mixture of nutrients, endocrine factors, trace minerals, and growth factors essential for gastrointestinal devel-

opment and function (Blum, 2006) but also immune-related factors (i.e., immunoglobulin, viable leukocytes, and cytokines) that afford protection against bacterial and viral infections during the first months of life. Colostral antibody, representing the antigenic experience of the dam, may also influence antibody responses of the calf to early vaccination (Husband and Lascelles, 1975; Endsley et al., 2003; Chase et al., 2008). Absorption of colostral immunoglobulin by the agammaglobulinemic newborn calf is considered adequate when

Figure 9. Mean ($\pm$ SEM) numbers of leukocytes in the circulation of colostrum-fed ($n = 7$) and colostrum-deprived ($n = 9$) calves (a), and numbers of granulocytes, lymphocytes, and monocytes comprising the blood leukocyte population (b). Total numbers of circulating leukocytes were determined weekly, and the composition of these populations was determined at wk 0 and at wk 7. Granulocyte, lymphocyte, and monocyte percentages were determined by flow cytometry and converted to actual numbers based on total numbers of circulating leukocytes. For a specific leukocyte subpopulation (b), treatment means that differed at a specific time are indicated by $*P < 0.05$.

serum IgG concentrations, consisting predominantly of IgG₁, exceed 10 mg/mL (McGuirk and Collins, 2004). This threshold concentration is associated with reduced mortality rates due to respiratory diseases and enteritis (Besser and Gay, 1994). Other immunoglobulin classes

(i.e., IgM and IgA) present in colostrum and absorbed into the circulation of the calf may also exert protective effects in the intestine and other tissues.

Results from the present study confirm the immediate positive effects of colostrum feeding not only on

Table 3. Mean ($\pm$ SEM) percentages of CD8⁺, CD4⁺, and $\gamma\delta$ TCR⁺ T cells; B cells (IgM⁺); and natural killer cells (NK) in blood lymphocyte populations harvested from colostrum-fed (n = 7) and colostrum-deprived (n = 9) calves at wk 0 and 7

Cell phenotype	Treatment	Wk 0 ¹	Wk 7 ¹
CD8 ⁺ T cell	Colostrum-fed	19.7 (3.0)	15.0 (1.1)
	Colostrum-deprived	15.7 (1.5)	15.0 (0.7)
CD4 ⁺ T cell	Colostrum-fed	15.4 (2.9)	16.0 (1.0)
	Colostrum-deprived	19.7 (1.9)	15.9 (1.5)
$\gamma\delta$ TCR ⁺ T cell	Colostrum-fed	34.6 (5.1)	36.7 (3.8)
	Colostrum-deprived	38.7 (1.8)	32.0 (2.7)
NK ⁺ cell	Colostrum-fed	4.3 (1.4)	11.5 (5.5)
	Colostrum-deprived	12.4 (2.9)*	25.9 (5.5)*
IgM ⁺ B cell	Colostrum-fed	6.9 (1.6)	9.3 (1.8)
	Colostrum-deprived	8.5 (1.3)	9.0 (2.1)

¹Average age at wk 0 was 1.9 d ($\pm$ 0.19 d) and at wk 7 was 51.1 d ($\pm$ 0.2 d).

* $P < 0.05$; treatment means that differed at specific time.

serum IgG₁ concentrations but also on IgG₂, IgA, and IgM concentrations. Not surprisingly, IgG₁ was the predominant immunoglobulin class in the circulation of CF calves throughout the study. Serum IgG₂, IgM, and IgA decreased with age and by the conclusion of the study, with the exception of IgM, were similar to concentrations in the circulation of adults (Butler, 1974). Although IgG₁, IgG₂, IgA, and IgM were not detected in the circulation of CD calves at wk 0, a pronounced increase was observed in the concentrations of IgG₁ and IgG₂, and to a lesser extent IgM, during the study, suggesting that immunoglobulins are produced endogenously in the preruminant calf, as reported previously (Husband et al., 1972). Previous research suggests that IgG₁ and IgG₂ do not reach appreciable concentrations in the CD calf until 16 to 32 d after birth and adult levels until 4 mo of age (Husband and Lascelles, 1975). Interestingly, IgG₂ concentrations in CD calves in the present study achieved adult concentrations (≥ 8 mg/mL, Butler, 1974) at approximately 1 mo of age.

In the present study, serum haptoglobin concentrations were assayed to provide an indirect method for estimating the effects of colostrum deprivation on calf health. Haptoglobin is one of several acute phase proteins produced in response to inflammatory changes associated with infection and, like serum amyloid A and C-reactive protein, has gained acceptance as a marker of systemic inflammation (Petersen et al., 2004). Haptoglobin concentrations are very low to undetectable in healthy calves and greater than 1 mg/mL during experimentally induced respiratory disease (Godson et al., 1996). The study also demonstrated strong correlations between haptoglobin concentrations and sick score (subjective clinical examination), body temperature, weight change, and plasma zinc concentration. In the present study, CD calves had markedly higher serum haptoglobin concentrations from wk 1 through wk 5 compared with CF calves. The sustained elevation

in haptoglobin concentrations in CD calves but not CF calves suggests that colostrum ingestion within the first hours after birth protects against inflammatory states associated with infections typically affecting the preruminant calf. Although health data were not recorded, the attending veterinarian's observations suggested CD calves were not as healthy as CF calves during the early weeks of the study.

Although transfer of maternal Ab associated with the ingestion of colostrum affords the newborn calf short-term protection against a wide range of potential pathogens, these antibodies influence the development of active (i.e., adaptive) immunity in the calf vaccinated at an early age. This is reflected by the fact that Ab responses of the colostrum-fed calves to early vaccination frequently are impaired relative to responses in older vaccinated animals or to the responses of the fetus exposed to antigen in utero (Husband and Lascelles, 1975; Hein et al., 1988; Endsley et al., 2003; Nonnecke et al., 2005). In the present study, effects of colostrum ingestion on serum Ab responses of CF and CD calves sensitized to BCG and OVA were evaluated using ELISA and immunoblot assays. To mimic the consequences of the dam's natural antigenic exposure, all dams were vaccinated with BCG within 2 mo of parturition. Results from both assays indicated anti-mycobacterial Ab in the circulation of BCG vaccinated dams at the time of calving. Anti-mycobacterial Ab was also detected in the circulation of CF calves but not in CD calves, suggesting that colostral anti-mycobacterial Ab was absorbed into the circulation of CF calves. Because all ungulates are born agammaglobulinemic and derive maternal immunoglobulins solely from the ingestion of colostrum (Butler, 1974), the absence of anti-mycobacterial Ab in CD calves born to BCG vaccinated dams was expected. Calves also were sensitized to OVA, a benign antigen not found in the natural environment of the dairy cow. Because of these char-

acteristics, OVA-specific Ab was not detectable or at extremely low levels in the circulation of the dams and calves at the time at parturition (dams) and before sensitization to OVA (calves), providing an opportunity to examine the Ab responses of calves not influenced by maternal Ab.

Historically, the role of antibody in determining the duration and severity of tuberculosis has been considered negligible. Several vaccine studies with cattle have demonstrated that T-cell central memory responses (Whelan et al., 2008) correlate negatively with mycobacterial burden (Waters et al., 2009) and tuberculosis-associated pathology (Vordermeier et al., 2009). As with T-cell central memory responses, IL-17 responses (as measured by real time reverse transcription-PCR) also appear to be associated with protection (Vordermeier et al., 2009). Antibody responses generally correlate positively to mycobacterial-elicited pathology (Lyashchenko et al., 2004) elicited by infection by *M. bovis*; however, antibody elicited by BCG vaccination has not been correlated to vaccine efficacy. In contrast, research by Glatman-Freedman (2006) suggests that mycobacteria-specific antibodies can modify various aspects of the infection process during tuberculosis, ultimately benefiting the host. Results from both ELISA and immunoblot assays indicate that colostrum ingestion influences Ab responses of BCG-vaccinated calves. Serum anti-mycobacterial Ab in CF calves, present at relatively high levels at wk 0, declined progressively during the study, indicating that endogenous Ab synthesis was not elicited in CF calves following BCG vaccination at wk 0. The decline may reflect the half-life (11–16 d) of colostrum-derived Ab in the neonate (Husband et al., 1972; Sasaki et al., 1976). In a related study (Nonnecke et al., 2005), preruminant calves vaccinated at 7 d postpartum with BCG failed to mount a measurable Ab response, whereas BCG-vaccinated steers had a pronounced Ab response (characterized by Ab in serum and in supernatants from antigen-stimulated PBMC cultures). Although serum Ab was absent in CD calves at wk 0, it was detected at wk 4 and 7 after vaccination, with the highest level occurring at wk 7, suggesting that the calf can produce an Ab to early vaccination when the inhibitory effects of colostrum, possibly associated with maternal Ab recognizing vaccinal antigens, are absent.

Results from ELISA and immunoblot assays indicated that Ab responses of CF and CD calves to OVA sensitization were comparable throughout the study, with very low amounts of Ab present at the time of vaccination (wk 0) followed by progressive increase in anti-OVA Ab in both groups during the postvaccination period. Because OVA-specific Ab were not detected in the maternal serum at the time of parturition and CF

calves Ab responses were robust, these results suggest that when maternal Ab are absent in colostrum the newborn calf can generate a vigorous humoral response to a protein antigen. This contention is supported by previous research (Husband and Lascelles, 1975) demonstrating that the neonate possesses the capacity to produce Ab to antigens not present in the natural environment of dairy cattle. Second, these data suggest that failure of the calf to mount measurable Ab responses to specific antigens is not necessarily due to immunological immaturity during the first weeks of life but rather due to the nature of the antigen and whether the dam's immunological experience in the form of colostrum Ab is exported to the newborn calf within hours after birth.

Previous studies suggest that other aspects of the adaptive immune response are not compromised to the same degree by colostrum ingestion as endogenous Ab synthesis. Endsley et al. (2003) evaluated the effects of maternal Ab on humoral and T cell responses of calves with high levels to BVD virus (BVDV). Results indicate that calves with high levels of Ab to BVDV 1 and BVDV 2 and challenged with virulent BVDV at 2 to 5 wk of age develop pronounced BVDV-specific CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ T-cell responses to challenge. Vaccination with a modified-live BVDV vaccine when high levels of maternal Ab are present induces antigen-specific T-cell responses and protection against experimental infection. Primates with high levels of pre-existing antibodies have minimal responses to active immunization with an attenuated respiratory syncytial virus vaccine; however, when subsequently challenged with a wild strain of respiratory syncytial virus they had enhanced antibody responses that exceeded those of animals vaccinated without pre-existing passively acquired antibody (Crowe, 2001). Taken together, these results suggest that vaccinating neonates with measurable amounts of circulating maternal Ab can induce memory T and B cells that may influence host responses to subsequent challenge.

With regard to the responses of newborn calves to BCG vaccination, Buddle et al. (2003) demonstrated that colostrum-fed calves vaccinated with BCG within 8 h of birth (when cellular immune responses to antigens of environmental mycobacteria are not detectable) had vigorous in vitro antigen-specific IFN- γ and IL-2 responses, antigen-responsive CD4⁺, CD8⁺ and $\gamma\delta$ TCR⁺ T cell populations, and a high level of immunity when challenged with virulent *M. bovis* at 14 to 17 wk of age. In a related study (Nonnecke et al., 2005), BCG vaccination of colostrum-fed newborn dairy calves elicited adult-like responses in ex vivo assays evaluating antigen-induced T-cell subset proliferation and antigen-induced IFN- γ , NO, and tumor necrosis factor- α secretion. In addition, cutaneous responses of

vaccinated calves and adults to intradermal injection of PPDb were pronounced and comparable, demonstrating the capacity of the young calf to develop a robust cell-mediated immune response.

Results confirm that the colostrum-fed preruminant calf is capable of generating robust cell-mediated immune responses to early vaccination. Responses of CF and CD calves to vaccination with BCG and OVA at <2 d of age were estimated by considering the effects of antigen- and mitogen-induced stimulation on cell functions associated with cell-mediated immunity (i.e., IFN- γ and NO secretion) and on the proliferation of peripheral blood T-cell subsets and B cells. The T/NK cell cytokine, IFN- γ , is produced during tuberculosis and is required for a protective response to this pathogen (Flynn and Chan, 2001). It plays a pivotal role in the activation of macrophages resulting in production of NO, a major contributor to the enhanced antimicrobial activity of the macrophage during active infections.

In the present study, Ag-specific IFN- γ and NO responses were evaluated at the time of vaccination and at 7 wk postvaccination. The magnitude of PPDb- and OVA-induced IFN- γ and NO responses of CF and CD calves were of similar magnitude at the time of vaccination and 7 wk later, suggesting that Ag-induced IFN- γ and NO responses of vaccinated calves were not influenced by colostrum ingestion or deprivation. The markedly higher Ag-elicited IFN- γ and NO responses of cells from both CF and CD at wk 7 relative to levels produced at wk 0 provide functional indication of a memory T-cell response generated by early vaccination. Previous studies (Rajaraman et al., 1997, 1998) indicate that stimulated IFN- γ and NO responses of PBMC from newborn calves are not as vigorous as those of adult PBMC, possibly due to the immunological immaturity of the newborn calf. Responses to PWM, a polyclonal stimulator of bovine lymphocyte function (Franklin et al., 1994), were also evaluated in both assays and served as a positive control for PBMC activity in these assays and the subset proliferation assays discussed below. Responses to PWM, lower at wk 0 than wk 7, support the contention that functional capacities of PBMC may be less developed in newborn calf.

Antigen (OVA and PPDA)-induced proliferation of CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells as well as IgM⁺ cells was comparable in CF and CD calves at the time of vaccination and 7 wk after vaccination. Vaccine reactivity of these populations has been demonstrated in vaccinated, colostrum-fed newborn calves even when evidence of antibody production is absent (Endsley et al., 2003; Nonnecke et al., 2005; Foote et al., 2007). When considering all calves, responses to both antigens were more vigorous at 7 wk postvaccination than at the time of vaccination. Responses of T subsets and B cells

to both PPDb and OVA were characterized by higher percentages of cells in generation 2 or higher at wk 7 than at wk 0, likely indicative of the development of an Ag-specific memory response to early vaccination. Evidence of priming of the lymphocyte population in the newborn calf following early vaccination may provide a basis for appropriate (i.e., protective) future responses. This contention is supported by a recent study (Waters et al., 2009) indicating that the development of memory T-cell responses because of vaccination of the newborn with BCG and newer TB vaccines (i.e., *M. bovis* Δ RD1 vaccine) is associated with reduced pathology or disease severity associated with subsequent experimental challenge. Buddle et al. (2003) also demonstrated that BCG vaccination of calves within 8 h after birth was associated with activation of CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ T cells and high degree of protection against development of lung pathology induced by challenge with virulent *M. bovis* at 6 wk of age. The B-cell (IgM⁺) populations in all calves were essentially unresponsive to antigen stimulation at the time of vaccination with very low percentages of cells in generations 2 through 5. By 7 wk postvaccination, responses to both antigens were characterized by marked increases in percentages in these generations, indicating that vaccination of the newborn calf promotes antigen-specific reactivity of this lymphocyte population. In a related study, Foote et al. (2007) showed increased antigen-induced expression of activation antigens on B cells from vaccinated preruminant calves. Although not evaluated in the present study, it would be informative to determine if enhanced antigen-specific B-cell responses postvaccination are associated with increased Ab production elicited by virulent *M. bovis* in the absence of maternal Ab.

Cutaneous delayed-type hypersensitivity responses of CD and CF calves to intradermal injection of PPDA or PPDb were comparable when evaluated 8 wk after vaccination. The more robust response of both CF and CD calves to PPDb versus PPDA was indicative of the development of T cell-mediated immune response by both groups to early BCG vaccination. As expected, in vivo reactivity to intradermal administration of antigen was associated with ex vivo antigen-induced proliferation of T-cell subsets harvested at approximately the same time. Previous research suggests that preruminant colostrum-fed calves vaccinated at an early age with BCG can develop adult-like antigen-specific responses in the cervical skin-fold test (Nonnecke et al., 2005).

Total numbers and types of cells comprising blood leukocyte populations in newborn calves frequently differ from blood leukocyte populations in adult cattle and these differences may contribute to the calf's increased susceptibility to diarrheal and respiratory infections (Chase et al., 2008). In the present study, total

numbers of leukocytes were unaffected by treatments or age; however, granulocyte numbers were higher in all calves at wk 0 with substantially higher numbers in newborn CD calves. Elevated numbers of granulocytes in the circulation of newborn CF and CD calves have been previously reported (Clover and Zarkower, 1980; Menge et al., 1999). Although monocyte numbers were unaffected by treatments, numbers were substantially lower in all calves shortly after birth than at 7 wk of age. Because monocytes contribute to innate immunity and are also Ag-presenting cells, increased numbers in older calves may indicate immune maturation in the preruminant calf. Total numbers of lymphocytes and percentages of CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, and B cells comprising the lymphocyte population were unaffected by treatment or age. These results differ from those of previous studies showing typical age-related changes in the percentages of CD8⁺ cells (increase), $\gamma\delta$ TCR⁺ cells (decrease), and B cell (increase) (Nonnecke et al., 2003). The relatively high degree of variation associated with data in the present study may have masked potential age-related differences in T-cell percentages. In both CF and CD calves, NK⁺ cell percentages were higher at wk 7 than at wk 0. Interestingly, NK⁺ cell percentages in CD calves were higher than in CF calves at both time points. Previous research suggests that the bovine neonate, like the human neonate, has a higher percentage of circulating NK cells compared with adults (Kulberg et al., 2004). Bovine NK cells can reduce numbers of *M. bovis* BCG in infected alveolar macrophages or macrophages derived from blood monocytes likely by their release of cytotoxic molecules (i.e., perforin and granulysin; Endsley et al., 2006). A subset of NK cells from the bovine neonate secretes significant quantities of IFN- γ when exposed to BCG-infected Ag-presenting cells (Hope et al., 2002). Based on these studies, high NK⁺ cell percentages in the newborn calves may influence immune response capacity during a period of increased risk of infection.

CONCLUSIONS

Colostrum, the only source of immunoglobulin for the newborn ruminant, provides an important and essential mechanism for transferring the immunological experience of the dam to the newborn calf. In the present study, effects of colostrum deprivation on functional aspects of the immune system of the preruminant calf, specifically those associated with development of the adaptive response to vaccination, were evaluated. Colostrum-fed and colostrum-deprived calves were vaccinated with 2 different antigens at less than 2 d of age to track the development of the adaptive immune response during the first 2 mo of life. Effects of

colostrum ingestion on the composition and functional capacities of blood-derived immune cell populations were evaluated at the time of vaccination and during the first 7 wk following vaccination. Results indicate that colostrum has the potential to inhibit endogenous antibody responses of the preruminant calf to early vaccination with little or no effect on cell-mediated responses. Although endogenous Ab responses were inhibited by colostrum, certain B-cell activities were retained. Results also suggest endogenous Ab response to early vaccination is likely antigen-dependent.

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