Effects of age and plane of nutrition on immune responsiveness of neonatal dairy calves

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Effects of age and plane of nutrition on immune responsiveness of neonatal dairy calves

by

Monica Ruth Foote

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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ABSTRACT

The neonatal calf has a heightened susceptibility to infectious disease. Traditional calf-rearing programs limit nutrient intake from milk or milk replacer during the first few weeks of life to promote dry-feed intake and early weaning. Dramatic improvements in growth performance achieved by feeding increased amounts of milk replacer with more protein is hypothesized to be associated with enhanced immune function and an increased resistance of the calf to infectious disease. Objectives of this dissertation were to characterize more extensively immune responses of the neonatal calf and to determine if intensified nutrition enhances immune function of milk replacer-fed calves. T-cell subsets from 1-wk-old calves showed decreased proliferative activity, a delayed increase in CD25 expression, and no demonstrable increase in CD44 expression or decrease in CD62L expression when compared with the mitogen-induced responsiveness of cells from steers. Mitogen-induced proliferation and expression of activation antigens by T cells from 8-wk-old, standard-fed calves, however, were similar to responses of cells from steers, indicating that T cell function during the neonatal period matures rapidly. Intensified nutrition during the neonatal period was associated with alterations in the responsiveness of T cell subsets to mitogenic stimulation, characterized by decreased proliferation, decreased expression of activation antigens (i.e., CD25 and CD44), decreased interferon-γ secretion, and increased nitric oxide production. These results suggest that animal maturity and neonatal nutrition influence functional activities of T lymphocyte subsets essential in the development of cell-mediated immunity. Feeding a milk replacer (30% crude protein/20% fat) at different rates to achieve no growth, low growth, and high growth did not affect antigen-specific recall responses of cells from vaccinated calves. These results suggest protein-energy malnutrition in the absence of weight loss does not affect adaptive immune responses of calves. Cells from high-growth rate calves had increased nitric oxide production and decreased viability. These alterations in cell function may have deleterious effects on resistance to infectious disease.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Neonatal animals are highly susceptible to bacterial and viral pathogens. This susceptibility increases the incidence of disease, leading to morbidity or mortality. The mortality rates of neonatal calves decline rapidly with age. The National Animal Health Monitoring Service (NAHMS) reported mortality of dairy heifers in the United States from birth to weaning to be 8.4% in 1991, 11% in 1995, and a range of 7.7% to 9.4% in 2002 that was dependent upon herd size (NAHMS, 2002). In a survey done in South Carolina, average mortality was measured at 19.1% for cattle up to six months of age, and 84% of the total mortality occurred within the first month (Jenny et al., 1981). The NAHMS also has reported that 34% of calves are reported as sickly or morbid, suggesting that over 40% of calves were considered to be ill prior to weaning. Roy (1990) made the observation that calf mortality has not declined since antibiotics have become available within the agricultural industry.

Traditional calf-rearing programs limit nutrient intake from milk or milk replacer during the first few weeks of life in order to promote dry feed (i.e., starter) intake and allow early weaning. Recent reports of the dramatic improvements in growth performance and feed efficiency by feeding greater amounts of milk replacer with higher protein concentrations has led to interest in intensified or accelerated feeding programs (Bartlett, 2001, Blome et al., 2003, Diaz et al., 2001, Tikofsky et al., 2001). It is believed that intensified or accelerated feeding programs increase the plane of nutrition to more “natural” levels and provide more “biologically appropriate” early growth (Drackley, 2005). Despite limited supportive information, it has been suggested that improving the plane of nutrition may similarly improve calf health and decrease morbidity and mortality (Drackley, 2005, Van Amburgh and Drackley, 2005). Thus, I have attempted to address the hypothesis that increasing plane of nutrition improves calf health by investigating the effects of plane of nutrition on immune
responses of the neonatal calf. The objectives of this dissertation were to describe immune responses of the neonatal calf and to examine the effects of neonatal nutrition on immune responses.

**Dissertation Organization**

This dissertation includes four manuscripts. Chapter 2 (Manuscript 1) was published in the Journal of Dairy Science and describes the effects of age and neonatal nutrition on immune responses to mitogen. Chapters 3 (Manuscript 2) and 4 (Manuscript 3) examine the effects of plane of nutrition on adaptive immune responses in vaccinated calves. Manuscript 2 has been accepted for publication in the International Journal of Vitamin and Nutrition Research, and Manuscript 3 will be submitted to the Journal of Nutrition. Chapter 5 (Manuscript 4) will be submitted to Veterinary Immunology and Immunopathology and describes neonatal calf immune responses to naturally occurring and non-naturally occurring antigen. A review of the literature precedes Manuscript 1, and general conclusion follows Manuscript 4. Articles cited in each chapter are included at the end of the respective chapters.

**Literature Review**

**Current perspectives on protein and energy requirements of the neonatal calf**

Rearing programs and nutritional management guidelines of the milk-fed calf have been described as static and nearly dogmatic despite the presence of available literature concerning the growth responses of calves to various dietary ingredients, amounts fed, environmental challenges, weaning age, rumen development, and infection (Van Amburgh and Drackley, 2005). Although Davis and Drackley (1998) summarized research elucidating the specific nutrient requirements of the young calf, it is evident that nutritional management of the dairy calf is driven by financial decisions rather than optimization of development (Van Amburgh and Drackley, 2005).
Although a huge step forward was made in collating knowledge of nutrient requirements of the young calf with the publishing of the Nutrient Requirements of Dairy Cattle, Seventh Ed. by the National Research Council (NRC, 2001), new data on calf growth and development have since been generated (Van Amburgh and Drackley, 2005). Data suggest that the composition of live-weight gain in the milk-fed calf, unlike the functioning ruminant, can be manipulated by diet, intake amount, and energy source (Bartlett, 2001, Bascom, 2003, Blome et al., 2003, Diaz et al., 2001, Donnelly, 1983, Donnelly and Hutton, 1976a, Donnelly and Hutton, 1976b, Tikofsky et al., 2001, Toullec, 1989). Recent work conducted by Diaz et al., (2001) highlights the inability of the existing nutrient requirement equations used in the United States (NRC, 1989, NRC, 1996, NRC, 2001) to predict the composition of gain in the milk-fed calf (Van Amburgh and Drackley, 2005). A new equation, developed by Van Amburgh and Drackley (2005), describes the composition of gain or retained energy of calves from birth to 105 kg of body weight.

Recent data support the concept that energy intake drives growth rate and protein requirements, and thus protein requirements vary depending on growth rate (Bartlett, 2001, Van Amburgh and Drackley, 2005). Increasing dietary protein decreases body fat deposition linearly while increasing protein deposition (Bartlett, 2001, Donnelly and Hutton, 1976a, Donnelly and Hutton, 1976b). Protein content of the diet needs to be approximately 280 g/kg dry matter (which is similar to the crude protein content of whole milk) in order to optimize protein accretion in the preweaned calf (Diaz et al., 2001, Van Amburgh and Drackley, 2005). Energy source also affects composition of gain. Increasing the fat to carbohydrate ratio in milk replacer increases body fat deposition (Tikofsky et al., 2001).

In addition to the early positive effects on growth, intensified early nutrition may benefit later performance as well. Although excess dietary energy during the period from 3 mo of age to puberty affects negatively mammary development and milk production (Sejrsen et al., 2000), other research indicates enhancing plane of nutrition earlier in life benefits subsequent milk production. In one
experiment, calves allowed to suckle nurse cows were compared with calves fed milk replacer [23% crude protein (CP), 18% fat] in restricted amounts. Suckled calves, consuming 14% more energy than milk-replacer-fed calves through 51 d of life, were 5 cm taller, calved 30 d earlier, and produced 453 kg more milk during the first lactation (Bar-Peled et al., 1997). In a related study, calves fed whole milk ad libitum produced 489 kg more milk during the first lactation compared with restricted-fed calves (Foldager et al., 1997). Although effects of early (prepubertal) mammary development on subsequent milk production have not been delineated, research indicates a positive effect of enhanced nutrition on early mammary development. Increasing protein and energy intake from 2 to 8 wk of age increases mammary parenchymal mass and parenchymal DNA and RNA without increasing parenchymal fat (Brown et al., 2005). Additionally, increasing plane of nutrition increases plasma insulin-like growth factor-1 (IGF-1) and leptin concentrations, and percentage body fat composition in preruminant calves and lambs, which may influence later development (Block et al., 2003, Ehrhardt et al., 2003). It has been hypothesized that improved nutritional status, resulting in increased insulin and IGF-1 concentrations (Diaz et al., 2001, Smith et al., 2002), may lead to greater long-term protein synthetic ability in muscle and thus may promote increased production and longevity (Drackley, 2005).

Recent data support the NRC (2001) maintenance energy requirements of calves under no-stress conditions (Van Amburgh and Drackley, 2005). Maintenance requirements of the young calf vary with age, weight, temperature, and other stressors (Schrama et al., 1993, Schrama et al., 1992, Scibilia et al., 1987). The thermo-neutral range of the neonatal calf is 15°C to 25°C with the lower critical temperature being 13°C (Arieli et al., 1995, Schrama et al., 1993). It has been reported that maintenance energy requirements of the very young calf increase 32% when housed at -4° compared with 10°C (Scibilia et al., 1987). The current NRC (2001) adopted an adjustment of 0.09 MJ/kg\(^{0.75}\) per day per degree below the lower critical temperature to account for the amount of variation in metabolizable energy available for growth during periods of cold stress. Because many areas of the
world experience temperatures capable of inducing severe cold stress in the neonatal calf, it has been suggested that increasing energy requirements to account for cold stress would decrease calf morbidity and mortality (Van Amburgh and Drackley, 2005).

**Nutrition and Immunity**

Since the 1950s, it has been understood that malnutrition and susceptibility to infectious disease are generally synergistic (Keusch, 2003, Scrimshaw, 2003, Scrimshaw et al., 1968). Although there are little data to date in livestock species, research conducted in the late 1950s and early 1960s indicated that improving the nutritional status of impoverished children decreases morbidity and mortality rates and improves growth (Scrimshaw, 2003, Scrimshaw and Guzman, 1995).

It is recognized today that nutrition (both macronutrients and micronutrients) influences the immune response. A brief list of examples from a variety of species, as previously reviewed (Alvarez and Mobarhan, 2003, Field et al., 2002), is as follows. Feeding of long-chain n-3 polyunsaturated fatty acids suppresses lymphocyte responses to mitogen, suppresses natural killer (NK) cell activity, suppresses delayed-type hypersensitivity (DTH) reactions, and decreases interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α cytokines. Vitamin A deficiency inhibits mitogen-stimulated T cell proliferation, decreases antigen-specific antibody production, decreases the ability of CD4 cells to provide help signals to B cells for antigen-specific IgG responses, limits Th2 cytokine gene expression, and decreases phagocytosis and oxidative burst capacities of the neutrophil. In addition, vitamin A supplementation improves antibody responses to measles vaccines, maintains gut integrity, lowers incidence of respiratory tract infections, and decreases mortality associated with diarrhea and measles. Ascorbic acid (vitamin C) is highly concentrated in leukocytes and is used rapidly during infection in order to prevent oxidative damage. Decreased concentrations of vitamin C in leukocytes are associated with decreased immune function and clinical vitamin C deficiency results in scurvy.
and increased incidence of infection and immunological anergy. In addition to alleviating oxidative stress, vitamin E (RRR-α-tocopherol) supplementation increases DTH skin test response, enhances mitogen-induced lymphocyte proliferation and IL-2 production, improves antibody responses to vaccines, and decreases synthesis of the immunosuppressive eicosanoid PGE₂. Selenium (acting synergistically with vitamin E to decrease oxidative stress) promotes lymphocyte proliferation, expression of the IL-2 receptor, cytolytic T lymphocyte tumor destruction, and NK-cell function. Iron deficiency impairs cell-mediated immunity, neutrophil function (decreased myeloperoxidase activity and bactericidal activity), NK activity, and macrophage bactericidal activity. 1,25-Dihydroxyvitamin D₃, the active form of vitamin D, is a potent immune modulator. 1,25-Dihydroxyvitamin D₃ can either prevent or markedly suppress experimental autoimmune encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, inflammatory bowel disease, and multiple sclerosis (Deluca and Cantorna, 2001). Vitamin D deficits increase risk of malignancies, particularly colon, breast, and prostate cancer (Peterlik and Cross, 2005). 1,25-Dihydroxyvitamin D₃ also induces antimycobacterial activity in monocytes (Sly et al., 2001).

Arginine and glutamine have been shown to influence immune function. Although a nonessential amino acid, arginine has been called conditionally essential because its availability is decreased under stressful situations such as trauma or sepsis (Alvarez and Mobarhan, 2003). Although arginine promotes wound healing, it is probably best known as the precursor for nitric oxide (NO), one of the products of macrophage activity. Increases in extracellular arginine above physiologic levels enhance NO-mediated killing of *Trypanosoma cruzi* by macrophages (Norris et al., 1995). Arginine supplementation in rats prevents age-related decreases in macrophage phagocytosis (Izgut-Uysal et al., 2004). In addition to its positive effects on macrophage activity, arginine increases lymphocyte proliferation and increases NK cell and neutrophil activity (Alvarez and Mobarhan, 2003). Glutamine, the most abundant amino acid in the body, is also a conditionally essential amino acid because it is depleted in cases of severe illness (Alvarez and Mobarhan, 2003).
Glutamine can be used as an energy source via partial oxidation. Resting lymphocytes use glutamine at a relatively high rate, and this rate is increased in activated lymphocytes (Calder, 1995, O'Rourke and Rider, 1989). Increased concentrations of glutamine in culture enhance lymphocyte proliferation and production of IL-2, IL-10, and IFN-γ (Ardawi and Newsholme, 1983, Yaqoob and Calder, 1997, Yaqoob and Calder, 1998). Increasing plasma glutamine concentrations by increasing glutamine concentration in the diet increase mitogen-stimulated lymphocyte proliferation, IL-2 receptor expression, and IL-2 production in mice (Kew et al., 1999). Taken together, these results provide substantial evidence indicating that micronutrients influence immune responses and therefore susceptibility to infectious disease.

Protein Energy Malnutrition and Immunity

Much of the information on the relationship between dietary protein and energy and immunocompetence pertains primarily to nutritional deficiency. Protein-energy malnutrition (PEM) is the major cause of immunodeficiency worldwide (Delafuente, 1991). Protein-energy malnutrition manifests as acute (wasting) and chronic (stunting) forms resulting in altered body composition and decreased linear growth, respectively (Suskind et al., 1990). Although some reports indicate that wasting but not stunting PEM influences cell-mediated adaptive immunity negatively (Rivera et al., 1986), additional reports suggest that stunting PEM likewise may depress cell-mediated immunocompetence and increase the risk of infection-related mortality in humans (Chandra and Sarchielli, 1996, Pelletier et al., 1995). It should be noted that most experiments have investigated effects of wasting, not stunting, PEM on immunity.

Protein-energy malnutrition has negative effects on both innate and adaptive immunity (reviewed by Woodward, 1998). Protein-energy malnutrition decreases the expression of membrane glycoprotein (mannose-fucose) receptors (Sordillo et al., 1991) which may account for the increased propensity of epithelial cells of PEM children to bind coliform bacteria in vitro (Chandra and Gupta,
Lytic activity of NK cells is decreased in cases of PEM, increasing susceptibility to viral diseases as well as some prokaryotic intracellular parasites (Ingram et al., 1995, Scott and Trinchieri, 1995). The acute-phase response to natural infection, vaccination, and sterile inflammatory stimulus is attenuated in human wasting PEM as well as in protein-deficient rodents (Doherty et al., 1993, Duran-Chavez et al., 1994, Jennings et al., 1992). Depression of the acute-phase protein response has been attributed to an attenuated TNF-α/IL-1/IL-6 cascade observed in wasting disease (Doherty et al., 1994, Hill et al., 1995, Munoz et al., 1994, Woodward, 1998). Negative effects of PEM on TNF-α and IL-1 also may explain the depressed mobilization of neutrophils from the marrow to inflammatory sites observed in cases of PEM (Borelli et al., 1995, Woodward, 1998).

Substantial evidence supports negative effects of PEM on adaptive immune responses. The lymphoid involution (decrease in size and cellularity of secondary lymphoid organs) that occurs in wasting PEM is considered contributory to PEM-associated immunodepression (Lee and Woodward, 1996, Woodward, 1998). Lymphoid involution associated with PEM is characterized by drastic decreases in mononuclear cell numbers in blood, spleen, and mesenteric lymph nodes (Lee and Woodward, 1996). Alterations in the proportions of lymphocyte subsets may contribute to the initiation or maintenance of PEM-induced immunodepression. A low CD4⁺/CD8⁺ T cell ratio in the blood commonly has been associated with PEM in humans and weanling mice and was widely accepted as a key indication of depressed T-cell dependent immunity in PEM (Chandra, 1991). The CD4⁺/CD8⁺ T cell ratio in the blood, however, does not always decrease in PEM, and PEM-induced depression in T-dependent adaptive responses may occur in the absence of a low CD4⁺/CD8⁺ T cell ratio (Lee and Woodward, 1996, Parent et al., 1994, Woodward and Miller, 1991). In addition, PEM-induced depression in the CD4⁺/CD8⁺ T cell ratio is only observed in blood and does not occur in the spleen or mesenteric lymph nodes, suggesting that the CD4⁺/CD8⁺ T-cell ratio in the secondary lymphoid organs is stable (Lee and Woodward, 1996). Imbalances within, rather than between, the
two main T cell subsets may be of greater importance. In mice, wasting PEM is associated with an overabundance of CD4⁺CD45RA⁺ T-cells (CD4⁺ naïve-phenotype) and CD8⁺CD45RA⁺CD62L⁺ T-cells (CD8⁺ naïve-phenotype) that are quiescent compared with CD45⁺ effector and memory phenotypes (ten Bruggencate et al., 2001, Woodward et al., 1999). In addition, wasting because of severe protein deficiency decreases proliferation and expression of IFN-γ, IL-2, and IL-2 receptor mRNA by splenic mononuclear cells (Mengheri et al., 1992). Protein-energy malnutrition also affects negatively humoral responses. Mucosal secretory IgA antibody responses are more sensitive to wasting than systemic humoral immunity, leading to the hypotheses that PEM affects epithelial IgA transport (Chandra, 1991, Chandra and Wadhwa, 1993).

*Obesity and Immunocompetence*

Obesity also has been associated with decreased immune function. Obesity, a disorder of energy balance in which energy intake exceeds energy expenditure, has been linked to a variety of health problems including cardiovascular diseases, diabetes mellitus, and cancer. The occurrence of obesity has been attributed to multiple factors, including genetics and lifestyle habits (i.e., diet, exercise). Obesity in humans is accompanied by a higher rate of infections and delayed wound healing (Khan and Bowman, 1999, Pi-Sunyer et al., 1999). It should be noted that the effects of obesity on immune function may be the result of adipokines (see section below: Leptin and Immune Function). Thirty-eight percent of obese children and adolescents show impairment of cell-mediated immune (CMI) responses including decreased DTH, abnormal lymphoproliferative responses to mitogens and decreases in bacterial killing by neutrophils (Chandra and Kutty, 1980). Obesity also has been related to elevated leukocyte and lymphocyte subsets (excluding NK and cytotoxic/suppressor T-cells), lower T- and B-cell mitogen-induced lymphocyte proliferation, but increased phagocytosis and oxidative burst activity by monocytes and neutrophils (Nieman et al., 1999). Additionally, an additive negative effect of obesity and old-age on immune function has been
reported (Moriguchi et al., 1995). Studies have been performed on genetically obese mice that have indicated obesity is associated with immunocompetence. Genetically obese mice however, are leptin deficient. Obesity-related immune defects in genetically obese animal models, therefore, are mainly attributable to a lack of leptin (Martí et al., 2001).

Generally speaking, protein and energy restriction affects positively immune function and longevity in rodents, whereas increased dietary fat has been linked to immunosuppression in rodents characterized by decreased lymphocyte responses to mitogenic stimulation and adhesiveness to endothelial cells (Erickson et al., 1980, Morrow et al., 1985, Sanderson and Calder, 1998). Mice (NZB strain; 7-10 mo of age) fed a low protein (6%) diet have more vigorous antibody responses to sheep red blood cells, graft vs. host reactions, and CMI after immunization against mastocytoma cells when compared with mice fed a normal protein (22%) diet (Fernandes et al., 1976). Feeding this low protein diet also abrogates age-related decrease in immune responsiveness to mitogenic stimulation. Similarly, lifelong caloric restriction of mice prevents age-associated decline in T-cell proliferation (Grossmann et al., 1990). Mechanisms by which life-long food restriction slows aging and decreases the rate of occurrence of age-associated disease processes are unknown. Expression of the IL-2 receptor by mitogen-stimulated lymphocytes from rats fed an ad libitum diet is decreased when compared with cells from rats fed a 40% food-restricted diet (Iwai and Fernandes, 1989). Similarly, high-fat diets suppress CD25 expression on splenic lymphocytes in mice (Peck et al., 2000). Feeding rats an ad libitum diet decreases mitogen-induced proliferative responses of splenic cells when compared with feed-restricted rats (Fernandes et al., 1997). The same study found a significant rise in memory CD4+ and CD8+ T-cell subsets in rats fed ad libitum but found no such shift in feed-restricted rats, supporting the observation that the ability of feed-restricted rats to recall antigens was lower but proliferative responses elicited by mitogen and superantigen were higher compared with ad libitum rats. Higher concentrations of IL-2 and lower concentrations of IL-6 and TNF-α also were observed in feed-restricted rats. The authors concluded that food restriction may selectively maintain lower
numbers of antigen-induced memory T cells with age, thereby assuring the production of higher concentrations of IL-2 with increasing age and that increased CMI function observed in aged feed-restricted rats is the result of the presence of a higher number of naïve T cells (Fernandes et al., 1997).

Energy Balance and Immunity in Cattle

The dairy cow experiences a state of immunosuppression characterized by a heightened susceptibility to infectious disease during the periparturient period (Kehrli and Harp, 2001). Periparturient immunosuppression has been characterized by decreased capacity of innate and adaptive immune functions, including decreased bactericidal capacity of blood neutrophils (Kehrli and Goff, 1989, Kehrli et al., 1989b), mitogen-elicited responsiveness of blood lymphocytes (Ishikawa, 1987, Kashiwazaki et al., 1985, Kehrli et al., 1989a), and serum IgG1 and IgM concentrations (Stabel et al., 1991). Percentages of CD3+, CD4+, CD8+, and γδTCR+ T cell in blood decrease while the percentage of monocytes increase at time of parturition (Kimura et al., 1999, Kimura et al., 2002). In addition, functional capacities of blood mononuclear leukocytes, including cytokine production (IFN-γ specifically), and secretion of IgM are decreased in the periparturient dairy cow (Ametaj et al., 2000, Nonnecke et al., 2003b). Factors contributing to parturition-related immunosuppression are likely diverse and interdependent. Theses factors include hormonal and metabolic demands of pregnancy and lactation as well as the physiologic stress and associated increase in blood cortisol that occurs at calving (Kehrli and Harp, 2001). Although corticosteroids are potent inducers of immunosuppression in cattle (Burton and Kehrli, 1996, Nonnecke et al., 1997), recent evidence suggests that the metabolic demands associated with the onset of lactation contribute to periparturient immunosuppression (Kimura et al., 2002, Nonnecke et al., 2003b). Mastectomy eliminates changes in leukocyte subset populations and functional capacities observed at time of parturition, suggesting metabolic demands of lactation contribute to periparturient immunosuppression (Kimura et al., 2002, Nonnecke et al., 2003b).
To date, research on the effects of energy balance on immunity in cattle has focused mainly on the periparturient cow. One study, however, investigated the effects of hypoalimentation (fed 0.5-0.3% of body weight per day resulting in weight loss of 0.52 kg/d), maintenance or control (fed 1.5% of body weight per day resulting in weight gain of 0.18 kg/d), and hyperalimentation (fed ad libitum resulting in weight gain of 1.07 kg/d) diets on immunological performance of yearling steers (Fiske and Adams, 1985). Hypoalimentation was associated with thymic atrophy, a decrease in numbers of circulating lymphocytes, and a decrease in serum antibody responses to chicken erythrocytes, whereas steers fed hyperalimentation diets had a depression in lymphocyte proliferation (Fiske and Adams, 1985). Plane of nutrition did not affect serum IgG and IgM concentrations, serum antibody to a *Brucella abortus* bacterin, or DTH responses to *M. bovis* BCG. In a separate study, *Mycobacterium* bovis-infected cattle subjected to protein energy malnutrition (17% body weight loss over 133 d) had decreased numbers of CD2⁺, CD8⁺, and γδTCR⁺ cells in the peripheral blood (Doherty et al., 1996). Despite these changes in blood lymphocyte populations, DTH responses, *in vitro* IFN-γ production, and lymphocyte proliferation to antigen were not affected by malnutrition. In addition, energy balance has no effect on CD62L (L-selectin) expression by circulating leukocytes (Perkins et al., 2001).

*Energy Status and Immunity in the Calf*

There is a dearth of information regarding effects of growth rate or nutritional status on immunity in the very young calf. Williams et al. (1981) observed that calves fed 600 g/d of milk replacer dry matter with ad libitum access to calf starter have greater daily gain and lower mortality compared with calves fed 300 g/d of milk replacer dry matter with restricted access to calf starter. Neonatal calves fed at 50% maintenance (110-130 g of milk replacer dry matter) from 2-28 d of age lose weight, have decreased IL-2 activity and lymphocyte proliferative responses to mitogen, and
have delayed primary humoral responses to K99 antigen compared with calves fed above maintenance requirements achieving 1 kg growth per week (Griebel et al., 1987).

Pollock et al. (1993) and Pollock et al. (1994) compared effects of weaning age (5, 9, and 13 weeks) and two levels of nutrition (400 g/d or 1000 g/d of milk replacer dry matter) on CMI and humoral immunity in young calves. Early weaning (5 wk) was associated with decreased serum anti-horse erythrocyte (HRBC) antibody responses and decreased lymphocyte proliferative responses to mitogen. Effects of weaning on immune responses may be the result of nutritional change or behavioral stress (Pollock et al., 1992). In these experiments, the high plane of nutrition was associated with decreased humoral responses (i.e., serum antibody responses to keyhole limpet haemocyanin (KLH) and anti-HRBC titres), although total serum immunoglobulin concentrations were unaffected (Pollock et al., 1994). In contrast, blood mononuclear cells from calves fed the high plane of nutrition had increased lymphocyte proliferative responses to mitogen but decreased DTH to KLH (Pollock et al., 1993). These results contrast to those of Griebel et al. (1987) and Fiske and Adams (1985). These apparent contradictions in humoral responses are probably the result of the levels of nutrition investigated. Griebel et al. (1987) and Fiske and Adams (1985) fed diets resulting in weight loss or wasting, whereas the low plane of nutrition diet offered by Pollock et al. (1993 and 1994) provided at least maintenance requirements.

Recent research (Nonnecke et al., 2003a) compared effects of feeding calves an intensified program (30% crude protein CP, 20% fat milk replacer fed at a rate of 2.4% body weight) with an industry standard programs (20% CP, 20% fat milk replacer fed at a rate of 1.4% body weight) on composition and functional capacities of peripheral blood mononuclear cell (PBMC) populations. The intensified program did not affect total numbers of blood leukocytes, composition of mononuclear leukocyte populations, mitogen-induced DNA-synthesis, or mitogen-induced IgM secretion. Blood mononuclear leukocytes from calves fed the intensified diet, however, produced less
IFN-γ and more inducible NO, suggesting plane of nutrition affects aspects of leukocyte function associated with CMI (Nonnecke et al., 2003a).

Adipokines and Immune Function

Immunosuppression induced by obesity or a high-fat diet may be attributable to adipokines produced by adipocytes, such as leptin and transforming growth factor-β. Leptin is a peptide hormone that affects energy metabolism by decreasing food intake, increasing energy expenditure, and decreasing metabolic efficiency. Leptin links nutritional status with immune functions, acting as a signal to the body of energy stores (Otero et al., 2005). Although exogenous leptin reverses starvation-induced immunosuppression (Lord et al., 1998), leptin inhibits lymphocyte proliferation and suppresses IL-2 production by lymphocytes (Lord et al., 2002). Interestingly, leptin enhances proliferation of naïve (CD4+CD45RA+ T-cells) T cells but inhibits proliferation of memory (CD4+CD45RO+ T-cells) T cells (Lord et al., 2002), which may have consequences on vaccine recall responses. Leptin also enhances IFN-γ-induced expression of NO synthase in macrophages (Raso et al., 2002). We have reported previously that high plane of nutrition results in increased in vitro NO production by PBMC (Nonnecke et al., 2003a). Obese mice also have elevated expression of transforming growth factor-β (Samad et al., 1997). Although transforming growth factor-β is known to play an important role in B-cell isotype switching and immune regulation, it also decreases IL-2 production and proliferative responses of CD4+ and CD8+ T-cells (McKarns and Schwartz, 2005, Wolfraim et al., 2004).

Effects of Infection on Nutritional Status

It is generally recognized that infection worsens nutritional status. The most obvious effect of infections is decreased growth in malnourished children (Mata, 1978, Mata et al., 1972). Infectious disease and increased pathogen load also have been shown to decrease performance (i.e.,
growth) in swine and poultry (Klasing, 1998, Williams et al., 1997a, Williams et al., 1997b). The effects of infections on growth may be attributed to increased nutrient demands by the immune system and to the anti-growth effects of cytokines (e.g., IL-1 and TNF-α) produced by the acute phase response of the host. Induction of an acute phase response in poultry by Escherichia coli lipopolysaccharide (LPS) injections produces a general catabolic state resulting in decreased weight gain, decreased feed efficiency, and loss of breast muscle as a result of diversion of amino acids to the liver for acute phase protein synthesis (Klasing, 1998, Klasing and Austic, 1984, Klasing and Johnstone, 1991, Mireles et al., 2005). In a related study in dairy cattle, LPS infusion resulted in decreased milk production, dry matter intake, plasma β-hydroxybutyrate, plasma insulin to glucagon molar ratio, serum calcium, serum phosphorus and serum 1,25-dihydroxyvitamin D₃, altered hepatic metabolic capacity, and increased plasma TNF-α, insulin, glucagon, and cortisol (Waldron et al., 2003a, Waldron et al., 2003b). Infection, regardless of strain virulence (i.e., infectious or vaccine strain), results in negative nitrogen balance in children and young adults that may last up to 21 d, even in the absence of disease symptoms or febrile responses (Beisel, 1972, Beisel et al., 1967, Gandra and Scrimshaw, 1961, Scrimshaw et al., 1960). Therefore, the inflammatory state likely induces partitioning of nutrients away from growth processes towards the metabolically consumptive immune system.

**Immunity of the Neonate**

Calves are born agammaglobulinemic and thus rely upon immunoglobulin provided by colostrum shortly after birth for protection against infection and disease. Several immunologically active maternal factors are present in colostrum and milk secretions in addition to immunoglobulin, including cytokines (Sordillo et al., 1991), immunologically active proteins (Ye and Yoshida, 1995), and immunologically active cells (Duhamel et al., 1987, Harp and Nonnecke, 1986, Riedel-Caspari and Schmidt, 1990). Studies in lambs and pigs have shown the uptake and distribution of maternal
cells by the neonate (Schnorr and Pearson, 1984, Tuboly et al., 1995, Williams, 1993). There is indirect indication of maternal cell transfer in the young calf. Rotavirus- and coronavirus-specific lymphocyte proliferation has been observed in PBMC from calves out of vaccinated dams but not from calves out of non-vaccinated calves (Archambault et al., 1988). Data provides genetic evidence for the transfer of maternal cells to the calf (Aldridge, 1999).

There are many factors that contribute to the physiologic immaturity of the neonatal immune system and the increased susceptibility of the calf to infectious disease. These factors include an increased percentage of naïve T cells (Clement et al., 1990) and γδT cells (Caro et al., 1998, Wilson et al., 1996) and a decreased percentage of B cells (Nagahata et al., 1991) in neonates compared with adults. Other differences include a decreased ability to make antibody (Nagahata et al., 1991), cytokines (Schibler et al., 1992), and complement (Mueller et al., 1983), and decreased neutrophil function (Dore et al., 1991, Higuchi et al., 1997) in neonates compared with that in adults. Factors exist in serum of neonatal calves, in addition to cortisol, that suppress lymphocyte proliferative response to mitogens (Manak, 1986). In addition, there may be potential negative effects of maternal antibody on antigen-specific immunoglobulin production by the calf (discussed below).

_Lymphocyte Subpopulation: Functional Differences Between Neonatal and Mature Animals_

Pronounced differences exist in lymphocyte subpopulation percentages and function between neonatal and mature livestock. Although this review emphasizes the bovine, other species are discussed to include differences not delineated in the bovine animal.

Interestingly, the composition of T cell subpopulations in the peripheral blood (i.e., CD2+, CD4+, CD8+, and γδ+ T cells) of the bovine fetus do not differ from T cell populations from adult cattle (Wilson et al., 1996). However, drastic changes occur at birth. Although the prevalence of the γδ+ T cell subpopulation decreases with age, the CD4+ T cell and CD8+ T cell subpopulations increase with time following birth (Caro et al., 1998). In cattle, proportions of CD4+ and CD8+ T cells peak in
the fetus, drop below adult proportions at birth and gradually increase to adult proportions by 120 d of age (Wilson et al., 1996). There is a drastic decrease in CD2⁺ circulating lymphocytes from 57% in fetal blood to 28% in calves 2 d of age (Wilson et al., 1996). In the goat, percentages of CD2⁺, CD4⁺, and CD8⁺ T cells in jejunal Peyer's patches increase from one month of age onwards (Caro et al., 1998). In spleens and jejunal Peyer's patches of goats and sheep, CD8⁺ T cells are more prevalent than CD4⁺ T cells and make up approximately 70 percent of total lymphocytes (Caro et al., 1998, Landsverk et al., 1991). It has been suggested that the CD8⁺ T cells in jejunal Peyer's patches may play a role as suppressors in the immunological tolerance mechanism in the gut (Caro et al., 1998).

The γδ⁺ T cell is more prevalent in cattle, sheep, goats, and swine adults than in humans and mice. This T cell population is even more prevalent in neonatal ruminants and swine (Hein and Mackay, 1991). In goats, the percentage of γδ⁺ T cells in peripheral blood can be 20 percent in the first week of life but declines to 4 percent by three months of age (Caro et al., 1998). In neonatal calves, γδ⁺ T cells are higher (in blood) at birth, making up approximately 40 percent of peripheral blood mononuclear cells. By 150 days of life, γδ⁺ T cells make up approximately 15 percent of the PBMC population (Wilson et al., 1996).

In pathogen-free pigs, the percentage of Ig⁺ and CD2⁺ cells increases with time in the spleen and blood (Joling et al., 1994). In thymic tissue of young pigs, CD4⁺ T cell percentages increase with time up to 10 wk of age, then decrease (Joling et al., 1994). Percentage of CD8⁺ T cells within the thymus, however, do not change in young pigs (Joling et al., 1994). Thymic tissue from neonatal calves has increased proportions of CD4⁺ and CD8⁺ T cells compared with adults (Wilson et al., 1996). Most species do not have double positive (CD4⁺CD8⁺) T lymphocytes in peripheral blood and spleen because they are presumed to die within the thymus. Swine, however, have detectable numbers of this double-positive T-cell population (Joling et al., 1994). There also is a greater frequency of thymic cells that are not positive for CD2 in swine (5-13 percent of monocytes) than in mice (3-4 percent) (Joling et al., 1994). This high number of CD2⁺Ig⁺ cells (i.e., null cells) in the pig
may represent epithelial lymphocytes with a wide antigen specificity similar to the γδ+ T cell (Joling et al., 1994).

Neonatal swine and ruminants rely on the γδ+ T cell subpopulation for immunity against infection. The large population of γδ+ T cells found in neonatal swine and ruminants may provide non-MHC-restricted cellular immunity until more mature αβ T cells become established (Hein and Mackay, 1991). Ruminants and other livestock species are often housed in pathogen-friendly environments and are thus constantly exposed to pathogens that infect mucosal surfaces and epithelial cells. The increased prevalence of γδ+ T cell populations in ruminants, therefore, may be beneficial to these species because these cells home in on epithelial surfaces (Hein and Mackay, 1991).

There is a rapid turnover of T cells shortly after birth. At birth, fetal T cells circulating through efferent lymph draining lymph nodes are lost rapidly from T cell pools in peripheral lymph nodes and are replaced by T cells formed after birth (Cahill et al., 1997). By the end of the first week of postnatal life, nearly three-quarters of the total T cell population circulating through peripheral lymph nodes have been formed after birth (Cahill et al., 1997).

**T cells as Suppressors of Immune Function**

Although CD4+ T cells are present in normal numbers in the human neonate, they lack the ability to provide help for antibody production (Clement et al., 1990). The dominant immunoregulatory function of CD4+ cells found in human umbilical cord blood is suppression, which is mediated by a subset of CD4+ T cells (Clement et al., 1990).

**B Cells**

Calves have low antibody-producing activity up to one month after birth (Nagahata et al., 1991). The total number of B lymphocytes increases from birth to four weeks of age (Nagahata et al., 1991, Nonnecke et al., 1999). Within the first week of life, B lymphocytes make up 5% of the total
mononuclear cell population (Senogles et al., 1978). In the calf, the prevalence of B lymphocytes increases steadily until 20 weeks of age when values reach 19% of the total mononuclear cell population, which is similar to adult levels (Senogles et al., 1978). Approximately 20% of splenic cells from one-week-old Cryptosporidium parvum and C. parvum-free mice are positive for Ig, and this percentage increases to 50% by two weeks of age (Harp and Sacco, 1996). In calves, B cell numbers and function increase with age. Antibody-producing activity of calf lymphocytes increases from 14 to 21 days of age (Nagahata et al., 1991). Feeding colostrum has negative effects on endogenous immunoglobulin production by the calf. Calves do not produce antibody in response to Brucella abortus vaccination if maternal B. abortus antibody is present in the circulation (Husband and Lascelles, 1975). Colostrum-deprived calves, however, do produce antibody to B. abortus vaccination. Colostrum-fed and colostrum-deprived calves have strong antibody responses to ovalbumin, to which there is no maternal antibody. Feeding colostrum decreases the number of Ig-positive cells in the lymph tissue of neonatal calves in an isotype-specific manner (Aldridge et al., 1998). Colostrum-fed calves have no IgG1 or IgG2 positive cells present in lymph tissue, whereas colostrum-deprived calves do. IgM and IgA positive cells are not affected by feeding of colostrum.

**Neutrophil Differences between Neonatal and Adult Cattle**

Differences in innate immune function also exist between neonates and adults. Differences exist in neutrophil number and function and in cytokine and complement production. At birth, the total number of neutrophils in the peripheral blood of calves is high but decreases within the first 20 d of life (Knowles et al., 2000). The number of neutrophils increases between the first six and 12 hours after birth in the colostrum-fed newborn calf but not in the colostrum-deprived newborn calf (LaMotte and Eberhart, 1976). Although total numbers may be high, there is evidence of decreased function. Neonatal calves have decreased protein kinase C-dependent O2- generation (Dore et al., 1991). Generation of O2- (free radical superoxide anion) is an essential part of the respiratory burst of
the neutrophil and is essential for destruction of the pathogen. The decreased killing capacity of calf neutrophils also may be the result of age-dependent changes in intracellular Ca\(^{2+}\) concentration (Higuchi et al., 1997). Fc receptor (FcR) expression and basal capping of Con A binding sites are decreased in neonatal bovine neutrophils compared with those of adult cattle (Zwahlen et al., 1992). Like human neutrophils, calf neutrophils have decreased myeloperoxidase and elevated alkaline phosphatase (Zwahlen et al., 1992). Decreased expression of FcR can contribute to impaired adherence and phagocytosis of bacteria, whereas lower concentrations of myeloperoxidase decrease the bactericidal capacity of neonatal neutrophils (Zwahlen et al., 1992). Phagocytic capacity of neutrophils increases after birth in calves and is more efficient in colostrum-fed calves than colostrum-deprived calves (LaMotte and Eberhart, 1976).

Cytokine and Complement Differences between Neonatal and Adult Cattle

Neonates have a decreased ability to produce cytokines and complement. Neonatal humans have a decreased ability to produce IL-6 (produced primarily from monocytes and macrophages) when compared with adults (Schibler et al., 1992). Neonatal calves produce less mitogen-stimulated IFN-\(\gamma\) than do adults (Nonnecke et al., 2003a). Neonates also have impaired Th1 memory effector function and are biased towards Th2 function at all phases of an immune response (Adkins et al., 2001). Polarized patterns of cytokine secretion by Th cells are essential for mounting effective immune responses. The development of a Th1 or Th2 response is dependent on the cytokines produced. Th1 cells secrete inflammatory cytokines, including IFN-\(\gamma\) and TNF-\(\alpha\). The cytokines produced by Th1 cells are necessary to activate macrophages promoting killing of intracellular pathogens. Th2 cells secrete cytokines (IL-4, IL-5, IL-10, and IL-13) important for fighting off extracellular pathogens by stimulating strong humoral immune responses. Neonates with decreased Th1 function may have an impaired capacity to activate macrophages.
Calves have a hemolytic complement titer of 99 at birth (Mueller et al., 1983). The hemolytic titer decreases to 39 after one day and increases to precolostral concentrations after four weeks of age. The precolostral concentration is still substantially lower in one-month-old calves compared with that in adults (Mueller et al., 1983). Similarly, complement 3 (C3) is present in the newborn calf at concentrations only 28 percent that of adults, decreases to 18 percent of adult concentrations one day after birth, and increases to 43 percent of adult concentrations by one month of age (Mueller et al., 1983).

Vaccination of the Neonate

The physiologic immaturity of the bovine neonatal immune system makes management of calves difficult. In an attempt to prevent respiratory and intestinal diseases, numerous vaccines have been developed for use in the neonatal calf. Efficacy of available vaccines, however, remains controversial.

The increased susceptibility of neonates to infection has been attributed to an intrinsic inability to mount effective (i.e., adult-like) Th1 responses. Recent studies in mice indicate that adult-level Th1 function can be achieved in newborns under Th1-promoting conditions such as complete Freund’s adjuvant (Adkins, 2000, Adkins et al., 2004). More often, however, the neonate develops Th2-dominant responses (Table 1), and immunization generally results in a Th2-biased secondary response (Adkins, 2000). The neonate is now considered to be immunodeviant rather than immunodeficient (Adkins et al., 2004).
Table 1. Th2-biased memory responses in neonates

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Adults</th>
<th>Responses*</th>
<th>Neonates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° Protein antigen in PBS(^1)</td>
<td>Mixed Th1/Th2</td>
<td>Mixed Th1/Th2</td>
<td></td>
</tr>
<tr>
<td>2° Protein antigen in PBS</td>
<td>Th1-dominant</td>
<td>Th2-dominant</td>
<td></td>
</tr>
<tr>
<td>1° Alum absorbed peptide + control oligonucleotides</td>
<td>Th2-dominant</td>
<td>Th2-dominant</td>
<td></td>
</tr>
<tr>
<td>2° Alum absorbed peptide + CpG oligonucleotides</td>
<td>Mixed Th1/Th2</td>
<td>Th2-dominant</td>
<td></td>
</tr>
<tr>
<td>1° Viral DNA vaccine</td>
<td>Mixed Th1/Th2</td>
<td>Mixed Th1/Th2</td>
<td></td>
</tr>
<tr>
<td>2° Live virus</td>
<td>Th1-dominant</td>
<td>Th2-dominant</td>
<td></td>
</tr>
</tbody>
</table>

*As determined by the production of IFN-\(\gamma\) (Th1), IL-4 (Th2), or their associated IgG\(_{2m}\) (Th1) or IgG\(_2\) (Th2) isotypes.

\(^1\)Phosphate buffered saline = PBS

Adapted from Adkins, 2000.

Vaccinating neonatal calves with three multiple-antigen vaccines simultaneously, however, does not affect absolute numbers of lymphocytes but does increase rectal temperatures, blood leukocyte counts, and neutrophil counts compared with nonvaccinated calves (Allen et al., 1996). It was concluded that simultaneous vaccination with multiple products induces an inflammatory response that is not beneficial and that may be harmful (Allen et al., 1996). Until recently, dogma suggested that vaccination of the young calf was not efficacious because of a lack of demonstrable antibody response. It has been hypothesized that maternal colostral-derived antibody blocks humoral responses of the calf to vaccines (Aldridge et al., 1998, Husband and Lascelles, 1975). Although the presence of specific maternal antibody blocks humoral responses to bovine viral diarrhea virus (BVDV) vaccination, antigen-specific CD4\(^+\), CD8\(^+\), and \(\gamma\delta\)TCR\(^+\) cells are developed and memory T and B cells may be generated by vaccination (Endsley et al., 2004, Endsley et al., 2003). These researchers suggest that calves with vaccination-induced memory T and B cells can be protected from virulent BVDV challenge (Endsley et al., 2003).
**Mycobacterium bovis bacillus Calmette-Guerin vaccination**

One-third of the world's population (i.e., about 1.7 billion people) is infected with *Mycobacterium tuberculosis*, and nearly 3 million people die each year from tuberculosis (WHO, 2001). *Mycobacterium bovis* and *tuberculosis* are intracellular pathogens that utilize the phagocytic macrophage as their host cell to live and replicate in the tissue of the alveolar wall of the lung. At the infection site, continual macrophage and lymphocyte recruitment leads to the formation of granulomas, which limit the spread of the infection. The macrophage combats its internal pathogen by generating reactive nitrogen intermediates (i.e., NO), generating reactive oxygen intermediates (i.e., superoxide), maturing and acidifying phagosomes, fusing phagosomes with lysosomes, exposing pathogens to defensins, and eliminating the niche required by the mycobacterium by macrophage host cell apoptosis. Immunologic control of *M. bovis* and *M. tuberculosis* infection is based on a Th1 response. Activation of bactericidal mechanisms of macrophages is dependent upon IFN-γ. In mice, strong IFN-γ and NO responses are essential for the establishment of resistance to tuberculosis (Chan et al., 2001, Flynn et al., 1993). Indeed, IFN-γ-knockout mice are more susceptible to *M. tuberculosis* infection, because of decreased macrophage activation and expression of inducible NO synthase (Flynn et al., 1993).

Immune responses to vaccination with *M. bovis* BCG have been characterized in the bovine (Maue et al., 2004, Nonnecke et al., 2003c, Waters et al., 2003). Sensitization to *M. bovis* BCG and elicitation of recall responses (i.e., IFN-γ and NO) by mycobacterial antigen (i.e., purified protein derivative; PPD) have been used to model CMI responses in cattle (Fiske and Adams, 1985, Nickerson and Nonnecke, 1986, Nonnecke et al., 1986).

Antigen-induced T cell subset proliferation and secretion of IFN-γ, NO, and TNF-α by PBMC from calves vaccinated with *M. bovis* BCG at less than 1 wk of age are comparable with or greater than responses of vaccinated adults (Nonnecke et al., 2005). In addition, DTH reactions to intradermal administration of PPD are similar in vaccinated calves and adults, demonstrating the
capacity of the bovine neonate to develop vigorous cell-mediated immune response in vivo (Nonnecke et al., 2005). Vaccinated calves, however, fail to demonstrate antigen-specific humoral responses as measured by antibody concentrations in sera and in supernatants from antigen-stimulated cultures (Nonnecke et al., 2005). *M. bovis* BCG vaccination of calves at birth results in protection against *M. bovis* challenge (Buddle et al., 2003). Results from studies evaluating effects of BVDV and *M. bovis* BCG vaccination suggest that early vaccination of newborn calves affords protection against certain infections dependent on Th1 responses for resolution.

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CHAPTER 2. EFFECTS OF AGE AND NUTRITION ON EXPRESSION OF CD25, CD44, AND L-SELECTIN (CD62L) ON T CELLS FROM NEONATAL CALVES

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ABSTRACT

Effects of the plane of nutrition and age on the proliferation and activation of lymphocyte subsets from milk replacer-fed calves were investigated. Holstein calves were fed a standard (0.45 kg/d of a 20% crude protein, 20% fat milk replacer, n=4) or intensified (1.14 kg/d of a 28% crude protein, 20% fat milk replacer, n=4) diet from 1- to 8-wk of age. Average daily weight-gain of intensified-diet (0.66 kg/d) calves was greater than standard-diet (0.27 kg/d) calves. Relative to the pokeweed mitogen-induced responses of CD4⁺ cells from steers (5 to 6 m of age), CD4⁺ cells from 1-wk old calves showed decreased proliferative activity, delayed increase in CD25 expression and no demonstrable increase in CD44 expression or decrease in CD62L expression. Calf CD8⁺ and γδT cell receptor⁺ cells, unlike T cells from the older animals, did not demonstrate decreased expression of CD62L after stimulation with mitogen. The increased expression of CD44 by mitogen-stimulated γδT cell receptor⁺ cells from older animals was not seen in γδT cell receptor⁺ cells from 1-wk-old calves. At wk 8 of age, mitogen-induced proliferation and expression of activation antigens by T cells from standard-fed calves were similar to responses of T cells from steers indicating rapid maturation of T cell function during the neonatal period. Feeding calves an intensified milk replacer was associated with decreased proliferation of mitogen-stimulated CD4⁺, CD8⁺, and γδT cell receptor⁺ cells; decreased CD25 expression by mitogen-stimulated CD4⁺ and CD8⁺ cells; and
decreased CD44 expression by mitogen-stimulated CD8\(^+\) cells. These results indicate that the functional capacity of the calf's T cell population becomes more adult-like during the first weeks of life and suggest that nutrition modulates T cell function during this period of immune maturation.

Key words: (calf, immune function, nutrition)

**Abbreviation key:** CD62L = L-selectin, CMI = cell-mediated immune, FBS = fetal bovine serum, IL = interleukin, PBMC = peripheral blood mononuclear cells, PEM = protein energy malnutrition, PI = proliferation index, PWM = pokeweed mitogen, TCR = T cell receptor.

**INTRODUCTION**

Activation, differentiation, trafficking, and migration of T cells through sites of inflammation or infection are essential for an effective immune response. The \(\alpha\) chain of the interleukin (IL)-2 receptor, CD25, is expressed on activated T cells, B cells, and monocytes. Formation of the high-affinity IL-2 receptor allows T cell proliferation and differentiation to be driven by IL-2. In cattle, proliferating CD4\(^+\), CD8\(^+\), and \(\gamma\delta\) T cell receptor (TCR)\(^+\) cells exhibit increased expression of CD25 (Waters et al., 2003b). Expression of CD25 on CD4\(^+\), CD8\(^+\), and \(\gamma\delta\) TCR\(^+\) T cells has been used to monitor responses of bovine T cells to inactivated bovine herpesvirus-1 (Endsley et al., 2002), *Mycoplasma bovis* (Vanden Bush and Rosenbusch, 2003), bovine respiratory syncytial virus (Sandbulte and Roth, 2002), *Mycobacterium bovis* (Nonnecke et al., 2005, Waters et al., 2003a; Waters et al., 2003b) and mitogenic stimulation (Franklin et al, 1994; Nonnecke et al., 1993).

Leukocyte trafficking is regulated by surface adhesive interactions between adjacent cells or between cells and the extracellular matrix. The leukocyte adhesion molecule, CD44, binds to components of the extracellular matrix and is considered essential for extravasation of T cells at sites of inflammation (Dailey, 1998). Expression of CD44 is up-regulated on antigen-activated murine T
cells and remains elevated on memory T cells (Dailey, 1998). In *Mycobacterium bovis*-infected cattle, expression is greater in CD4\(^+\), CD8\(^-\), and γδTCR\(^+\) T cells proliferating in response to antigen than in T cells unresponsive to antigen (Waters et al., 2003b).

The lymph node homing receptor, L-selectin (CD62L), is required for entry of cells into lymph nodes through high endothelial venules. Its expression on murine lymphocytes is down-regulated after polyclonal and antigenic stimulation in vivo and in vitro (Dailey, 1998). Activated T cells with reduced CD62L expression do not adhere to lymph node high endothelial venules in vitro or traffic to lymph nodes in vivo in the mouse (Dailey, 1998). In *Mycobacterium bovis* infected cattle, CD62L expression is decreased in antigen activated CD4\(^+\), CD8\(^+\), and γδTCR\(^+\) T cells (Waters et al., 2003b). Decreased expression reduces clearance of activated cells from the blood by lymph nodes allowing them to traffic to sites of activated endothelium.

Although the effects of nutrient supply on body composition and performance of neonatal calves are established (Diaz et al., 2001; NRC, 2001), few studies have considered effects of nutrient supply on immune function of the young calf (Foote et al., 2005; Nonnecke et al., 2003). Nutritional status influences broad aspects of immune function (Scrimshaw et al., 1968; Sullivan et al., 1993; Watson and McMurray, 1979). In prepubescent mice, protein-energy malnutrition (PEM) depresses thymus-dependent immunity (Woodward, 1998). In mice, PEM is associated with an overabundance of CD4\(^-\)CD45RA\(^+\) T cells (CD4\(^+\) naïve-phenotype) and CD8\(^-\)CD45RA\(^+\)CD62L\(^+\) T cells (CD8\(^+\) naïve-phenotype) that are quiescent compared with CD45\(^-\) effector and memory phenotypes (ten Bruggencate et al., 2001; Woodward et al., 1999). Similarly, feed-restricted rats have a decreased capacity to respond to recall antigens when compared to ad libitum-fed rats (Fernandes et al., 1997). Depressed humoral and cell-mediated immune (CMI) responses in calves (Griebel et al., 1987) and decreased numbers of circulating CD8\(^{+}\) and γδTCR\(^+\) T cells in cattle experimentally infected with virulent *Mycobacterium bovis* (Doherty et al., 1996) have also been associated with PEM.
Excess nutrition also modulates immune function. Expression of IL-2 receptor (CD25) by mitogen-stimulated lymphocytes from rats fed an ad libitum diet is reduced when compared to cells from rats fed a 40% food-restricted diet (Iwai and Fernandes, 1989). Similarly, high-fat diets suppress CD25 expression on splenic lymphocytes in mice (Peck et al., 2000). Although CD62L expression by circulating leukocytes from adult cattle is not affected by energy balance (Perkins et al., 2001), effects of energy on CD25, CD44, and CD62L expression on activated bovine lymphocytes have not been reported.

The objective of this study was to evaluate effects of age and increased nutrient supply provided by intensified milk replacer on the functional capacity of peripheral blood T cell subsets from young dairy calves. We hypothesized that increasing dietary protein and energy would enhance proliferative responses and activation antigen (CD25, CD44, and CD62L) expression on mitogen stimulated T cell subsets from neonatal calves.

**MATERIALS AND METHODS**

**Animals and Treatments**

All procedures involving animals were approved by the National Animal Disease Center, Animal Care and Use Committee. Holstein bull calves (n = 8) were housed at the Land O' Lakes, Inc., Research Farm, Webster City, IA and four Holstein steers (5 to 6 m of age) were housed at the National Animal Disease Center, Ames, IA. Calves were housed on raised stalls in an environmentally controlled building. Steers were housed on straw in a building with a southern exposure. Calves were allotted randomly to a standard (n = 4) or intensified (n = 4) diet. The standard diet consisted of a 20% CP, 20% fat milk replacer fed at 0.45 kg/d with calf starter (18% CP, Land O'Lakes, Inc., St. Paul, MN) offered ad libitum. The intensified diet consisted of a 28% CP,
20% fat milk replacer fed at 1.14 kg/d with calf starter (22% CP, Land O'Lakes, Inc.) offered ad libitum. Calves were fed these diets from 1- to 8-wk of age.

**Blood Mononuclear Cell Recovery and Enrichment**

Blood was collected from calves at 1-wk (prior to initiation of dietary treatments) and at 8-wk of age (7 wk after initiation of dietary treatments) by jugular venipuncture. Blood was collected from the steers at the same times. Sixty mL of blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose [a sterilized solution containing sodium citrate (77 μmol/L), citric acid (38 μmol/L) and dextrose (122 μmol/L).

Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation as described previously (Nonnecke et al., 1991). The PBMC-enriched populations were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Sigma, St. Louis, MO) antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin, Sigma), 50 μM 2-mercaptoethanol (Sigma), 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT).

**Blood Mononuclear Cell Cultures**

Staining of isolated PBMC with PKH67 green fluorescent dye (Sigma) for flow cytometric analysis of lymphocyte subset proliferation was performed following manufacturer directions as described previously (Waters et al., 2003a). Briefly, 4 × 10^7 PBMC were centrifuged (10 min, 400 × g) supernatants were decanted, and cells resuspended in 2 mL of diluent C that was provided in the PKH67 kit. Diluted cells were added to 1 mL of diluted (4 μM) PKH67 green fluorescent dye, incubated for 5 min and then incubated 1 min with 4 mL of inactivated FBS to adsorb excess dye and
stop further uptake of dye. Cells were then washed twice with RPMI 1640 medium. Individual wells of 96-well tissue culture plates were seeded with $2 \times 10^5$ PKH67-stained PBMC in a total volume of 200 μL (10 replicates for each treatment) in 96-well round-bottom microtiter plates. Cultures were nonstimulated (media only) or stimulated with pokeweed mitogen (PWM, 1 μg/mL) and incubated for 3 and 6 d at 39°C in a humidified atmosphere with 5% CO₂.

**Analysis of Proliferation and Expression of Activation Markers**

Culture replicates were pooled after 3 and 6 d incubation periods. Approximately $2 \times 10^5$ pooled PKH67 stained cells in 150 μL of culture medium were added to individual wells of 96-well round-bottomed microtiter plates. PKH67-stained cells were double-labeled with one of three phenotype makers (CD4, CD8, or γδTCR) and one of three activation markers (CD25, CD44, or CD62L). Primary and secondary antibodies used in the analyses are listed in Table 1. Monoclonal primary antibody (1 μg/well) diluted in phosphate buffered saline (PBS) containing 0.02% NaN₃ and 1% inactivated FBS was added to individual wells in 25 μL aliquots. Cells were incubated for 15 min at room temperature and centrifuged (400 x g at room temperature for 2 min). Supernatant was decanted and cells were double-labeled with 100 μL of a cocktail containing two secondary isotype-specific antibodies conjugated to fluorochromes (IgM-allophycocyanin and IgG₁-phycoerythrin; listed in Table 1). Secondary antibodies were diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were incubated for 15 min at room temperature in the dark and then centrifuged as described above. Cells were resuspended in 200 μL of PBS and examined by flow cytometry the same day.

Ten thousand cells exhibiting light scattering properties consistent with bovine PBMC were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson, San Jose, California). Lymphocyte proliferation (CD4⁺, CD8⁺, and γδTCR⁺ cells) data were acquired using
CellQuest (Becton Dickinson, San Jose, California) software and analyzed using Modfit LT™ (Verity Software House, Inc. Topsham, ME) software. Proliferation profiles were determined for the gated CD4⁺, CD8⁺, and γδTCR⁺ T cell subsets within the PBMC population. Lymphocyte proliferation was estimated by determining the fluorescence intensity of PKH67-stained cells. As cells proliferate, fluorescence intensity decreases, and cell generation can be mathematically determined by fluorescence intensity using Modfit LT™ software. Data are presented as the mean (± SEM) percentage of T cells proliferating beyond the parent population, as determined by Modfit LT™ software, and as a proliferation index (PI), also determined by Modfit LT™ software. The PI is the sum of cells in all generations divided by the theoretical number of parent cells present at the beginning of the incubation period. Thus, PI is a measure of the increase in cell number in culture over the course of the incubation period.

Activation marker (i.e. CD25, CD44, CD62L) data were acquired using CellQuest (Becton Dickinson, San Jose, California) software and were analyzed using FlowJo (Tree Star Inc., San Carlos, CA) software. Data are presented as geometric mean fluorescence intensities (mean ± SEM) of gated cells (i.e. CD4⁺, CD8⁺, and γδTCR⁺ cells) within the PBMC population.

Statistical Analysis

Data were analyzed as a completely randomized design using Statview software (version 5.0, SAS Institute, Inc., Cary, NC) using the general linear model for all analysis. Animal served as the experimental unit in the analysis of all data. Growth was analyzed as a split-plot with repeated measures ANOVA. The model included the fixed effects of dietary treatments (standard vs. intensified diet), time (wk on experiment), and the treatment × time interaction, and calf was included in the model as the random effect. Fisher’s protected-LSD test was applied when significant effects (P < 0.05) were detected. Proliferative responses (i.e. percent proliferating and PI) and activation marker expression (i.e. mean fluorescence intensity of activation antigens on specific T cell subsets)
were analyzed as a split-plot with factorial ANOVA. Model included fixed effects of dietary treatments (standard vs. intensified diet), age (calf vs. steer), and stimulation (nonstimulated vs. stimulated cultures). Significance was declared at $P < 0.05$ and trends from $P > 0.05$ to $< 0.10$. Fisher’s protected-LSD test was applied when trends or significant ($P < 0.05$) effects were detected.

**RESULTS**

**Growth Performance of Standard-Diet and Intensified-Diet Calves**

Although mean BW of standard-diet calves and intensified-diet calves were not different ($P > 0.05$) at the beginning of the trial, weights of calves on the intensified diet were greater ($P < 0.01$) than weights of calves on the standard diet from wk 2 through 7 of the experimental period. The average daily weight-gain of intensified-diet calves (0.66 kg/d) exceeded ($P < 0.0001$) the daily gain of standard-diet calves (0.27 kg/d) during the experimental period.

**Proliferative Responses of T cells Subsets from Steers and 1-wk Old Calves**

Proliferative responses of PWM stimulated lymphocytes from steers and calves at 1-wk of age, are shown in Figure 1. Percentages of CD4$^+$ cells proliferating in response to PWM did not differ between the two age groups (Figure 1a), although the PI of CD4$^+$ cells from steers was greater than the PI of calf CD4$^+$ cells (Figure 1b). Proliferative responses of CD8$^+$ cells from steers and 1-wk old calves were not different; however, responses (i.e. percentage proliferating and PI) of calf $\gamma\delta$TCR$^+$ T cells were greater than responses of steer $\gamma\delta$TCR$^+$ cells (Figure 1a). Responses of mitogen stimulated PBMC from calves and steers were similar to the corresponding responses of their CD4$^+$ T cell populations (data not shown).
Proliferation of T cell Subsets from Steers and 8-wk Old Calves

Proliferative responses of mitogen stimulated lymphocytes from 8-wk old calves (i.e. 7 wk after initiation of dietary treatments) are shown in Figure 2. The hyporesponsiveness of T cell subsets from 1-wk old calves to mitogenic stimulation (Figure 1) was not observed in cultures consisting of PBMC from 8-wk old calves (Figure 2). Percentages of CD4+, CD8+ and γδTCR+ cells proliferating in response to PWM from standard-diet calves and steers were not different (Figure 2a). Additionally, PI of CD4+, CD8+ and γδTCR+ cells from standard-diet calves and steers were not different (Figure 2b). In PWM stimulated cultures, percentages of proliferating CD8+ and γδTCR+ cells were greater in PBMC populations from steers than in PBMC populations from intensified-diet calves. The PI of PWM stimulated, steer CD4+ and CD8+ cells were higher than PI of CD4+ and CD8+ cells from intensified-diet calves. The PI of stimulated γδTCR+ cells was not affected (P > 0.05) by age or by diet (Figure 2b). The PWM induced proliferative responses of complete PBMC populations were similar to responses of individual T cell subsets (data not shown).

Dietary treatments also affected mitogen induced proliferative responses of calf T cell subsets. In PWM stimulated cultures, percentages of proliferating CD4+, CD8+ and γδTCR+ cells was greater in cultures of PBMC from standard-diet calves than in cultures of PBMC from intensified-diet calves (Figure 2a). Proliferation indices of stimulated CD4+, CD8+ and γδTCR+ cells from standard-diet and intensified-diet calves; however, were not different (Figure 2b).

Expression of Activation Antigens by T cell Subsets from Steers and 1-wk Old Calves

Expression of CD25, CD44, and CD62L by nonstimulated and PWM stimulated T cell subsets from calves less than 1-wk of age and steers is shown in Tables 2, 3, and 4. Expression of CD25 by CD4+, CD8+, and γδTCR+ T cells from steers was higher in PWM stimulated cultures than in nonstimulated cultures (Table 2). The only exception was CD25 expression on steer γδTCR+ T
cells which was of similar magnitude in stimulated and nonstimulated, 6-d cultures. Expression of CD25 on CD4^+ cells from 1-wk old calves differed from expression by CD4^+ cells from steers. Unlike CD4^+ cells from steers, stimulated cells from calves tended to demonstrate increased CD25 expression in 6-d but not 3-d cultures. In addition, CD25 expression on calf CD4^+ cells tended to be lower than steer CD4^+ cells in stimulated 6-d cultures. Expression of CD25 on calf and steer CD8^+ and γδTCR^+ T cells was similar in nonstimulated and stimulated cultures. The only exception was the higher CD25 expression by calf CD8^+ cells in nonstimulated, 6-d cultures.

Expression of CD44 on CD4^+, CD8^+, and γδTCR^+ T cells from steers was higher in PWM stimulated than in nonstimulated cultures (Table 3). The exception was the lower CD44 expression by γδTCR^+ T cells in stimulated relative to nonstimulated, 6-d cultures. Expression of CD44 by T cell subsets from calves differed markedly from CD44 expression by T cells from steers. Although CD44 expression by T cell subsets from steers increased after PWM stimulation, CD44 expression by calf CD4^+, CD8^+ and γδTCR^+ T cells did not increase after stimulation. The only exception was CD44 expression on calf CD8^+ cells tended to be higher in PWM-stimulated than in nonstimulated 3-d cultures. Surprisingly, CD44 expression by calf γδTCR^+ T cells was lower in PWM-stimulated than in nonstimulated, 6-d cultures. Age-related differences of CD44 mean values also were observed. In nonstimulated 6-d cultures, CD44 expression by calf CD8^+ cells was higher than expression by steer CD8^+ cells. Expression of CD44 by calf γδTCR^+ cells was lower than steer γδTCR^+ cells in PWM stimulated 3-d cultures.

Expression of CD62L by γδTCR^+ T cells from steers increased in 3-d, mitogen stimulated cultures (Table 4). In 6-d cultures, CD62L expression by CD4^+, CD8^+, and γδTCR^+ T cells was lower in PWM-stimulated than in nonstimulated cultures. In 3- and 6-d cultures of calf PBMC, CD62L expression by CD4^+, CD8^+, and γδTCR^+ cells was similar in nonstimulated and PWM-stimulated cultures.
**Activation Antigen Expression by T cell Subsets from Steers and 8-wk Old Calves**

Expression of CD25, CD44, and CD62L by nonstimulated and PWM stimulated T cell subsets from steers and 8-wk old calves is shown in Tables 5, 6, and 7. As observed at the beginning of the trial (Table 2), CD25 expression by steer CD4, CD8, and γδTCR cells was higher in PWM stimulated than nonstimulated, 3- and 6-d cultures (Table 5). For 8-wk old calves, CD25 expression by CD4, CD8 and γδTCR cells was higher in PWM stimulated than nonstimulated, 3- and 6-d cultures (Table 5). The delayed expression of CD25 by stimulated CD4 cells from 1-wk old calves (Table 2) was not observed in PBMC cultures from standard- and intensified-diet, 8-wk old calves (Table 5).

Expression of CD25 was affected by the plane of nutrition. In PWM stimulated 3-d cultures, CD25 expression by CD4 and CD8 cells from standard-diet calves was higher than the CD25 expression by the same T cell subsets from steers and intensified-diet calves. In PWM stimulated 6-d cultures, CD25 expression by γδTCR T cells from intensified-diet calves tended to be lower (P < 0.1) than the CD25 expression by γδTCR cells from steers.

As observed at the beginning of the trial (Table 3), expression of CD44 by CD4, CD8, and γδTCR T cells from steers was higher in PWM stimulated than in nonstimulated cultures (Table 6). The exception was the lack of an effect of PWM on CD44 expression by CD4 and γδTCR cells in 3-d cultures. Mitogen induced expression of CD44 by CD4, CD8, and γδTCR cells from 8-wk old standard-diet calves was similar to expression by stimulated, steer CD4, CD8 and γδTCR subsets.

Expression of CD44 was affected by dietary treatment with responses of stimulated T cell subsets from intensified-diet calves frequently being different or tending to be different from responses of cells from standard-fed calves or steers (Table 6). Unlike CD4 cells from standard-diet calves, stimulated cells from intensified-fed calves did not demonstrate increased CD44 expression in
3-d cultures. In PWM stimulated 6-d cultures, CD44 expression by CD4+ from intensified-fed calves tended to be lower than standard-fed calves. Expression of CD44 on CD8+ cells from intensified-fed calves tended to be lower than standard-fed calves in mitogen stimulated 3-d cultures. When compared to level of CD44 expression on γδTCR+ and CD4+ cells from steers, CD44 expression on the same T cell subsets from intensified-fed calves were lower or tended to be lower in mitogen stimulated 6-d cultures.

Effects of animal maturity and dietary treatment on CD62L expression by T cell subsets are presented in Table 7. As observed at the beginning of the trial (Table 4), CD62L expression on CD4+, CD8+, and γδTCR+ T cells from steers was lower in PWM-stimulated than in nonstimulated cultures (Table 7). The only exception was the lack of an effect of stimulation on CD62L expression by steer γδTCR+ cells in 6-d cultures.

Several age-related differences in CD62L expression by cells from 8-wk old calves and steers were evident (Table 7). Unlike steers, CD62L expression by resting and PWM stimulated γδTCR+ cells from standard-diet calves was not different in 3-d cultures. In stimulated 3-d cultures, expression of CD62L was higher on CD4+ cells from standard-fed calves than from steers and tended to be higher on CD8+ cells from standard-diet calves compared with steers. In addition, CD8+ cells from standard-diet calves had higher CD62L expression compared with steers in nonstimulated 6-d cultures. Expression of CD62L by stimulated γδTCR+ cells from standard-fed calves and steers tended to be different in 3- and 6-d cultures. Similarly, nonstimulated γδTCR+ cells from standard-diet calves tended to be higher compared with steers in 6-d cultures.

Dietary treatments also affected CD62L expression. In PWM stimulated 3-d cultures, CD62L expression by CD4+ cells from intensified-diet calves was lower than the expression by cells from standard-diet calves. In nonstimulated 3- and 6-d cultures, CD62L expression by CD8+ cells from intensified-diet calves was lower than from standard-fed calves. Similarly, γδTCR+ cells from
intensified-diet calves had lower CD62L expression compared with standard-diet calves in nonstimulated 6-d cultures. Unlike T cells from standard-fed calves, PWM stimulated CD8$^+$ and γδTCR$^+$ cells from intensified-fed calves did not demonstrate decreased CD62L expression in 3-d or 6-d cultures, respectively.

**DISCUSSION**

This study describes, for the first time, the expression of activation antigens (i.e., CD25, CD44, and CD62L) on nonstimulated and mitogen-stimulated CD4$^+$, CD8$^+$, and γδTCR$^+$ cells from neonatal calves. This study also compared the expression of these antigens on T cell subsets from calves and steers, and examined the influence of neonatal nutrition on their expression.

Although age-related changes in circulating mononuclear leukocyte population composition have been described in young and adult dairy cattle (Ayoub and Yang, 1996; Hein and Mackay, 1991; Nonnecke et al., 2003), functional changes in these populations are poorly described. Proliferation and expression of activation markers in response to polyclonal stimulation (i.e., PWM) were analyzed before initiation of dietary treatments in order to compare responses of adult and neonatal T cell subsets to activation. The PWM elicited responses of lymphocytes from steers in the current experiment were similar to antigen specific responses previously reported in adult cattle (Endsley et al., 2002; Sandbulte and Roth, 2002; Waters et al., 2003b). Neonates were generally hyporesponsive to PWM stimulation when compared to older cattle. Studies in humans indicate CD25 is expressed on more circulating T cells in older (7-14 d of age) than in younger (1-7 d of age) infants and that CD62L expression is lower on cord blood B cells than on adult B cells (Hodge et al., 2004; Tasker and Marshall-Clarke, 2003). Together, these results suggest that within the first wk of life neonatal lymphocytes may exhibit defects in activation and homing mechanisms. Interestingly, nonstimulated CD8$^+$ cells from calves had a higher CD25 and CD44 expression compared with adults after 6-d of culture, suggesting that this cell phenotype may be activated nonspecifically in the young calf.
Decreased or delayed expression of CD25 on CD4+ and γδTCR+ from neonates could result in reduced differentiation and proliferation of these subsets. Decreased CD44 expression on activated CD4+ cells could decrease extravasation of this subset to sites of inflammation. A failure to decrease CD62L expression on activated CD4+, CD8+, and γδTCR+ cells may promote clearance of these subsets from the circulation by lymph nodes decreasing their trafficking to sites of activated endothelium.

The age-related differences observed when calves were 1-wk of age were not observed in 8-wk old calves. In particular, PWM induced CD25 and CD44 expression by CD4+, CD8+, and γδTCR+ cells from standard-diet calves was similar to or higher when compared with the level of expression by T cells from older cattle. The PWM induced decrease in CD62L expression by CD4+, CD8+, and γδTCR+ cells from 8 wk old-standard-fed calves was more characteristic of responses of adult cattle than the 1-wk old calf.

The neonatal calf has a heightened susceptibility to a variety of infectious diseases. The developmental immaturity of the neonate’s immune system is considered contributory to its increased susceptibility. New strategies are needed to enhance the functional capacity of the calf’s immune system. One possible strategy is to optimize the calf’s protein and energy status through dietary manipulation. In the current experiment, we analyzed the effects of increased dietary protein and energy on mitogen induced CD25, CD44, and CD62L expression by T cell subsets from neonatal calves. Increased dietary protein and energy decreased PWM induced proliferative responses of CD4+, CD8+, and γδTCR+ cells when compared to responses of the same cell populations from standard-fed calves and steers. In addition, the magnitude of PWM induced CD25 expression by CD4+ and CD8+ cells from intensified calves was lower than in CD4+ and CD8+ populations from standard-fed calves. These data suggest that alterations in dietary protein and energy influence markedly CD25 expression by T cell subsets. Although this does not prove a causal relationship, these data suggest that a possible explanation for the differences in proliferative responses may be due
to dietary effects on CD25 expression. Reduced expression of the IL-2 receptor on T cells resulting from increased dietary protein and energy has been described previously in rodents (Iwai and Fernandes, 1989; Peck et al., 2000).

Increased dietary fat has been linked to immunosuppression in rodents characterized by reduced lymphocyte responses to mitogenic stimulation and adhesiveness to endothelial cells (Erickson et al., 1980; Morrow et al., 1985; Sanderson and Calder, 1998). High fat diet-induced immunosuppression may be an effect of certain adipokines, such as leptin and transforming growth factor-β, produced by adipocytes. Leptin links nutritional status with immune functions, acting as a signal to the body of energy stores (reviewed by Otero et al., 2005). Although exogenous leptin has been shown to reverse starvation-induced immunosuppression, (Lord et al., 1998), leptin inhibits proliferative responses of and suppresses IL-2 production by PBMC in vitro (Lord et al., 2002). Interestingly, leptin enhances naïve (CD4+ CD45RA+ T cells) T cell proliferation but inhibits memory (CD4+ CD45RO+ T cells) T cell proliferation (Lord et al., 2002), which may have consequences on vaccine recall responses. In addition, leptin enhances interferon-γ-induced expression of nitric oxide synthase in macrophages (Raso et al., 2002). We have reported previously that high plane of nutrition results in increased in vitro nitric oxide production by PBMC (Nonnecke et al., 2003; Foote et al., 2005). It has been shown that obese mice also have elevated expression of transforming growth factor-β (Samad et al., 1997). Although transforming growth factor-β is known to play an important role in B cell isotype switching and immune regulation, it has been shown to decreases IL-2 production and proliferative responses of CD4+ and CD8+ T cells (McKarns and Schwartz, 2005; Wolfrain et al., 2004). Although neither plasma leptin concentrations or body fat composition were not measured in the current experiment, it has recently been reported that enhanced plane of nutrition increases plasma leptin concentrations and percent body fat composition in preruminant calves and lambs (Block et al., 2003; Ehrhardt et al., 2003).
In the current experiment, feeding an intensified diet to calves decreased CD44 expression on PWM stimulated CD8^+ cells compared with standard-diet calves, and delayed the increase of CD44 expression on PWM stimulated CD4^+ and γδTCR^+ cells PWM compared with standard-diet calves. Diet also affected CD62L expression. Intensified-diet calves had lower CD62L expression on CD8^+ and γδTCR^+ cells in non-stimulated cultures compared with standard-diet calves, suggesting that these two populations may not be cleared efficiently from the circulation when compared with the same populations from standard-diet calves. Decreased CD62L expression on certain lymphocyte subsets from intensified-diet calves may explain the previous observation that intensified-diet calves have a higher percentage of circulating CD4^+ and γδTCR^+ cells compared with standard-diet calves (Foote et al., 2005). Following mitogenic stimulation, however, CD62L expression on CD8^+ cells from intensified-diet calves did not decrease until 6 d of culture, and CD62L on γδTCR^+ cells did not decrease at either time-point. These data suggest that nonstimulated lymphocytes may not be properly cleared from circulation, and supports previous data (Iwai and Fernandes, 1989; Fernandes et al., 1997; Sanderson and Calder, 1998) suggesting increased dietary energy affects negatively responses of PBMC to mitogenic stimulation. This also suggests that although previous data suggest intensified-diet calves have a higher percentage of circulating T cells, the functions of these populations may be compromised. In addition, our observations indicating that proliferative responses and expression of CD25, CD44, and CD62L by T cells from 8 wk-old, standard-diet calves were similar to those of the older cattle suggests that feeding a standard milk replacer (i.e. 20% CP, 20% fat, 0.45 kg/d) does not produce a state of protein and energy malnutrition. The relationship between neonatal nutrition and immune competency and adaptive immune responses, however, requires further investigation.

Lymphocytes from 1-wk old calves exhibited reduced proliferative responses, reduced expression of CD25 and CD44, and increased expression of CD62L following in vitro stimulation with PWM. In addition, neonatal nutrition influenced mitogen induced proliferative responses and
expression of the activation antigens, CD25, CD44, and CD62L. In conclusion, these results suggest that animal maturity and neonatal nutrition influence functional activities of T lymphocyte subsets essential in the development of CMI with possible consequences to the calf's susceptibility to infectious disease. A fine line may exist between adequate nutrition and over nutrition in the neonatal calf. Care must be taken to ensure dairy calves, born with little body fat, receive adequate nutrients for maintenance, especially in cold weather to avoid protein energy malnutrition. However, rapid growth rates or over nutrition may not be beneficial to the developing immune system.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Primary and secondary antibodies used in the flow cytometric analysis of PBMC populations from neonatal calves and steers.

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<th>Primary antibody specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Secondary antibody$^{2,3}$</th>
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<td>CD4$^+$</td>
<td>GC50A1</td>
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<td>IgM-APC</td>
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<td>BAQ111A</td>
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<td>CD62L</td>
<td>BAQ92A</td>
<td>IgG1</td>
<td>IgG1-PE</td>
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1 Primary mouse-generated monoclonal antibodies from VMRD, Pullman WA
2 Rat anti-mouse IgM-allophycocyanin (APC) [~0.2 μg/well] from Pharmingen, San Diego, CA
3 Goat anti-mouse IgG1-phycoerythrin (PE) [1 μg/well] from Southern Biotechnology Associates, Birmingham, AL
Table 2. Expression (mean fluorescence intensity) of CD25 on T cell subsets from steers and 1-wk-old calves.\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>3 d cultures</th>
<th>6 d cultures</th>
<th>Mitogen Effect\textsuperscript{3}</th>
<th>P &gt; F\textsubscript{i}</th>
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<td>PWM</td>
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<tr>
<td>Steers</td>
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<td>120 ± 4</td>
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<td>74 ± 24</td>
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<td>CD8\textsuperscript{+}</td>
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<td>Steers</td>
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<td>γδTCR\textsuperscript{+}</td>
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\textsuperscript{1}Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) and stimulated with pokeweed mitogen (PWM) for 3 and 6 d.
\textsuperscript{2}Values represent mean fluorescence intensity (mean ± SE).
\textsuperscript{3}P values
\textsuperscript{*}Values for a specific T cell subset within a column differ, P < 0.05.
\textsuperscript{†}Values for a specific T cell subset within a column tend to differ, P < 0.10.
Table 3. Expression (mean fluorescence intensity) of CD44 on T cell subsets from steers and 1-wk-old calves.  

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<th>6 d cultures</th>
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<td>Mitogen Effect</td>
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<td>Calves</td>
<td>144 ± 15</td>
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<sup>1</sup>Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) and stimulated with pokeweed mitogen (PWM) for 3 and 6 d.

<sup>2</sup>Values represent mean fluorescence intensity (mean ± SE).

<sup>3</sup>P values

<sup>4</sup>Values for a specific T cell subset within a column differ, P < 0.05.
Table 4. Expression (mean fluorescence intensity) of CD62L on T cells subsets from steers and 1-wk-old calves.$^{1,2}$

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<th>Mitogen Effect$^3$</th>
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<tr>
<td></td>
<td>NS</td>
<td>PWM</td>
<td>P &gt; F$_i$</td>
<td>NS</td>
</tr>
<tr>
<td>CD4$^+$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>41 ± 3</td>
<td>34 ± 1</td>
<td>0.099</td>
<td>92 ± 19</td>
</tr>
<tr>
<td>Calves</td>
<td>47 ± 16</td>
<td>49 ± 14</td>
<td>0.92</td>
<td>124 ± 76</td>
</tr>
<tr>
<td>CD8$^+$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>83 ± 12</td>
<td>96 ± 16</td>
<td>0.13</td>
<td>165 ± 24</td>
</tr>
<tr>
<td>Calves</td>
<td>81 ± 18</td>
<td>161 ± 42</td>
<td>0.54</td>
<td>172 ± 68</td>
</tr>
<tr>
<td>$\gamma$$\delta$TCR$^+$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>153 ± 34</td>
<td>294 ± 40</td>
<td>&lt; 0.05</td>
<td>193 ± 17</td>
</tr>
<tr>
<td>Calves</td>
<td>233 ± 37</td>
<td>268 ± 57</td>
<td>0.63</td>
<td>308 ± 86</td>
</tr>
</tbody>
</table>

$^1$Cells from calves (n = 4) and steers (n = 4) were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d.

$^2$Values represent mean fluorescence intensity (mean ± SE).

$^3$P values
Table 5. Expression (mean fluorescence intensity) of CD25 on T cell subsets from steers and calves 7 wk after initiation of dietary treatments.\(^1,2\)

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>3 d cultures</th>
<th>Mitogen Effect(^3)</th>
<th>6 d cultures</th>
<th>Mitogen Effect(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>PWM</td>
<td>P &gt; F(_i)</td>
<td>NS</td>
</tr>
</tbody>
</table>

- **CD4\(^+\)**
  - Steers: 131 ± 14, 371 ± 35\(^b\), < 0.001, 391 ± 97\(^b\), 1567 ± 208, < 0.01
  - Standard calves: 122 ± 16, 749 ± 77\(^a\), < 0.001, 776 ± 135\(^a\), 1659 ± 121, < 0.01
  - Intensified calves: 102 ± 19, 477 ± 119\(^b\), < 0.05, 662 ± 125\(^b\), 1331 ± 174, < 0.05

- **CD8\(^+\)**
  - Steers: 22 ± 2, 464 ± 50\(^b\), < 0.0001, 46 ± 7\(^b\), 1065 ± 130, < 0.001
  - Standard calves: 33 ± 9, 708 ± 26\(^a\), < 0.0001, 86 ± 20\(^b\), 1194 ± 270, < 0.01
  - Intensified calves: 45 ± 19, 364 ± 111\(^b\), < 0.05, 129 ± 9\(^a\), 829 ± 128, < 0.01

- **γδTCR\(^+\)**
  - Steers: 51 ± 10, 297 ± 24, < 0.0001, 40 ± 4\(^b\), 928 ± 224\(^f\), < 0.01
  - Standard calves: 86 ± 44, 347 ± 34, < 0.01, 69 ± 18\(^a\), 627 ± 87, < 0.001
  - Intensified calves: 127 ± 33, 282 ± 45, < 0.05, 95 ± 8\(^a\), 496 ± 81, < 0.01

\(^1\)Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves (n = 4) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves (n = 4) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers (n = 4) were fed a typical diet to allow for moderate growth.

\(^2\)Values represent mean fluorescence intensity (mean ± SE).

\(^3\)P values

\(^{a,b}\) For a specific T cell subset within a column, values with differing superscript differ, P < 0.05.

\(^{f}\)CD25 mean fluorescence intensity on nonstimulated CD8\(^+\) cells from steers tends to differ from standard-diet calves, P < 0.1.

\(^{f}\)CD25 mean fluorescence intensity on PWM-stimulated γδTCR\(^+\) from steers tends to differ from intensified-diet calves, P < 0.1.
Table 6. Expression (mean fluorescence intensity) of CD44 on T cell subsets from steers and calves 7 wk after initiation of dietary treatments.\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>3 d cultures</th>
<th>Mitogen Effect\textsuperscript{3}</th>
<th>6 d cultures</th>
<th>Mitogen Effect\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>PWM</td>
<td>P &gt; F\textsubscript{i}</td>
<td>NS</td>
</tr>
<tr>
<td>CD4\textsuperscript{+}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>1244 ± 126\textsuperscript{a} 1241 ± 143</td>
<td>0.99 1033 ± 165\textsuperscript{b} 3060 ± 88</td>
<td>&lt;0.0001</td>
<td>1241 ± 143</td>
</tr>
<tr>
<td>Standard calves</td>
<td>959 ± 99\textsuperscript{ab} 1661 ± 160</td>
<td>&lt;0.01 2010 ± 207\textsuperscript{a} 3071 ± 196</td>
<td>&lt;0.01</td>
<td>2010 ± 207\textsuperscript{a} 3071 ± 196</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>634 ± 182\textsuperscript{b} 1278 ± 356</td>
<td>0.16 1824 ± 193\textsuperscript{a} 2665 ± 108\textsuperscript{b}</td>
<td>&lt;0.01</td>
<td>1824 ± 193\textsuperscript{a} 2665 ± 108\textsuperscript{b}</td>
</tr>
<tr>
<td>CD8\textsuperscript{+}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>500 ± 62</td>
<td>1365 ± 181\textsuperscript{ab} &lt;0.01</td>
<td>627 ± 81\textsuperscript{b} 2717 ± 444</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Standard calves</td>
<td>535 ± 105</td>
<td>1643 ± 78\textsuperscript{a} &lt;0.001</td>
<td>925 ± 240</td>
<td>2757 ± 246</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>511 ± 130 1088 ± 166\textsuperscript{b} &lt;0.05</td>
<td>1040 ± 898</td>
<td>1983 ± 140</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>γδTCR\textsuperscript{+}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>677 ± 79\textsuperscript{a} 636 ± 31</td>
<td>0.65 402 ± 28\textsuperscript{b} 2310 ± 434\textsuperscript{a} &lt;0.01</td>
<td>&lt;0.05</td>
<td>636 ± 31</td>
</tr>
<tr>
<td>Standard calves</td>
<td>446 ± 33</td>
<td>628 ± 9</td>
<td>&lt;0.01</td>
<td>672 ± 118</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>614 ± 123 646 ± 28</td>
<td>0.81 589 ± 90</td>
<td>1315 ± 178\textsuperscript{b} &lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves (n = 4) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves (n = 4) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers (n = 4) were fed a typical diet to allow for moderate growth.

\textsuperscript{2}Values represent mean fluorescence intensity (mean ± SE).

\textsuperscript{3}Values represent P values

\textsuperscript{ab} For a specific T cell subset within a column, values with differing superscript differ, P < 0.05.

\textsuperscript{*}CD44 mean fluorescence intensity on nonstimulated γδTCR\textsuperscript{+} cells from steers tends to differ from standard-diet calves, P < 0.1.

\textsuperscript{†}CD44 mean fluorescence intensity on nonstimulated CD8\textsuperscript{+} cells from steers tends to differ from intensified-diet calves, P < 0.1.

\textsuperscript{‡}CD44 mean fluorescence intensity on PWM-stimulated CD4\textsuperscript{+} cells from intensified-diet calves tends to differ from steers and standard-diet calves, P < 0.1.
Table 7. Expression (mean fluorescence intensity) of CD62L on T cell subsets from steers and calves 7 wk after initiation of dietary treatments.\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>3 d cultures</th>
<th>6 d cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitogen Effect\textsuperscript{1}</td>
<td>P &gt; F\textsubscript{i}</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>PWM</td>
</tr>
<tr>
<td>CD4\textsuperscript{+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>269 ± 44\textsuperscript{a}</td>
<td>55 ± 7\textsuperscript{b}</td>
</tr>
<tr>
<td>Standard calves</td>
<td>210 ± 47\textsuperscript{ab}</td>
<td>108 ± 10\textsuperscript{a}</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>114 ± 16\textsuperscript{b}</td>
<td>48 ± 8\textsuperscript{b}</td>
</tr>
<tr>
<td>CD8\textsuperscript{+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>529 ± 71\textsuperscript{a}</td>
<td>130 ± 12</td>
</tr>
<tr>
<td>Standard calves</td>
<td>449 ± 71\textsuperscript{a}</td>
<td>234 ± 36\textsuperscript{*}</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>222 ± 50\textsuperscript{b}</td>
<td>136 ± 45</td>
</tr>
<tr>
<td>(\gamma\delta)TCR\textsuperscript{+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>623 ± 59</td>
<td>324 ± 42</td>
</tr>
<tr>
<td>Standard calves</td>
<td>561 ± 84</td>
<td>616 ± 108\textsuperscript{f}</td>
</tr>
<tr>
<td>Intensified calves</td>
<td>450 ± 82</td>
<td>483 ± 110</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Cells were nonstimulated (NS) or stimulated with pokeweed mitogen (PWM) for 3 and 6 d. Standard-diet calves (n = 4) were fed a 20% CP, 20% fat milk replacer fed at 0.45 kg/d and intensified-diet calves (n = 4) were fed a 28% CP, 20% fat milk replacer fed at 1.14 kg/d. Steers (n = 4) were fed a typical diet to allow for moderate growth.

\textsuperscript{2}Values represent mean fluorescence intensity (mean ± SE).

\textsuperscript{3}Values represent P values.

\textsuperscript{ab} For a specific T cell subset within a column, values with differing superscript differ P < 0.05.

\textsuperscript{*} CD62L mean fluorescence intensity on PWM-stimulated CD8\textsuperscript{+} cells from Standard-diet calves had a tendency to be higher than Steers and Intensified-diet calves, P < 0.1.

\textsuperscript{1}CD62L mean fluorescence intensity on \(\gamma\delta\)TCR\textsuperscript{+} T cells from standard-diet calves tends to differ from steers, P < 0.1.

\textsuperscript{1}CD62L mean fluorescence intensity on CD8\textsuperscript{+} cells from intensified-diet calves tends to differ from steers, P < 0.1.
Figure 1. Pokeweed mitogen induced proliferative responses of peripheral blood CD4⁺, CD8⁺, and γδTCR⁺ T lymphocytes from 1-wk old calves (n = 4) and steers (5 to 6 months of age, n = 4). T cell responses are presented as percentages proliferating (panel a) and as proliferation indices (panel b). Mononuclear cell cultures were incubated for 6 d. Values represent proliferative responses (mean ± SEM) of PWM stimulated cultures minus responses of nonstimulated cultures.

Figure 2. Pokeweed mitogen induced proliferative responses of peripheral blood CD4⁺, CD8⁺, and γδTCR⁺ lymphocytes from standard- (n = 4) and intensified-diet (n = 4) calves, and steers (n = 4) at wk 7 of the trial. Calves were 8 wk of age at the time of blood collection. T cell responses are presented as percentages proliferating (panel a) and as proliferation indices (panel b). Mononuclear cell cultures were incubated for 6 d. Values represent proliferative responses (mean ± SEM) of PWM stimulated cultures minus responses of nonstimulated cultures.
Figure 1. Pokeweed mitogen induced proliferative responses of peripheral blood CD4⁺, CD8⁺, and γδTCR⁺ T lymphocytes from 1-wk old calves (n = 4) and steers (5 to 6 months of age, n = 4).
Figure 2. Pokeweed mitogen induced proliferative responses of peripheral blood $\text{CD}^+$, $\text{CD}^8^+$, and $\gamma\delta$TCR$^+$ lymphocytes from standard- ($n = 4$) and intensified-diet ($n = 4$) calves, and steers ($n = 4$) at wk 7 of the trial.
CHAPTER 3. EFFECTS OF INCREASED DIETARY PROTEIN AND ENERGY ON COMPOSITION AND FUNCTIONAL CAPACITIES OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM VACCINATED NEONATAL CALVES

A paper accepted by the International Journal of Vitamin and Nutrition Research

Monica R. Foote¹, Brian J. Nonnecke², W. Ray Waters, Mitchell V. Palmer, Donald C. Beitz, Mike A. Fowler, Bill L. Miller, Tom E. Johnson, H. Bruce Perry
¹Primary author, ²Corresponding author

Abstract

Effects of increased protein and energy provided by an intensified milk replacer on antigen specific, cell-mediated immune response of the neonatal calf were examined. Calves were fed a standard (0.45 kg/d of a 20% crude protein, 20% fat milk replacer; n = 11) or intensified (1.14 kg/d of a 28% crude protein, 20% fat milk replacer; n = 11) diet from 1 to 6 wk of age. All calves were vaccinated with Mycobacterium bovis, bacillus Calmette-Guerin (BCG) at 1 wk of age. The daily weight gain of intensified-diet calves (0.62 kg/d) was greater than the weight gain of standard-diet calves (0.29 kg/d). Liver, kidney, heart, thymus, and subcervical lymph node from intensified-diet calves were heavier than the same organs from standard-diet calves. Flow cytometric analysis of peripheral blood mononuclear cell (PBMC) populations indicated that CD4⁺ cell, γδTCR⁺ cell, and monocyte percentages, although unaffected by diet during the first 5 wk of the study, were higher in intensified-diet calves at wk 6. The decline in γδTCR⁺ cell percentages and increase in B cell percentages with increasing age seen in all calves are characteristic of the maturing immune system of the calf. CD8⁺ T cell or B cell percentages were not affected by diet. In intensified-diet calves, percentages of CD4⁺ expressing interleukin-2 receptor increased and percentages of γδTCR⁺ cells expressing interleukin-2 receptor decreased with time. The same populations in standard-diet calves did not change with time.
Percentages of CD4+ and CD8+ T cells, and B cells expressing MHC class II antigen were unaffected by diet or age. Although mitogen-induced interferon (IFN)-γ and nitric oxide (NO) secretion increased with age for all calves, PBMC from intensified-diet calves produced less IFN-γ and more NO than did cells from standard-diet calves at wk 6 of the study. Antigen-induced secretion of IFN-γ and NO also increased with age but was unaffected by diet. Antigen-elicited delayed-type hypersensitivity was unaffected by diet suggesting increased dietary protein and energy did not alter adaptive immunity in vivo. Overall, these results suggest that feeding calves a commercially available, intensified milk replacer affects minimally the composition and functional capacities of PBMC populations. Additional research is necessary to determine whether these subtle effects influence the calf's susceptibility to infectious disease.

**Key words:** preruminant calf, cell-mediated immunity, milk replacer, *Mycobacterium bovis* bacillus Calmette-Guerin

**Introduction**

Current recommendations for protein and energy requirements of the young calf have been shown to be inadequate for optimal growth [1]. Based on controlled studies [1-3] and in-house trials demonstrating the growth-promoting effects of increased dietary protein and energy, several companies now market intensified milk replacer in the United States (e.g., Cows Match, Land O'Lakes, Inc., Milk Products Co, Fort Dodge, IA; Advance Excelerate Calf Milk Replacer, Milk Specialties, Dundee, IL; and Super Star 25-15, Merrick's, Inc., Middleton, WI). Although the beneficial effects of intensified nutrition on growth performance of neonatal calves are documented; effects on immune function are not well characterized.

Nutritional status influences immune function [4-6]. Prepubescent protein-energy malnutrition depresses thymus-dependent immunity [7]. Evidence suggests that protein energy...
malnutrition results in reduced cell-mediated immune (CMI) responses in young calves [8]. There is little information available, however, on the effects of protein and energy provided in amounts exceeding NRC recommendations on the immune system of the neonatal calf. Peripheral blood mononuclear cells (PBMC) from calves fed increased dietary energy and protein produce less interferon (IFN)-γ and more nitric oxide (NO) in response to polyclonal stimulation in vitro than do cells from calves fed less energy and protein [9]. Both functions are intimately associated with development of CMI.

Effects of plane of nutrition on antigen-specific CMI responses of vaccinated, milk replacer-fed calves, however, have not been investigated. Vaccination with Mycobacterium bovis bacillus Calmette-Guerin (BCG) is commonly used in the prevention of tuberculosis. In mice, strong IFN-γ and NO responses are essential in resistance to tuberculosis [10, 11]. Sensitization to M. bovis BCG and elicitation of recall responses (i.e., IFN-γ and NO) by mycobacterial antigen have been used to model CMI responses in cattle [12, 13]. We hypothesized that increasing dietary protein and energy would enhance antigen-specific, CMI responses in vaccinated calves. The objective of the present study was to determine whether increased nutrient supply provided by milk replacer alters growth performance, in vitro antigen-induced T cell function, and cutaneous delayed-type hypersensitivity reactions in young calves vaccinated with M. bovis BCG.

Materials and Methods

Calves and dietary treatments: Twenty-two Holstein bull calves weighing 43-47 kg and ranging from 3-10 d of age were housed at the Land O’ Lakes, Inc. Research Farm, Webster City, IA. All animals were cared for following institutional animal care and use guidelines. Calves were housed on raised stalls in an environmentally controlled building. Upon arrival, adequate colostrum intake was
verified by serum gamma globulin analysis (Sure-Tech Labs, Fort Dodge, IA). Only calves demonstrating adequate passive immunity were utilized.

Calves were allotted randomly to a standard (n = 11) or intensified (n = 11) diet. Calves allotted to the standard diet were fed 0.45 kg each day (in dry matter) of a 20% crude protein (CP), 20% fat industry-standard milk replacer. Standard-diet calves were also offered calf starter (18% CP) on a restricted basis. The intensified diet is representative of commercially available “intensified” milk replacers in the USA. Calves allotted to the intensified diet were fed 1.14 kg each day (in dry matter) of a 28% CP, 20% fat milk replacer. Intensified-diet calves were also offered calf starter (22% CP) ad libitum. Fresh water was available at all times. Dietary treatments began within 24 h after arrival and immediately after vaccination with \( M. \text{bovis} \) BCG. Dietary treatments were administered until calves were 6 wk of age. Calves were weighed upon arrival, before initiation of the trial, and weekly thereafter.

Calves were euthanized (Sleepaway®, Fort Dodge Laboratories, Fort Dodge, IA) at the end of the experiment and the weights of liver, adrenal, pancreas, thyroid, kidney, heart, thymus, subcervical lymph node, and prefemoral lymph node were recorded.

**Vaccine preparation and administration:** The \( M. \text{bovis} \) BCG (Pasteur strain) was grown in Middlebrook’s 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO). Mid-log phase grown bacilli were pelleted by centrifugation at 750 × g, washed twice with phosphate-buffered saline (PBS; 0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 mL of PBS. Enumeration of bacilli was by serial dilution plate counting on Middlebrook’s 7H11 selective media (Becton Dickinson, Cockeysville, MD). At approximately 1 wk of age, all calves (n = 22) were vaccinated subcutaneously in the right mid-cervical region with a single dose containing \( 1 \times 10^5 \) colony-forming units of \( M. \text{bovis} \) BCG.
Blood mononuclear cell recovery and enrichment: Peripheral blood was collected from calves immediately before vaccination and initiation of treatments (approximately 1 wk of age) and at 3, 5, and 6 wk of age. Sixty milliliters of blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose [a sterilized solution containing sodium citrate (77 µmol/L), citric acid (38 µmol/L), and dextrose (122 µmol/L)] by jugular venipuncture. Smaller, anticoagulated (anticoagulant: potassium ethylenediaminetetraacetic acid) blood samples were collected into 5-mL vacutainers (Becton Dickinson, Franklin Lakes, NJ).

Cells used in IFN-γ and NO secretion assays were isolated from peripheral blood and enriched by density gradient centrifugation as described previously [14]. The PBMC-enriched populations were resuspended in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Sigma, St. Louis, MO) antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin, Sigma), 50 µM 2-mercaptoethanol (Sigma), 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT).

Analysis of blood mononuclear leukocyte population composition: A subset of calves (n = 16, 8 calves per treatment) was used for collecting blood composition data. The composition of PBMC populations was evaluated by flow cytometry as described [9]. Approximately $5 \times 10^5$ cells in 100 µL of potassium ethylenediaminetetraacetic acid-anticoagulated blood were added to each of 8 wells of a 96-well, round-bottom microtiter plate (Costar, Cambridge, MA). Individual wells were preloaded with a 50-µL aliquot of monoclonal antibody (Table 1) diluted in PBS containing 0.02% NaN₃ and 1% inactivated fetal bovine serum. After the cells were incubated for 15 min at room
temperature, the plate was centrifuged (400 × g for 2 min at room temperature). Supernatant was
decanted, contaminating erythrocytes were removed from wells by hypotonic lysis, and the plate was
centrifuged again as described above. Supernatant was decanted, and cells were resuspended in 100 
µL of each of two isotype-specific antibodies conjugated to fluorochromes (Table 1). Secondary
antibodies were diluted in PBS containing 0.02% NaN₃ and 1% inactivated fetal bovine serum. Cells
were incubated for 15 min at room temperature in the dark and were centrifuged as described above.
Cells were resuspended in 200 µL of FacsLyse buffer (Becton Dickinson, San Jose, CA) and stored in
the dark at 4°C until examined on the flow cytometer. Five thousand cells exhibiting light scattering
properties consistent with bovine mononuclear cells were analyzed. Data were acquired using a
BDLSR flow cytometer (Becton Dickinson, San Jose, California) and analyzed using FlowJo (Tree
Star Inc., San Carlos, CA), and CellQuest software (Becton Dickinson, San Jose, California).
Variables recorded for each marker were percentages of cells that stained positive.

In vitro production and measurement of IFN-γ: A subset of calves (n = 16, 8 calves per treatment)
was used for collecting in vitro IFN-γ secretion data. IFN-γ secretion by nonstimulated, antigen- and
mitogen-stimulated PBMC cultures was evaluated in 96-well, flat-bottom tissue culture plates. Wells
were seeded with 4 × 10⁵ cells in a total culture volume of 200 µL (final concentration of 2 × 10⁶
cells/mL in culture). Culture medium consisted of supplemented RPMI 1640 (Gibco Laboratories) as
described for PBMC isolation and enrichment. Cultures were either nonstimulated (medium alone),
stimulated with PWM (1 µg/mL, Sigma) or with M. bovis PPD (a filtrate of virulent M. bovis, 10 
µg/mL, CSL Ltd.). Triplicate wells were prepared for each in vitro treatment. Plates were incubated
at 39°C in a humidified atmosphere of 5% CO₂ for 72 h. Cultures were centrifuged (400 × g for 2
min at room temperature), supernatants were harvested (100 µL/well) and stored at −80°C until later
analysis for IFN-γ. The IFN-γ concentration (ng/mL) in culture supernatants was determined by using an IFN-γ capture ELISA as reported previously [15].

In vitro production and measurement of inducible NO: A subset of calves (n = 16, 8 calves per treatment) was used for collecting in vitro NO secretion data. Secretion of nitrite, the stable oxidation product of NO, by nonstimulated and antigen- and mitogen-stimulated PBMC cultures was evaluated in 96-well flat-bottom tissue culture plates. Wells were seeded with $4 \times 10^5$ cells in a total culture volume of 200 μL (final concentration of $2 \times 10^6$ 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 1 μg/mL PWM (Sigma) or with 10 μg/mL M. bovis PPD (CSL Ltd.). Triplicate wells were prepared for each treatment. N^G- Monomethyl-L-arginine (L-NMMA), a competitive inhibitor of inducible NO synthase, was used as a negative control. The L-NMMA was added to parallel nonstimulated or stimulated cultures to verify that the nitrite production was due to inducible NO synthase. Plates were incubated at 39°C in a humidified atmosphere of 5% CO₂ for 72 h. The amount of nitrite in culture supernatants was measured by a modification of the method of Green et al. [16] as described previously by [17].

Measurement of delayed-type hypersensitivity in vivo: In vivo sensitization to M. bovis BCG was evaluated by using the comparative cervical skin test following Bovine Tuberculosis Eradication Uniform Methods and Rules (Animal and Plant Health Inspection Service brochure #91-45-011). This test utilizes intradermal injection of biologically balanced United States Department of Agriculture bovine and avian PPD tuberculin. Calves (n=22) were injected intradermally with 100 μL of M. bovis and M. avium PPD at separate sites in the mid-cervical region 5 wk following M. bovis BCG vaccination (6 wk of age). Skin-fold thickness was measured immediately before
administration of PPD and 72 h later. Responses were estimated by subtracting pre-test skin fold thickness from skin fold thickness at 72 h after administration of antigen.

**Statistical analysis:** 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. Growth, composition of PBMC populations, and *in vitro* functional capacities of PBMC populations were analyzed as a split-plot with repeated measures ANOVA. The model included the fixed effects of dietary treatments (standard vs. intensified diet), time (wk on experiment), and the treatment × time interaction, and calf was included in the model as the random effect. Fisher's protected-LSD test was applied when significant effects ($P < 0.05$) and trends ($P < 0.10$) were detected by the model. Delayed-type hypersensitivity and organ weight data were analyzed as a split-plot with factorial ANOVA. Dietary treatment and calf were included in the model as fixed and random effects, respectively. Fisher's protected-LSD test was applied when significant effects ($P < 0.05$) and trends ($P < 0.10$) were detected by the model.

**Results and Discussion**

**Growth performance of calves:** Mean body weights of standard-diet calves and intensified-diet calves were not different ($P > 0.05$) at the beginning of the study (wk 1) (Fig. 1). Mean body weights of intensified-diet calves were higher ($P < 0.01$) than mean body weights of standard-diet calves by wk 2 of age. From wk 3 to wk 6, intensified-diet calves were heavier ($P < 0.001$) than standard-diet calves. The average daily gain of intensified-diet calves (0.62 kg) was greater ($P < 0.0001$) than that of standard-diet calves (0.29 kg) across the experimental period. It has been shown previously that calves fed intensified milk replacers have greater average daily weight gain and deposit more lean tissue than do calves fed diets following current recommendations of the NRC [1-3]. In the current experiment, daily growth rates (0.29 kg and 0.62 kg for standard and intensified diets, respectively)
were not as high as observed previously (0.55 kg and 1.2 kg for standard and intensified diets, respectively) [9]. These differences can be explained by the fact that calves in the current experiment were fed a static amount of milk replacer, whereas calves in the previous experiment were fed on a body weight basis.

Effects of intensified nutrition on weights of several organs are shown in Table 2. Increased dietary protein and energy resulted in heavier \((P < 0.05)\) weights of metabolically important organs including liver, adrenal gland, kidney, and heart, and immunologically important organs including subcervical lymph node and thymus. Weights of prefemoral lymph node, pancreas, or thyroid gland were unaffected \((P > 0.05)\) by dietary treatment. In neonatal sheep, it has been shown recently that rapidly reared lambs have a larger liver, kidney, and thymus than do slowly reared lambs [18]. Thymic involution occurs in the young animal and is expected. It is unknown whether the effects of diet on thymus weight affect thymus-dependent immunity in these young animals.

**Composition of blood mononuclear cell populations:** The relative contribution of CD4\(^+\) cells to the total PBMC population and the expression of the activation marker IL-2\(r\) are provided in Figure 2. Percentages of CD4\(^+\) cells in standard-diet and intensified-diet calves increased \((P < 0.01 \text{ and } P < 0.0001, \text{ respectively})\) with time (Fig. 2a), suggesting maturational changes in the composition of blood mononuclear cell populations with time. Percentages of CD4\(^+\) cells in intensified-diet calves increased \((P < 0.01)\) from wk 5 to wk 6, whereas percentages of CD4\(^+\) cells in standard-diet calves did not change \((P > 0.1)\) from wk 5 to wk 6. By wk 6, percentages of CD4\(^+\) cells in intensified-diet calves were higher \((P < 0.05)\) than in standard-diet calves. Dual-color flow cytometric analysis to assess the expression of the activation markers IL-2\(r\) and MHC class II indicated that, although percentages of CD4\(^+\), \(\gamma \delta \text{TCR}^+\), B cells, and monocytes expressing the activation marker MHC class II were not affected by dietary treatment or time (data not shown), percentages of IL-2\(r^+\), CD4\(^+\) cells in intensified-diet calves increased \((P < 0.01)\) with time whereas the percentage of IL-2\(r^+\), CD4\(^+\) cells in
standard-diet calves did not change with time (Fig. 2b). This suggests that feeding an intensified diet early in life may enhance early maturation of the CD4\(^+\) subset and may increase the capacity of CD4\(^+\) cells to respond to IL-2, which is necessary for lymphocyte proliferative responses.

The relative contribution of CD8\(^+\) cells to the total PBMC population was not affected by diet (data not shown). Percentages of CD8\(^+\) cells, however, did change with time (data not shown). Percentages of CD8\(^+\) cells in milk replacer-fed calves increased \((P < 0.01)\) with time and are higher at wk 5 than any other time-point (data not shown). More detailed examination of the phenotypes of circulating CD4\(^+\) and CD8\(^+\) cells may have revealed even further insight into the abundance of naïve versus memory T cells as seen in experiments with rodents suggesting an overabundance of naïve versus memory/effector T cell phenotypes in the blood and lymphoid tissues during protein and energy malnutrition [19, 20]. The effect of providing protein and energy in amounts exceeding NRC recommendations on the abundance of naïve versus memory T cells needs to be investigated.

Percentages of \(\gamma\delta\text{TCR}^+\) cells in standard-diet and intensified-diet calves decreased \((P < 0.0001)\) with time (Fig. 3a). Percentages of \(\gamma\delta\text{TCR}^+\) cells in standard-diet calves decreased \((P < 0.05)\) from wk 5 to wk 6, whereas percentages of \(\gamma\delta\text{TCR}^+\) cells in intensified-diet calves did not change \((P > 0.05)\). By wk 6 percentages of \(\gamma\delta\text{TCR}^+\) cells in intensified-diet calves were higher \((P < 0.05)\) than in standard-diet calves. Although intensified-diet calves had higher percentages of circulating \(\gamma\delta\text{TCR}^+\) cells, percentages of IL-2r\(^+\), \(\gamma\delta\text{TCR}^+\) cells from intensified-diet calves decreased \((P < 0.05)\) whereas percentages of IL-2r\(^+\), \(\gamma\delta\text{TCR}^+\) cells from standard-diet calves did not change with time (Fig. 3b). By wk 6 there was a trend for percentages of IL-2r\(^+\), \(\gamma\delta\text{TCR}^+\) cells from intensified-diet calves to be lower \((P < 0.1)\) than standard-diet calves (Fig. 3b). Percentages of \(\gamma\delta\text{TCR}^+\) cells are higher in neonatal cattle and decreases with age [21, 22], and thus a decrease in the presence of \(\gamma\delta\text{TCR}^+\) cells suggests maturation of PBMC populations. Although the exact role and function of the \(\gamma\delta\text{TCR}^+\) cell remains uncertain, it has been suggested that the \(\gamma\delta\text{TCR}^+\) cell plays an important role in the anti-
tubercular response [23, 24]. Protein energy malnutrition has been shown to decrease circulating $\gamma\delta$TCR$^+$ T cells in cattle experimentally infected with virulent $M. bovis$, suggesting that protein and energy status affects adaptive immune responses important in antimycobacterial activity [25]. Results from the present study suggest that providing protein and energy in amounts exceeding NRC recommendations increases a cell population ($\gamma\delta$TCR$^+$ T cell) in the neonatal BCG-vaccinated calf that is important in antimycobacterial activity. The activity and/or functional capacity of this population in the intensified-diet calves, however, may be hindered by the reduced expression of IL-2r.

Relative contributions of B cells and monocytes to the total PBMC population are provided in Figure 4. Because the dietary treatment and dietary treatment $\times$ time effects were not significant, only age-related changes in percentages of B cells in milk replacer-fed calves are shown in Figure 4a. Percentages of B cells in standard-diet and intensified-diet calves increased ($P < 0.05$) with time. Similar age-dependent increases in the proportion of B cells in young calves have been reported [26-28] and are indicative of the maturation of the calf's immune system.

Monocytes, the circulating form of macrophages, are phagocytic cells capable of producing reactive oxygen and nitrogen (i.e. NO) species. Percentages of monocytes in standard-diet and intensified-diet calves decreased ($P < 0.01$) with time (Fig. 4b). Higher ($P < 0.05$) percentages of monocytes in standard-diet calves compared with intensified-diet calves during wk 3 and wk 5 may be indicative of a state of immune activation due to exposure to the range of microbes present in the environment. Percentages of monocytes in standard-diet calves decreased ($P < 0.05$) from wk 5 to wk 6, whereas percentages of monocytes in intensified-diet calves did not change from wk 5 to wk 6, resulting in a trend ($P < 0.1$) for lower percentages of monocytes in standard-diet calves compared with intensified-diet calves at wk 6.
Interferon-γ and NO production by blood mononuclear cells: It has been shown previously that *M. bovis* vaccinated calves exhibit strong CMI responses to recall antigen PPD [29, 30]. In the present study, a BCG sensitization and challenge model [29] was used to evaluate effects of increased dietary protein and energy on adaptive, cell-mediated immune responses of milk replacer-fed dairy calves. Using this model, we evaluated IFN-γ and NO production in antigen-stimulated PBMC cultures, functions intimately associated with CMI. Because responses of adult PBMC to PWM are typically vigorous [31-33], the response to PWM served as a positive control for cell function in each assay.

Effects of dietary treatments on secretion of IFN-γ in PWM- and PPD-stimulated cell cultures are shown in Figure 5. Values represent the amount of IFN-γ secreted in stimulated cell cultures minus the amount of IFN-γ secreted in nonstimulated cell cultures. Before initiation of dietary treatments and vaccination (wk 1), secretion of IFN-γ by PWM-stimulated PBMC from standard-diet and intensified-diet calves was not different (*P* > 0.05) (Fig. 5a). PWM-induced interferon (IFN)-γ secretion increased (*P* < 0.05) with age for all calves, suggesting maturity of the immune response. Split-plot analysis indicates that secretion of IFN-γ by PWM-stimulated PBMC from standard-diet calves increased with time (*P* < 0.05), whereas IFN-γ secretion by PBMC from intensified-diet calves did not change (*P* > 0.05) with time. By wk 6, PBMC from intensified-diet calves secreted less (*P* < 0.05) IFN-γ in response to PWM stimulation than did cells from standard-diet calves. Secretion of IFN-γ by PWM-stimulated PBMC from milk replacer-fed calves is not affected by BCG vaccination [29]. The increased capacity for PBMC from standard-diet calves to secrete IFN-γ can not be explained by the diet-induced PBMC compositional changes resulting in increased presence of circulating CD4+ cells, γδTCR+ cells, and monocytes in intensified-diet calves. More detailed examination of functional capacities of specific phenotypes (i.e. intracellular IFN-γ staining of T cell subsets) would provide further insight. These results, combined with the observation of decreased IL-
2r⁺, γδTCR⁺ cells, suggest that although intensified nutrition results in the increased representation of certain PBMC subsets, specific functional capacities may be hindered.

Dietary treatment did not affect (P > 0.05) antigen (i.e. PPD)-induced, IFN-γ secretion (Fig. 5b). Secretion of IFN-γ by PPD-stimulated PBMC from standard-diet and intensified-diet calves increased (P < 0.05) with time and was greater (P < 0.05) at wk 5 and wk 6 compared with wk 1. The diet x time interaction for PPD-induced IFN-γ was not significant (P > 0.05) suggesting that differences in protein and energy between the two treatments were not sufficient to influence antigen-specific IFN-γ production.

Effects of dietary treatments on NO secretion in PWM- and PPD-stimulated cell cultures are shown in Figure 6. Values represent the amount of nitrite produced in stimulated cell cultures minus the amount of nitrite produced in nonstimulated cell cultures. Prior to vaccination (wk 1), cells from all calves secreted minimal amounts of nitrite in response to PWM and PPD. Nitrite secretion by PWM-stimulated PBMC cultures from standard-diet (P < 0.05) and intensified-diet (P < 0.01) calves increased with time (Fig. 6a), suggesting maturation of the immune response. By 6 wk after vaccination and initiation of dietary treatments, PBMC from intensified-diet calves produced more nitrite (P < 0.05) in response to PWM stimulation than did PBMC from standard-diet calves (Fig. 6a). The increased capacity for PBMC from intensified-diet calves to secrete NO may be partially explained by the diet-induced increase in the percentage of monocytes, a potent producer of reactive oxygen and nitrogen species. It is not known whether these effects of diet on in vitro NO secretion by PWM-stimulated PBMC reflect a beneficial or detrimental response in vivo. Formation of reactive nitrogen intermediates is important for intracellular killing of pathogens by bovine macrophages. However, reactive nitrogen species in combination with reactive oxygen species also can be damaging to host tissues if their production and secretion remain unchecked. Nitric oxide and superoxide anion can form peroxynitrite. This compound contributes to tissue toxicity by nitrating
tyrosine, blocking processes important in cell regulation, including the regulation of insulin-like growth factor 1 by growth hormone [34].

Antigen (i.e. PPD) elicited NO production was apparent by 2 wk after BCG vaccination (wk 3 of age; Fig. 6b). Nitrite secretion by PPD-stimulated PBMC cultures from intensified-diet \( P < 0.05 \) and standard-diet \( P = 0.05 \) calves increased with time. At wk 2 post-vaccination, PBMC from intensified-diet calves tended to produce more \( P = 0.08 \) NO in response to PPD-stimulation than did PBMC from standard-diet calves. At wk 5, PPD-induced NO production by both groups of calves was not different \( P > 0.05 \). The diet \( \times \) time interactions for nitrite secretion by PWM- or PPD-stimulated PBMC were not significant \( P > 0.1 \). These results suggest that differences in protein and energy between the two treatments were not sufficient to influence antigen-specific NO production.

**Cutaneous delayed-type hypersensitivity:** Responses to intradermal administration of *M. bovis* PPD and *M. avium* PPD 5 wk following vaccination (i.e. 6 wk of age) are shown in Figure 7. Diet did not affect \( P > 0.05 \) responses to cutaneous administration of the antigens, *M. bovis* PPD and *M. avium* PPD, suggesting differences in protein and energy between the two treatments is not sufficient to influence antigen-specific recall responses. In contrast, Pollock et al. [35] observed that feeding young calves lower levels of nutrition results in increased skin sensitivity to keyhole limpet haemocyanin. Differences in antigens, ages of the animals, and dietary treatments between the current study and the previous study by Pollock [35] may explain the divergent results. In the present study, changes in skin fold thickness were greater \( P < 0.05 \) in response to *M. bovis* PPD than to *M. avium* PPD administration, indicating the specificity of the response to vaccination with *M. bovis* BCG.
Conclusions

In the present study, a recently established antigen sensitization and challenge model [29] was used to evaluate the effects of increased dietary protein and energy on adaptive, cell-mediated immune responses in milk replacer-fed dairy calves. Using this model, we have shown that providing increased dietary protein and energy to the young calf affects the composition of PBMC populations, specifically the percentages of CD4+ cells, γδTCR+ cells, and monocytes. Antigen-induced IFN-γ or NO secretion by PBMC cultures and in vivo reactivity to antigen (i.e. cutaneous delayed-type hypersensitivity reaction) were not affected, suggesting differences in protein and energy between the two treatments were not sufficient to influence antigen-specific recall responses. Despite the lack of a dietary-effect on antigen-induced recall responses, mitogen-induced IFN-γ and NO secretion by PBMC were affected by feeding an intensified milk replacer. Additional research is necessary to determine whether these subtle effects on the composition and functional capacities of PBMC populations influence the susceptibility of the calf to infectious disease.

Acknowledgments

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References


secretion by blood leukocytes from young and adult cattle vaccinated with *Mycobacterium bovis* BCG. Int. J. Vitam. Nutr. Res. 73, 235-244.


Table 1. Primary and secondary antibodies used in the flow cytometric analysis of blood mononuclear cell populations from calves fed a standard (n=11) or intensified (n=11) diet.

<table>
<thead>
<tr>
<th>Primary antibody specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Working concentration</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cell</td>
<td>GC50A1</td>
<td>IgM</td>
<td>21 μg/mL</td>
<td>αIgM-PE</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>CACT80C</td>
<td>IgG1</td>
<td>7 μg/mL</td>
<td>αIgG1-PerCP</td>
</tr>
<tr>
<td>γδ TCR^+ T cell</td>
<td>CACT61A</td>
<td>IgM</td>
<td>14 μg/mL</td>
<td>αIgM-PE</td>
</tr>
<tr>
<td>B cell</td>
<td>BAQ155A</td>
<td>IgG1</td>
<td>3.5 μg/mL</td>
<td>αIgG1-PE</td>
</tr>
<tr>
<td>Monocyte</td>
<td>CAM36A</td>
<td>IgG1</td>
<td>7 μg/mL</td>
<td>αIgG1-PE</td>
</tr>
<tr>
<td>MHC^1 class II antigen</td>
<td>TH14B</td>
<td>IgG2a</td>
<td>3.5 μg/mL</td>
<td>αIgG2-PerCP</td>
</tr>
<tr>
<td>IL-2^2 receptor</td>
<td>CACT108A</td>
<td>IgG2a</td>
<td>28 μg/mL</td>
<td>αIgG2-PerCP</td>
</tr>
</tbody>
</table>

^1 MHC = major histocompatibility complex.
^2 IL-2r = interleukin-2 receptor.
^3 Primary antibodies were from VMRD, Pullman, WA.
^4 Phycoerythrin (PE)-conjugated secondary antibodies were from Southern Biotechnology Associates, Birmingham, AL, and were used at a concentration of 1 μg/mL. Peridinin chlorophyll protein (PerCP) labeled secondary antibodies were from Becton Dickinson, San Jose, CA, and were used at a 1:15 dilution.
Table 2. Weights of organs (means ± SE) of immunological and metabolic importance from calves fed standard (n=11) and intensified diets (n=11) from 1 to 6 wk of age.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Standard Diet</th>
<th>Intensified Diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcervical Lymph Node, g</td>
<td>7.75 ± 0.55</td>
<td>9.90 ± 0.70</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Prefemoral Lymph Node, g</td>
<td>4.82 ± 0.88</td>
<td>6.75 ± 1.06</td>
<td>NS¹</td>
</tr>
<tr>
<td>Thymus, g</td>
<td>178.39 ± 22.58</td>
<td>337.68 ± 37.88</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Liver, kg</td>
<td>1.12 ± 0.04</td>
<td>1.67 ± 0.08</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Adrenal Gland, g</td>
<td>4.49 ± 0.24</td>
<td>5.92 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>48.95 ± 8.85</td>
<td>45.64 ± 3.30</td>
<td>NS</td>
</tr>
<tr>
<td>Thyroid Gland, g</td>
<td>12.81 ± 1.49</td>
<td>16.12 ± 2.16</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney, g</td>
<td>309.27 ± 11.95</td>
<td>433.43 ± 27.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Heart, g</td>
<td>420.60 ± 16.29</td>
<td>544.81 ± 36.73</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

¹NS = Not significant (P > 0.1).
Figure 1. Effect of nutritional plane on growth performance of milk replacer-fed calves. Shown are the body weights (mean ± SEM) of calves fed standard (n = 11) and intensified (n = 11) milk replacers at weekly intervals during the period of study. At approximately 1 wk of age, calves were vaccinated with *Mycobacterium bovis*, strain bacillus Calmette Guerin.

Figure 2. Percentages of CD4⁺ T cells in peripheral blood mononuclear cell (PBMC) populations and expression of interleukin-2 receptor (IL-2r⁺) by CD4⁺ T cells from calves fed standard (n = 8) and intensified (n = 8) milk replacers. Shown are the percentages (mean ± SEM) of CD4⁺ T cells (panel a) and percentages of CD4⁺ T cells expressing IL-2r (panel b). The P values within the legend are from ANOVA of the effect of time split by treatment. At the beginning of the experiment, all calves were approximately 1 wk of age and were vaccinated with *Mycobacterium bovis*, strain bacillus Calmette Guerin.

a,b,c Within a treatment group, means with different letter superscript differ, P < 0.05.

1 In panel a, percentages of CD4⁺ T cell in PBMC populations were higher (P < 0.05) in intensified-diet calves than standard-diet calves at 6 wk of age.

Figure 3. Percentages of γδTCR⁺ cells in peripheral blood mononuclear cell (PBMC) populations and expression of interleukin-2 receptor (IL-2r⁺) by γδTCR⁺ cells from calves fed standard (n = 8) and intensified (n = 8) milk replacers. Shown are the percentages of (mean ± SEM) γδTCR⁺ cells (panel a) and percentages of γδTCR⁺ cells expressing IL-2r (panel b). The P values within the legend are from ANOVA of the effect of time split by treatment. At the beginning of the experiment, all calves were approximately 1 wk of age and were vaccinated with *Mycobacterium bovis*, strain bacillus Calmette Guerin.

a,b,c Within a treatment group, means with different letter superscript differ, P < 0.05.
Figure 4. Percentages of B cells (panel a) and monocytes (panel b) in peripheral blood mononuclear cell (PBMC) populations from calves fed standard (n = 8) and intensified (n = 8) milk replacers. Because treatment and treatment × time effects were not significant for the B cell phenotype, data shown represent age-related changes observed in all calves (n = 16). The P values within the legend are from ANOVA of the effect of time split by treatment. At the beginning of the experiment, all calves were approximately 1 wk of age and were vaccinated with Mycobacterium bovis, strain bacillus Calmette Guerin.

Within a treatment group, means with different letter superscript differ, P < 0.05.

In panel b, percentages of monocytes in PBMC populations were higher (P < 0.05) in standard-diet calves than in intensified-diet calves at 3 and 5 wk of age.

Figure 5. In vitro interferon (IFN)-γ secretion by peripheral blood mononuclear cells (PBMC) from calves fed standard (n = 8) and intensified (n = 8) milk replacers. Shown are IFN-γ responses (mean ± SEM) of pokeweed mitogen (panel a) and antigen (i.e. purified protein derivative, panel b) stimulated PBMC cultures. Values represent responses by stimulated cell cultures minus responses by nonstimulated (i.e. resting) cell cultures. The P values within the legend are from ANOVA of the effect of time split by treatment. At the beginning of the experiment, all calves were approximately 1 wk of age and were vaccinated with Mycobacterium bovis, strain bacillus Calmette Guerin.

In panel a, means within a treatment group with different letter superscript differ, P < 0.05.

In panel a, PBMC from standard-diet calves secreted more (P < 0.05) IFN-γ than PBMC from intensified-diet calves at 6 wk of age.
In panel b, responses at 5 and 6 wk differ from responses at wk 1 within a treatment, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 6.** *In vitro* nitric oxide (NO) secretion by peripheral blood mononuclear cells (PBMC) from calves fed standard (n = 8) and intensified (n = 8) milk replacers. The amount of nitrite, a stable product of nitric oxide production, in culture supernatants is a correlate of the amount of nitric oxide produced. Shown are nitrite (mean ± SEM) concentrations in pokeweed mitogen (panel a) and antigen (i.e. purified protein derivative, panel b) stimulated PBMC cultures. Values represent responses by stimulated cell cultures minus responses by nonstimulated (i.e. resting) cell cultures. The $P$ values within the legend are from an ANOVA of the effect of time split by treatment. At the beginning of the experiment, all calves were approximately 1 wk of age and were vaccinated with *Mycobacterium bovis*, strain bacillus Calmette Guerin.

$^{a,b}$ Within a treatment group, means with different letter superscript differ, $P < 0.05$.

$^{1}$ In panel a, PBMC from intensified-diet calves secreted more ($P < 0.05$) nitrite than PBMC from standard-diet calves at 6 wk of age.

**Figure 7.** *In vivo* delayed-type hypersensitivity reactions of calves fed standard (n = 11) and intensified (n = 11) milk replacers. Shown are changes in cervical skin fold thickness (mean ± SEM) 72 h after intradermal administration of *M. avium* and *M. bovis* purified protein derivative (PPD). Test was performed 5 wk after vaccination and the initiation of dietary treatments. Calves were vaccinated subcutaneously with *Mycobacterium bovis*, strain bacillus Calmette Guerin.
Figure 1. Effect of nutritional plane on growth performance of milk replacer-fed calves.
Figure 2. Percentages of CD4+ T cells in peripheral blood mononuclear cell (PBMC) populations and expression of interleukin-2 receptor (IL-2r+) by CD4+ T cells from calves fed standard (n = 8) and intensified (n = 8) milk replacers.
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Figure 7. *In vivo* delayed-type hypersensitivity reactions of calves fed standard (*n* = 11) and intensified (*n* = 11) milk replacers.
CHAPTER 4. HIGH-GROWTH RATE FAILS TO ENHANCE ADAPTIVE IMMUNE RESPONSES IN NEONATAL CALVES AND DECREASES IMMUNE CELL VIABILITY

A paper to be submitted to the Journal of Nutrition

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ABSTRACT

The objective of the current study was to investigate the effects of different feeding rates achieving three targeted growth rates (No Growth, Low Growth, and High Growth) on adaptive immune responses of neonatal calves vaccinated with \textit{Mycobacterium bovis} bacillus Calmette-Guerin (BCG) and ovalbumin 3 wks after initiation of dietary treatments. The daily growth rates for No-, Low-, and High-growth calves were different throughout the experimental period and averaged 0.11 \pm 0.02 kg, 0.58 \pm .02, and 1.16 \pm 0.04 kg, respectively. Although relative proportions of certain leukocytes in blood were affected by growth rate, adaptive immune responses generally were not affected. High-growth rate calves had lower proportions of blood mononuclear leukocytes (MNL) and higher proportions of polymorphonuclear leukocytes compared with calves fed at maintenance (i.e., no growth). Relative proportions of CD4\textsuperscript{+} cells increased with age in No- and Low-growth calves, characteristic of the maturing calf, but failed to increase in High-growth calves. Proportions of CD4\textsuperscript{+} and CD8\textsuperscript{+} cells expressing CD45RO (memory phenotype) and ovalbumin-specific IgG\textsubscript{1} and IgG\textsubscript{2} concentrations after vaccination were not affected by growth rate. Interferon (IFN)-\textgamma and nitric oxide (NO) secretion by PPD-stimulated MNL were not affected by growth rate. Antigen (i.e., PPD)-elicited delayed-type hypersensitivity in No-growth calves was greater than Low-growth but similar to High-growth calves. Viability of MNL, CD4\textsuperscript{+}, CD8\textsuperscript{+}, and \gamma\delta TCR\textsuperscript{+} cells in stimulated and non-stimulated cultures from High-growth calves was substantially lower compared with viability of cells...
from No- and Low-growth calves. The increased NO production by non-stimulated MNL from High-growth calves may have contributed to this effect. In conclusion, responses to ovalbumin and M. bovis BCG vaccination were affected minimally by growth rate. These results suggest PEM in the absence of weight loss does not affect negatively adaptive immune responses of calves and that increasing growth rate or plane of nutrition above maintenance requirements does not benefit adaptive immune responses. High rates of growth, however, may affect negatively immune cell viability, with potentially deleterious effects on the calf’s resistance to infectious disease.

**Abbreviations used:** 7-AAD, 7-amino actinomycin D; CMI, cell-mediated immune; DTH, delayed-type hypersensitivity; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; MNL, mononuclear leukocytes; NO, nitric oxide; PBS, phosphate buffered saline; PEM, protein-energy malnutrition; PMN, polymorphonuclear leukocytes; PPD, purified protein derivative; OVA, ovalbumin; RNI, reactive nitrogen intermediates; TCR, T-cell receptor.

**INTRODUCTION**

Neonatal animals are highly susceptible to bacterial and viral pathogens. Traditional calf-rearing programs limit nutrient intake from milk or milk replacer during the first few weeks of life in order to promote dry feed (i.e., starter) intake and allow early weaning. Recent reports of dramatic improvements in the growth performance and feed efficiency resulting from feeding greater amounts of milk replacer with higher protein concentrations has led to interest in intensified or accelerated feeding programs (1-4). It is believed that intensified or accelerated feeding programs increase the plane of nutrition to more “natural” levels and provide more “biologically appropriate” early growth (5). Despite limited supportive information, improving the plane of nutrition may also improve calf health and decrease morbidity and mortality (5,6).
Protein-energy malnutrition (PEM) is the major cause of immunodeficiency worldwide (7). Protein-energy malnutrition manifests as acute (wasting) and chronic (stunting) forms resulting in altered body composition and reduced linear growth, respectively (8). Although some reports indicate that wasting but not stunting PEM influences cell-mediated adaptive immunity negatively (9), additional reports suggest that stunting PEM likewise may depress cell-mediated immunocompetence and increase the risk of infection-related mortality in humans (10,11). Most experiments to date have investigated effects of wasting-, not stunting-, PEM on immunity. Substantial evidence supports negative effects of PEM on adaptive immune responses. Malnutrition accounts for tuberculin anergy in underprivileged children, despite vaccination with \( M. \text{bovis} \) BCG, resulting in high rates of fatal tuberculous infections (12). A low CD4\(^+\)/CD8\(^+\) T cell ratio in the blood has commonly been associated with PEM in humans and weanling mice and was widely accepted as a key indication of depressed T-cell dependent immunity in PEM (13). Imbalances within, rather than between, the two main T-cell subsets may be of greater importance. In mice, wasting PEM is associated with an overabundance of CD4\(^+\)CD45RA\(^-\) T-cells (CD4\(^+\) naïve-phenotype) and CD8\(^-\)CD45RA\(^+\)CD62L\(^+\) T-cells (CD8\(^+\) naïve-phenotype) that are quiescent compared with CD45\(^-\) effector and memory phenotypes (14,15). In addition, wasting due to severe protein deficiency decreases proliferation and expression of IFN-\( \gamma \), IL-2, and IL-2 receptor mRNA expression by splenic mononuclear cells (16).

Generally speaking, restricting dietary protein and energy affects positively immune function and longevity in rodents. Mice fed a low protein (6%) diet have more vigorous antibody responses and CMI after immunization compared with mice fed a normal protein (22%) diet (21). A low protein diet also abrogates age-related decreases in immune responsiveness to mitogenic stimulation. Similarly, lifelong caloric restriction of mice prevents age-associated decline in T-cell proliferation (22). Mechanisms by which life-long food restriction slows aging and reduces the rate of occurrence of age-associated disease processes are unknown. Expression of the IL-2 receptor by mitogen-
stimulated lymphocytes from rats fed an *ad libitum* diet is reduced when compared with cells from rats fed a 40% food-restricted diet (23). Feeding rats an *ad libitum* diet decreases mitogen-induced proliferative responses of splenic cells when compared with feed-restricted rats (24). The increase in memory CD4+ and CD8+ T-cell subsets in rats fed *ad libitum* was not observed in feed-restricted rats. Higher levels of IL-2 and lower levels of IL-6 and TNF-α were also observed in feed-restricted rats. The authors concluded that increased CMI function observed in aged feed-restricted rats is due to the presence of a higher number of naïve T cells (24).

Effects of an increased plane of nutrition on composition and functional capacities of blood mononuclear leukocyte (MNL) populations in young calves have been reported recently (25,26). Mitogen-stimulated blood MNL from calves fed the enhanced plane of nutrition diet produced less IFN-γ and more inducible NO, suggesting plane of nutrition affects aspects of leukocyte functions associated with CMI in calves (26). Enhanced plane of nutrition also was associated with decreased immune responses to mitogen stimulation, including decreased proliferation of CD4+, CD8+, and γδTCR+ cells; decreased CD25 expression by CD4+ and CD8+ cells; and decreased CD44 expression by CD8+ cells (25). Results from investigations of the effects of neonatal nutrition on adaptive immune responses of calves vaccinated with *Mycobacterium bovis bacillus* Calmette-Guerin (BCG) indicate that antigen-specific recall responses are minimally affected by increased nutrition (27). In these previous experiments, however, the low plane of nutrition diet did not induce PEM (25-27). In these studies, calves were vaccinated prior to initiation of dietary treatments (27).

We hypothesize that increasing plane of nutrition above maintenance requirements does not enhance adaptive immune responses in vaccinated neonatal calves. The objective of the current study was to investigate the effects of three different growth rates (no growth), low growth, and high growth, on adaptive immune responses of neonatal calves vaccinated 3 wks after initiation of dietary treatments.
MATERIALS AND METHODS

Animals. Animal procedures were approved by the Animal Care and Use Committee of the National Animal Disease Center, ARS, USDA, Ames, IA. Twenty-four Holstein bull calves were acquired from a single Wisconsin dairy herd over a 2-wk period. All calves were given 3.9 L each of colostrum within 6 h of birth. At birth, navels were dipped in iodine and an Escherichia coli vaccine (Genecol-99, Shering Plough Animal Health, Union, NJ) was administered orally. After birth, calves were transported to the National Animal Disease Center, ARS, USDA, Ames, IA, where they were housed individually in elevated pens in a temperature-controlled (64°C) barn. Upon arrival at the National Animal Disease Center, calves were given 2 mL iron dextran (100 mg/mL; AmTech, Phoenix Scientific, Inc., St. Joseph, MO) intramuscularly, 2.5 mL of BoSe (2.19 mg sodium selenite and 50 mg of RRR-α-tocopherol/mL, Schering-Plough Animal Health Corp) intramuscularly, and 2 mL of a vitamin B complex (Phoenix Scientific, Inc., St. Joseph, MO) subcutaneously.

Dietary treatments. Before the trial, calves were fed twice daily 0.3 kg of a 20% crude protein (CP), 20% fat (F) milk replacer (Land O’ Lakes, Inc.) reconstituted to 15% dry matter. On the first Monday after arrival (average age 9.1 d; wk 0 of experiment), calves were weighed and assigned randomly to one of three treatment groups (8 calves per treatment). Treatments were designed to achieve three targeted daily rates of gain (No Growth = 0.0 kg, Low Growth = 0.55 kg, or High Growth = 1.2 kg) in live weight over a 7-wk period. The NRC Nutrient Requirements of Dairy Cattle calf model computer program (28) was used to estimate milk replacer intakes needed to achieve target growth rates. All calves were fed a 30% CP, 20% F all-milk protein milk replacer (Land O’ Lakes, Inc.) reconstituted to 14% dry matter. The diet was formulated to ensure that protein would not be the most limiting nutrient. Composition of the milk replacer is provided in Table 1. Calves were weighed each Monday at midday, and diets were reformulated to allow for changes in live weight. Because the vitamin concentrations in the milk replacer were based on the expected dry-matter intake
of the High-growth calves, supplements were given to No-growth and Low-growth calves once weekly to compensate for reduced milk replacer consumption. Calves were fed individually using buckets twice a day (0700 and 1800 h) and were offered water *ad libitum*. No starter grain was offered. Amounts of feed offered and refused were recorded at each feeding.

**Preparation and administration of *M. bovis* BCG vaccine.** The BCG (Pasteur strain) was grown in Middlebrook’s 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) as described for virulent *M. bovis* (Bolin et al., 1997). At wk 3 of the experiment, all calves (*n* = 24) were vaccinated subcutaneously in the right midcervical region with $10^7$ cfu of *M. bovis* BCG.

**Preparation and administration of ovalbumin.** Crystallized ovalbumin (OVA; Grade V, Sigma) was dissolved 2:1 (mg/mL) in sterile PBS and then diluted 1:1 (vol/vol) in incomplete Freund’s adjuvant (MP Biomedicals, Inc., Aurora, Ohio). The mixture was emulsified and administered to calves within an hour of preparation. At wk 3 and wk 5 of the experiment, 4 mL of adjuvanted OVA (4 mg OVA) was administered to all calves (*n* = 24) subcutaneously in the left midcervical region.

**Blood collection.** Peripheral blood (90 mL) was collected via jugular venipuncture at wk 0, before the initiation of dietary treatments, wk 3 (time of primary sensitization), 5 (time of OVA secondary vaccination), 6 and 7 of the experimental period. Blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose [sodium citrate (77 μM), citric acid (38 μM), and dextrose 122 μM)]. Blood samples were collected into 10-mL vacutainers with potassium ethylenediaminetetraacetic acid (EDTA; Becton Dickinson, Franklin Lakes, NJ) at the same timepoints. Coagulated (no additive) blood samples were collected once weekly during the experimental period for quantification of antigen-specific IgG concentrations. Harvested serum was frozen (-20°C) until needed.
The MNL used in functional assays were isolated and enriched by density gradient centrifugation as described previously (Nonnecke et al., 1991). The MNL-enriched populations were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), antibiotics (100 U/mL of penicillin G and 100 μg/mL of streptomycin sulfate, Sigma), 50 μM 2-mercaptoethanol (Sigma), 1% nonessential amino acids (Sigma), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT).

Recall antigens. Recall antigens used were crystallized ovalbumin (OVA; Grade V, Sigma) and M. bovis-derived purified protein derivative (PPD; Pfizer, Kalamazoo, MI).

Analysis of composition of MNL populations. The composition of MNL populations was evaluated by flow cytometry as described previously (26). Approximately $5 \times 10^5$ cells in 100 μL of anticoagulated blood were added to each of 8 wells of a 96-well, round-bottom microtiter plate (Costar, Cambridge, MA). Individual wells were preloaded with a 50-μL aliquot of monoclonal antibody (Table 2) diluted in PBS containing 0.02% NaN₃ and 1% inactivated fetal bovine serum. Cells were incubated for 15 min at room temperature, and then centrifuged (400 × g for 2 min at room temperature). Supernatant was decanted, contaminating erythrocytes were removed by hypotonic lysis, and the plate was centrifuged again as described above. Supernatant was decanted, and cells were resuspended in 100 μL each of two isotype-specific antibodies conjugated to fluorochromes (Table 2). Secondary antibodies were diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were incubated for 15 min at room temperature in the dark and were centrifuged as described above. Cells were resuspended in 200 μL of FacsLyse buffer (Becton Dickinson, San Jose, CA) and stored in the dark at 4°C until examined on the flow cytometer. Five thousand cells...
exhibiting light scattering properties consistent with bovine MNL were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson, San Jose, California) and analyzed using FlowJo (Tree Star Inc., San Carlos, CA), and CellQuest software (Becton Dickinson, San Jose, California). Percentages of cells staining positive for each marker were recorded.

Measurement of antibody in serum and in culture supernatants. OVA-specific IgG\textsubscript{1} and IgG\textsubscript{2} concentrations in serum samples collected weekly before and after vaccination were determined by a capture ELISA. The concentration of OVA used in the ELISA was 1.56 μg/mL diluted in PBS. Microtiter plates (96-well; Immulon II, Dynatech) were coated with OVA (100 μL/well). Plates, including control wells containing PBS alone, were incubated for 15 h at 4°C. Plates were washed 3× with PBS with 0.05% Tween 20 (PBST; 200 μL/well), and blocked with a commercial milk diluent/blocking solution (200 μL/well; Kirkegaard and Perry Laboratories, Gaithersburg, MD). After incubation for 1 h at 37°C in the blocking solution, wells were washed in PBST and test sera were added to wells (100 μL/well). Test and control sera were diluted in PBS containing 0.1% gelatin. Optimal dilutions of test sera were determined by evaluation of the reactivity of 2-fold serial dilutions ranging from 1:6 to 1:800. After incubating for 20 h at 4°C with test samples, wells were washed with PBST and incubated for 1 h at 37°C with horseradish peroxidase-conjugated, anti-bovine IgG heavy and light chains (Kirkegaard and Perry Laboratories) in PBS plus 0.1% gelatin. Wells were then washed with PBST and incubated for 4.5 min at room temperature with substrate (3,3′,5,5′-tetramethylbenzidine; Kirkegaard and Perry Laboratories). The reaction was stopped by addition of sulfuric acid (0.18 M) and absorbencies (450 nm) of individual wells measured using an ELISA plate reader (Molecular Devices, Menlo Park, CA). The change in optical density readings was calculated by subtracting the mean optical density readings for wells receiving PBS alone from the mean optical density readings for antigen-coated wells receiving the same test sample.
**Interferon-γ secretion.** Cells used in interferon IFN-γ assays were from blood samples collected at wk 3 (before vaccination), 5, and 7 of the experimental period. Wells were seeded with $4 \times 10^5$ cells in a total culture volume of 200 μL (final concentration of $2 \times 10^6$ cells/mL in culture). Culture medium consisted of supplemented RPMI 1640 (Gibco Laboratories) as described for MNL isolation and enrichment. Triplicate cultures were either non-stimulated (medium alone) or stimulated with PPD ($10 \mu g/mL$) or OVA ($10 \mu g/mL$). Cultures were incubated at 39°C in a humidified atmosphere of 5% CO$_2$ for 72 h. Supernatants were subsequently harvested from centrifuged plates ($400 \times g$, 5 min, room temperature) and stored at -80°C. The IFN-γ concentration (ng/mL) in culture supernatants was determined by using an IFN-γ capture ELISA as reported previously (29). The IFN-γ concentrations (ng/mL) in non-stimulated cultures were subtracted from IFN-γ concentrations in stimulated cultures.

**In vitro production and measurement of inducible NO.** Secretion of nitrite, the stable oxidation product of NO, by non-stimulated and OVA- and PPD-stimulated blood MNL cultures was evaluated in 96-well flat-bottom tissue culture plates. Wells were seeded with $4 \times 10^5$ cells in a total culture volume of 200 μL (final concentration of $2 \times 10^6$ cells/mL in culture). Culture medium consisted of supplemented RPMI 1640 (Gibco Laboratories) as described above for MNL isolation and enrichment. Cultures were non-stimulated (medium alone), stimulated with 10 μg/mL OVA or with 10 μg/mL *M. bovis* PPD. Wells were prepared in triplicate for each treatment. N^6^-Monomethyl-L-arginine (L-NMMA), a competitive inhibitor of inducible NO synthase (iNOS), was used as a negative control and was added to parallel non-stimulated or stimulated cultures to verify that nitrite production was due to iNOS. Plates were incubated at 39°C in a humidified atmosphere of 5% CO$_2$. 
for 48 h. The amount of nitrite in culture supernatants was measured by a modification of the method of Green et al. (30) as described previously (31).

Measurement of delayed-type hypersensitivity in vivo. In vivo sensitization to *M. bovis* BCG was evaluated using the comparative cervical skin test following Bovine Tuberculosis Eradication Uniform Methods and Rules (Animal and Plant Health Inspection Service brochure #91-45-011). Calves (n = 24) were injected intradermally with 100 µL of *M. bovis* PPD in the mid-cervical region 5 wk following *M. bovis* BCG vaccination. Skin-fold thickness was measured immediately before administration of PPD and 72 h later. Responses were estimated by subtracting pre-test skin fold thickness from skin fold thickness 72 h after administration of antigen.

Viability of MNL. Viability of blood MNL in non-stimulated and OVA- and PPD-stimulated cultures was evaluated when calves had been on dietary treatments for 7 wk. Blood was collected from a subset (n = 4 per treatment) of calves. Wells of 96-well round-bottom plates were seeded with $4 \times 10^5$ PKH67-stained cells in a total culture volume of 200 µL (final concentration of $2 \times 10^5$ cells/mL in culture; 10 replicates per stimulation). PKH67-staining of cells was performed following manufacturer’s directions as reported previously (25). Cultures were non-stimulated (media only) or stimulated with OVA (10 µg/mL) or PPD (10 µg/mL) and incubated for 3 d at 39°C in a humidified atmosphere with 5% CO₂. Culture replicates were pooled after 3-d incubation periods. Approximately $2 \times 10^3$ pooled PKH67 stained cells in 150 µL of culture medium were added to individual wells of 96-well round-bottomed microtiter plates. Cells were labeled with one of three phenotype makers (CD4, CD8, or γδTCR). Primary and secondary antibodies used in the analyses are listed in Table 2. Monoclonal primary antibody (1 µg/well) diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS was added to individual wells in 25 µL aliquots. Cells were incubated
for 15 min at room temperature and centrifuged (400 × g at room temperature for 2 min). Supernatant was decanted, and cells were labeled with 100 μL of αlgM-allophyocyanin (APC). Secondary antibody was diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were incubated for 15 min at room temperature in the dark and then centrifuged as described above. Cells were resuspended (200 μL of PBS) and 7-amino actinomycin D (7-AAD) was added to each well (1 μg; Molecular Probes, Eugene, OR). Cells were incubated for 15 min at room temperature in the dark and centrifuged (400 × g at room temperature for 2 min). Cells were washed with 200 μL of PBS, centrifuged as described above, and then resuspended in 200 μL of FacsLyse buffer (Becton Dickinson, San Jose, CA). Cells were stored in the dark at 4°C until examined on the flow cytometer. Ten thousand cells exhibiting light scattering properties consistent with bovine MNL were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson, San Jose, California) and analyzed using FlowJo (Tree Star Inc., San Carlos, CA) software. Cells without 7-AAD labeling were considered viable, whereas apoptotic and dead cells showed low and high 7-AAD staining, respectively (32).

**Statistical analysis.** 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. Body weight, composition of blood MNL populations, serum antibody concentration, secretion of IFN-γ, and production of NO were analyzed as a split-plot with repeated measures ANOVA. The model included the fixed effects of growth rate (No, Low, or High), time (wk on experiment), and the treatment × time interaction, and calf was included in the model as the random effect. Fisher's protected-LSD test was applied when significant effects (P < 0.05) and trends (P < 0.10) were detected by the model. Average daily gain, cell viability, and delayed-type hypersensitivity were analyzed as a split-plot with factorial ANOVA. Growth rate and calf were included in the model as
fixed and random effects, respectively. Fisher’s protected-LSD test was applied when significant effects \( P < 0.05 \) and trends \( P < 0.10 \) were detected by the model.

**RESULTS**

*Growth performance.* Average weights of calves were not different before initiation of dietary treatments and averaged 45.0 ± 0.7 kg, 46.0 ± 2.1 kg, and 46.1 ± 1.3 kg for No, Low, and High Growth treatments, respectively (Figure 1). Mean body weights of calves from the three treatment groups were different \( P < 0.05 \) by wk 1 and remained different throughout the experimental period. Daily growth rates for No- and Low-growth calves exceeded whereas daily growth rates for High-growth calves were lower than targets. The daily growth rates for No-, Low-, and High-growth calves were different \( P < 0.0001 \) throughout the experimental period and averaged 0.11 ± 0.02 kg, 0.58 ± .02, and 1.16 ± 0.04 kg, respectively. Before vaccination (wk 0-3), daily growth rates were different and averaged 0.06 ± 0.03 kg, 0.53 ± 0.02 kg, and 1.16 ± 0.08 kg for No, Low, and High Growth treatments, respectively.

*Composition of blood cell populations.* Effects of growth rate, time, and their interaction on the composition of blood cell populations from calves are summarized in Table 3. The relative contribution of polymorphonuclear leukocytes (PMN) and MNL cells to the total blood cell population is shown in Figure 2. No-growth calves had higher \( P < 0.05 \) percentages of MNL and lower \( P < 0.05 \) percentages of PMN compared with High-growth calves across time. Percentages of PMN cells decreased \( P < 0.05 \) with time in No-growth and High-growth, but not Low-growth calves. In addition, percentages of MNL cells increased \( P < 0.05 \) with time in No-growth and High-growth, but not Low-growth calves.
The contribution of \( \gamma \delta TCR^+ \) cells and B cells to the MNL cell population is provided in Figure 3. Percentages of \( \gamma \delta TCR^+ \) cells increased \((P < 0.05)\) with time in No-growth but not Low- or High-growth calves. At wk 5, percentages of \( \gamma \delta TCR^+ \) cells were lower \((P < 0.05)\) in High-growth calves compared with No- and Low-growth calves. Percentages of \( \gamma \delta TCR^+ \) cells also were lower \((P < 0.05)\) in High-growth calves compared with No-growth calves at wk 6 and 7. Growth rate did not affect \((P > 0.05)\) percentages of B cells, but B cell percentages did change \((P < 0.05)\) across time for all treatment groups. In addition, CD4:CD8 ratio was not affected by growth rate \((P > 0.05)\) but did change \((P < 0.05)\) with time.

The relative contribution of CD4\(^+\) cells to the MNL cell population was not affected \((P > 0.05)\) by treatment across time (Figure 4a). Percentages of CD4\(^+\) cells increased \((P < 0.05)\) with time in No-growth and Low-growth calves but failed to increase \((P > 0.05)\) with time in High-growth calves. Percentages of CD8\(^+\) cells were not affected by treatment, but did change \((P < 0.05)\) with time (Figure 4b). The proportion of CD4\(^+\) and CD8\(^+\) cells expressing a memory phenotype (i.e., CD45RO positive) was not affected \((P > 0.05)\) by growth rate across time (Figure 4d and 4d, respectively). Percentages of CD4\(^+\) and CD8\(^+\) cells expressing CD45RO increased \((P < 0.05)\) with time for all treatment groups. At wk 5, percentages of CD8\(^+\) cells expressing CD45RO were higher \((P < 0.05)\) in High-growth compared with No-growth and Low-growth calves.

**Antibody response to ovalbumin vaccination.** Serum OVA-specific IgG\(_1\) and IgG\(_2\) increased \((P < 0.05)\) following vaccination (Figures 5a and 5b, respectively). Growth rate did not affect \((P > 0.5)\) OVA-specific IgG\(_1\) or IgG\(_2\) concentration in serum across time (Figures 5a and 5b). Two wk following primary vaccination (wk 5), concentration of OVA-specific IgG\(_2\) was higher \((P < 0.05)\) in High-growth than in Low-growth calves. Concentration of OVA-specific IgG\(_2\) was similar \((P > 0.05)\) in High- and No-growth calves.
**Secretion of IFN-γ and NO by blood MNL in response to antigen.** Effects of growth rate on secretion of IFN-γ in OVA- and PPD-stimulated cultures are shown in Figure 6. Secretion of IFN-γ by OVA-stimulated blood MNL from No- and High-growth calves increased ($P < 0.01$) with time following vaccination whereas IFN-γ responses to OVA stimulation by MNL from Low-growth calves did not change ($P > 0.05$) with time. Growth rate did not affect ($P > 0.05$) secretion of IFN-γ by PPD-stimulated blood MNL from calves. Secretion of IFN-γ by PPD-stimulated MNL did increase ($P < 0.01$) with time following vaccination.

Effects of growth rate on NO production are shown in Figure 8. Blood MNL from calves did not produce NO in response to OVA (data not shown). Growth rate did not affect ($P > 0.05$) NO production by PPD-stimulated MNL. Production of NO by PPD-stimulated blood MNL from High-, Low-, and NO-growth calves increased ($P < 0.05$, $P < 0.05$, and $P = 0.05$, respectively) with time following vaccination (Figure 7). Blood MNL isolated from High-growth calves ($20.89 \pm 2.3$) produced more NO ($P < 0.05$) in non-stimulated cultures compared with MNL from Low-($13.90 \pm 1.7$) and NO-growth ($12.34 \pm 1.8$) calves.

**Cutaneous delayed-type hypersensitivity.** Responses to intradermal administration of *M. bovis* PPD 5 wk following vaccination are shown in Figure 8. Change in skin fold thickness was greater ($P < 0.05$) in No-growth calves compared with Low-growth but not High-growth calves.

**Blood MNL viability in culture.** Growth rate affected the viability of blood MNL in non-stimulated and antigen-stimulated cultures (Figure 9). Percentages of viable MNL, CD4$^+$, CD8$^+$, and $\gamma\delta$TCR$^+$ cells in non-stimulated (Figure 9a) and PPD-stimulated cultures (Figure 9b) established from cells isolated from high-growth calves were lower ($P < 0.01$) compared with No- and Low-growth calves.
DISCUSSION

This study describes, for the first time, the effects of feeding at different rates to achieve targeted daily growth rates on adaptive immune responses in neonates. Although the effects of wasting PEM on immune function has been described, the effects of stunting PEM (i.e., no growth) or alternatively very high rates of growth on immune function in the neonate have not been characterized extensively.

Growth rate affected the composition of blood MNL populations. Calves fed at rates to achieve no growth actually had higher percentages of circulating MNL compared with calves fed at rates achieving high growth rates. In addition, percentages of circulating MNL and CD4 cells increased with age in calves fed at rates to achieve no growth, confirming maturational changes in the composition of MNL populations with time that have been reported (26,27). Percentages of γδTCR+ cells in No-growth calves, however, did not decrease with time as would be expected in the maturing calf, but actually increased. Percentages of γδTCR+ cells are higher in neonatal cattle and decreases with age (33,34), and thus a decrease in the presence of γδTCR+ cells would suggest maturation of MNL populations. Although the role of the γδTCR+ cell remains uncertain, it has been suggested that this T-cell subset is important in the protective response to tuberculosis (35,36). Wasting PEM decreases the circulating numbers of γδTCR+ cells in cattle experimentally infected with virulent M. bovis, suggesting that nutritional status affects cell populations having antimycobacterial activity (37). These results suggest that stunting PEM in the neonatal calf may affect maturational changes in a T cell subset (i.e., γδTCR+) important in early adaptive immune responses, but does not affect proportions of other T cell subsets (i.e. CD4+ and CD8+ cells). The failure of CD4+ cell percentages to increase in High-growth calves suggests neonatal nutrition affects maturational changes in T cell subsets.

Feeding calves at different rates to achieve three different growth rates did not affect CD4:CD8 ratio or proportions of CD4+ and CD8+ cells expressing a memory phenotype. Proportions
of memory CD4+ and CD8+ T cells increased with time, suggesting maturational changes and responses to vaccination and presence of environmental pathogens. Imbalances within the two main T cell subsets may be of great importance in PEM-induced immunosuppression. In mice, wasting PEM is associated with an overabundance of CD4+CD45RA+ T-cells (CD4+ naïve-phenotype) and CD8+CD45RA+CD62L+ T-cells (CD8+ naïve-phenotype) that are quiescent compared with CD45+ effector and memory phenotypes (14,15). Results from the current experiment suggest that stunting PEM in the neonatal calf does not affect relative proportions of memory CD4+ and CD8+ T cells. Therefore, weight loss (i.e., wasting PEM) may be necessary to affect naïve:memory cell ratios in young calves.

Because neonatal calves have strong antibody responses following sensitization to OVA (38), serum antibody responses to OVA were used to evaluate effects of growth rate on adaptive, humoral immune responses of milk replacer-fed dairy calves. In the current experiment, growth rate did not affect OVA-specific antibody concentrations in serum, suggesting calves fed a diet resulting in no growth have normal in vivo antibody responses. In addition, it has been shown previously that M. bovis BCG-vaccinated calves exhibit strong CMI responses to PPD (39,40). In the present study, a BCG sensitization and challenge model (27,39) was used to evaluate effects of growth rate on adaptive, CMI responses of milk replacer-fed calves. Using this model, we evaluated IFN-γ and NO production in antigen-stimulated blood MNL cultures, functions intimately associated with CMI. In the current experiment, growth rate of neonatal calves did not affect secretion of IFN-γ and production of NO in PPD-stimulated cultures. Calves fed a maintenance diet (i.e., no growth) however, had greater DTH responses to intradermal administration of PPD, suggesting that calves fed a diet resulting in no growth have normal DTH reactions indicative of competent in vivo CMI responses. The greater (numerically) production of IFN-γ by MNL from No- and High-growth calves compared with Low-growth calves may explain the stronger DTH responses of these calves.
Research indicates that neonatal calves fed at 50% maintenance from 2-28 d of age lose weight and have delayed primary humoral responses to K99 antigen compared with calves fed above maintenance requirements achieving 1 kg growth per week (41). High plane of nutrition in calves, however, has been associated with decreased serum antibody responses to keyhole limpet haemocyanin (KLH) and anti-horse red blood cell titres and decreased DTH to KLH (42,43). Weight loss in older cattle has been associated with a decrease in serum antibody responses to chicken erythrocytes (44). Feeding cattle to lose (-0.52 kg/d), maintain (+0.18 kg/d), or gain (1.07 kg/d) body weight does not affect serum antibody to *Brucella abortus* bacterin or DTH responses to *M. bovis* BCG (44). In addition, wasting PEM (17% body weight loss over 133 d) in adult cattle infected with *Mycobacterium bovis* does not affect DTH responses, *in vitro* IFN-γ production, or lymphocyte proliferation to antigen (37). Discrepancies in adaptive responses are probably the result of the levels of nutrition investigated (i.e., some diets fed resulted in weight loss or wasting, whereas the low plane of nutrition in others provided at least maintenance requirements). Taken together, dietary extremes causing weight loss or high rates of weight gain may influence adaptive immunity in cattle.

In the current experiment, MNL isolated from high-growth rate calves produced elevated concentrations of inducible NO in non-stimulated cultures and had very low viability. Nitric oxide produced by activated macrophages, in addition to other cell types, is an important mediator of immune responses. Enhanced expression of inducible NO in human macrophages increases killing of *Mycobacterium tuberculosis* (45). Excess production or sustained release of NO, however, can be detrimental to tissues and immune responses. Nitric oxide is a potent inhibitor of lymphocyte activation and proliferation and can induce lymphocyte apoptosis (46-48). Synthesis of nitric oxide from L-arginine by iNOS is induced mainly by Thelper1 cytokines such as IFN-γ. Under certain conditions, iNOS can produce O$_2^-$ in addition to NO. The concomitant generation of NO and O$_2^-$ favors the production of peroxynitrite, a highly reactive oxidizing agent capable of nitrating tyrosines.
on proteins (48). Peroxynitrite can induce apoptosis in lymphocytes via mitochondrial processes and can directly cause oxidative DNA damage (46,48-51).

High glucose induces generation of reactive oxygen species and reactive nitrogen intermediates (52). Thus, situations of hyperglycemia can lead to excess free-radical generation and subsequent oxidative stress. High glucose induces oxidative and nitrosative stress-induced apoptosis in many cell types (52). Several dietary factors influence inducible NO production (53). Protein energy malnutrition and arginine deficiency both reduce NO synthesis, whereas increasing concentrations of free fatty acids or feeding a high saturated-fat diet increases iNOS expression and activity (53). Our results suggest that feeding young calves to achieve high growth rates also increases NO production. Feeding neonatal calves an enhanced plane of nutrition is associated with decreased proliferation of mitogen-stimulated CD4\(^+\), CD8\(^+\), and \(\gamma\delta\) TCR\(^+\) cells and decreased CD25 (IL-2r) expression by mitogen-stimulated CD4\(^+\) and CD8\(^+\) cells (25). It has been shown that NO acts at the level of IL-2r signaling, blocking the phosphorylation and activation of several signaling molecules (54,55). Although no causal relationship has been proven, together these results suggest that the negative effects of enhanced plane of nutrition and high growth rate in neonatal calves on lymphocyte proliferation, activity, and viability may be a result of excess production of NO.

In conclusion, the effects of growth rate on adaptive immune responses to OVA and M. bovis BCG vaccination in calves were minimal, suggesting PEM in the absence of weight loss does not affect adaptive immune responses of the neonate. Weight loss may be a requirement to achieve PEM-induced immunodeficiency. High growth rates, however, decreased immune cell viability, having potentially deleterious effects on disease resistance. Further investigation is required to determine if high rates of growth induce metabolic/oxidative stress on cells of the immune system.
ACKNOWLEDGEMENTS

Milk replacer was generously donated by Land O'Lakes, Inc., Minneapolis MN; special thanks go to Mike Fowler, Dr. Bill Miller, Tom Johnson, and Bruce Perry. Authors thank Nancy Eischen and Donald McDorman in the Periparturient Diseases of Cattle Research Unit, National Animal Disease Center, Jody Mentele in the Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Bruce Pesch in the Central Flow Cytometry Laboratory, National Animal Disease Center, and Dr. Alex Maue, University of Missouri, for technical support. Authors also thank Emily Miller, Andy Moser, and Paul Amundson for excellent animal care.

LITERATURE CITED


TABLE 1. Chemical composition of milk replacer fed to calves at three levels of intake to achieve three targeted daily rates of gain.

<table>
<thead>
<tr>
<th>Component</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Dry Matter (DM)</td>
<td>97.0</td>
</tr>
<tr>
<td>Crude Protein, % of DM</td>
<td>30.0</td>
</tr>
<tr>
<td>Fat, % of DM</td>
<td>20.0</td>
</tr>
<tr>
<td>Lactose, % of DM</td>
<td>30.6</td>
</tr>
<tr>
<td>Calcium, % of DM</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphorus, % of DM</td>
<td>0.7</td>
</tr>
<tr>
<td>Ash, % of DM</td>
<td>11.4</td>
</tr>
</tbody>
</table>
**TABLE 2.** Primary and secondary antibodies used in the flow cytometric analysis of blood cell populations from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain.

<table>
<thead>
<tr>
<th>Primary antibody specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Working concentration</th>
<th>Secondary antibody (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cell</td>
<td>GC50A1</td>
<td>IgM</td>
<td>14 µg/mL</td>
<td>αIgM-FITC, αIgM-APC</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>CACT80C</td>
<td>IgG1</td>
<td>14 µg/mL</td>
<td>αIgG1-FITC</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>BAQ111A</td>
<td>IgM</td>
<td>14 µg/mL</td>
<td>αIgM-APC</td>
</tr>
<tr>
<td>γδ TCR(^+) T cell</td>
<td>CACT61A</td>
<td>IgM</td>
<td>14 µg/mL</td>
<td>αIgM-PE, αIgM-FITC</td>
</tr>
<tr>
<td>B cell</td>
<td>BAQ155A</td>
<td>IgG1</td>
<td>3.5 µg/mL</td>
<td>αIgG1-FITC</td>
</tr>
<tr>
<td>CD45RO</td>
<td>GC44A</td>
<td>IgG3</td>
<td>20 µg/mL</td>
<td>αIgG3-FITC</td>
</tr>
</tbody>
</table>

\(^1\)Primary antibodies were from VMRD, Pullman, WA.

\(^2\)Phycoerythrin (PE)-conjugated secondary antibodies were from Southern Biotechnology Associates, Birmingham, AL, and were used at a concentration of 1 µg/mL. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Southern Biotechnology Associates, Birmingham, AL, and were used at a concentration of 5 µg/mL. Allophycocyanin (APC)-conjugated secondary antibodies were from Pharmingen, San Diego, CA, and used at a concentration of 2 µg/mL.
TABLE 3. Results ($P$ values) of repeated measures ANOVA for phenotype variables describing blood cell populations from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>Treatment Effect</th>
<th>Time Effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphonuclear leukocytes, %</td>
<td>$&lt; 0.05$</td>
<td>$&lt; 0.0001$</td>
<td>NS (0.55)</td>
</tr>
<tr>
<td>Mononuclear leukocytes, %</td>
<td>$= 0.05$</td>
<td>$&lt; 0.0001$</td>
<td>NS (0.55)</td>
</tr>
<tr>
<td>Contribution to MNL population, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma\delta$ TCR$^+$ cells</td>
<td>NS (0.09)</td>
<td>$&lt; 0.01$</td>
<td>NS (0.06)</td>
</tr>
<tr>
<td>CD$^+$ T cells</td>
<td>NS (0.78)</td>
<td>$&lt; 0.0001$</td>
<td>NS (0.11)</td>
</tr>
<tr>
<td>CD$^-$ T cells</td>
<td>NS (0.51)</td>
<td>$&lt; 0.0001$</td>
<td>NS (0.72)</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>NS (0.18)</td>
<td>$&lt; 0.0001$</td>
<td>NS (0.78)</td>
</tr>
<tr>
<td>B cells</td>
<td>NS (0.48)</td>
<td>$&lt; 0.001$</td>
<td>NS (0.96)</td>
</tr>
</tbody>
</table>

1 NS = Not significant, $P > 0.05$
FIGURE 1. Growth performance of calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain. Shown are the body weights (mean ± SEM) of calves fed to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates at weekly intervals during the period of study. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin.

FIGURE 2. Percentages of mononuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMN) in blood from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain. Shown are the percentages (mean ± SEM) of MNL (panel a) and percentages of PMN (panel b) in blood from calves fed to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin.

*ab* Percentages of MNL or PMN are different between treatments across time, *P* < 0.05.

ANOVA results (*P* values) of the effect of time split by treatment on percentages of MNL were No-growth (< 0.01), Low-growth (0.14), and High-growth (< 0.01). ANOVA results (*P* values) of the effect of time split by treatment on percentages of PMN were No-growth (< 0.01), Low-growth (0.17), and High-growth (< 0.01).

FIGURE 3. Percentages of γδTCR$^+$ cells and B cells and CD4:CD8 ratio in blood mononuclear leukocyte (MNL) populations from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain. Shown are the percentages (mean ± SEM) of γδTCR$^+$ cells (panel a), percentages of B cells (panel b), and CD4:CD8 ratio in blood MNL from calves fed to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves
received a secondary vaccination of ovalbumin. ANOVA results (P values) of the effect of time split by treatment on percentages of γδTCR⁺ cells were No-growth (< 0.01), Low-growth (0.22), and High-growth (0.24). Due to technical difficulty, B cell data for wk 7 is excluded. ANOVA results (P values) of the effect of time split by treatment on percentages of B cells were No-growth (0.14), Low-growth (0.09), and High-growth (< 0.01). ANOVA results (P values) of the effect of time split by treatment on CD4:CD8 ratio were No-growth (< 0.05), Low-growth (0.08), and High-growth (< 0.001).

* Single asterisk indicates that percentages of γδTCR⁺ T cells were lower (P < 0.05) in High-growth calves compared with No-growth calves at wk 6 and 7.

** Double asterisk indicates that percentages of γδTCR⁺ T cells were lower (P < 0.05) in High-growth calves compared with No- and Low-growth calves at wk 5.

FIGURE 4. Percentages of CD4⁺ cells, CD8⁺ cells, and relative proportion of CD4⁺ and CD8⁺ cells expressing CD45RO (memory phenotype) in blood mononuclear cell (MNL) populations from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain. Shown are the percentages (mean ± SEM) of CD4⁺ cells (panel a) and percentages of CD8⁺ cells (panel b) in blood MNL from calves fed to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. Also shown are the percentages (mean ± SEM) of CD4⁺ cells and CD8⁺ cells expressing CD45RO (panels c and d, respectively). At 3 wk of the experimental period, calves were vaccinated with Mycobacterium bovis, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin.

* At wk 5 (2 wk following primary vaccination), percentages of CD8⁺ cells expressing CD45RO were higher (P < 0.05) in High-growth compared with No-growth and Low-growth calves. ANOVA results (P values) of the effect of time split by treatment on percentages of CD4⁺ cells were No-growth (< 0.0001), Low-growth (< 0.001), and High-growth (0.07). ANOVA results (P values) of
the effect of time split by treatment on percentages of CD8+ cells were No-growth (< 0.01), Low-growth (< 0.0001), and High-growth (< 0.0001). ANOVA results (P values) of the effect of time split by treatment on percentages of CD4+ cells and CD8+ cells expressing CD45RO were (< 0.01) for all treatments.

**FIGURE 5.** Relative amounts of ovalbumin-specific IgG1 (panel a) and IgG2 (panel b) in serum from calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. Data represent mean difference (mean ± SEM) between test and background absorbance values. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin. " At wk 5 (2 wk following primary vaccination), concentration of OVA-specific IgG2 was higher (P < 0.05) in High-growth calves compared with Low-growth calves. ANOVA results (P values) of the effect of time split by treatment on ovalbumin-specific IgG1 and IgG2 were P < 0.01 for all treatments.

**FIGURE 6.** In vitro interferon (IFN-γ) secretion by blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. Shown are IFN-γ responses (mean ± SEM) of ovalbumin-(panel a) and purified protein derivative-stimulated (panel b) blood MNL cultures. Values represent responses by stimulated cell cultures minus responses by non-stimulated cell cultures. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin. ANOVA results (P values) of the effect of time split by treatment on IFN-γ responses in OVA-stimulated cultures were No-growth (< 0.001), Low-growth (0.42), and High-growth (< 0.0001). ANOVA results (P values) of the effect of time split by
treatment on IFN-γ responses in PPD-stimulated cultures were No-growth (< 0.001), Low-growth (< 0.01), and High-growth (< 0.001).

**FIGURE 7.** In vitro nitric oxide (NO) secretion by blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. The amount of nitrite, a stable product of nitric oxide production, in culture supernatants is a correlate of the amount of nitric oxide produced. Shown are nitrite (mean ± SEM) concentrations in purified-protein derivative-stimulated MNL cultures. Values represent responses by stimulated cell cultures minus responses by non-stimulated cell cultures. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin. ANOVA results (*P* values) of the effect of time split by treatment on NO responses in PPD-stimulated cultures were No-growth (= 0.05), Low-growth (< 0.01), and High-growth (< 0.01).

*ab* Values in panel b with different superscripts are different (*P* < 0.05).

**FIGURE 8.** *In vivo* delayed-type hypersensitivity reactions of calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates. Shown are changes in cervical skin fold thickness (mean ± SEM) 72 h after intradermal administration of *M. bovis* purified protein derivative. Test was performed 5 wk after vaccination and 8 wk after initiation of dietary treatments. At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin.

*ab* Values with different superscripts are different (*P* < 0.05).
FIGURE 9. Viability of blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no (n = 4), low (n = 4), or high (n = 4) growth rates for 7 wk. Data represent percentages (mean \pm SEM) of viable cells (MNL, CD4\(^+\), CD8\(^+\), and \(\gamma\delta\)TCR\(^+\) cells) in non-stimulated (panel a) and purified protein derivative-stimulated 3-d cultures (panel b). At 3 wk of the experimental period, calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin and ovalbumin. At 5 wk, calves received a secondary vaccination of ovalbumin.

\(^{ab}\) Values within a cell phenotype with different superscripts are different \((P < 0.05)\)
FIGURE 1. Growth performance of calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain.
FIGURE 2. Percentages of mononuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMN) in blood from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain.
FIGURE 3. Percentages of $\gamma^\delta$TCR$^+$ cells and B cells and CD4:CD8 ratio in blood mononuclear leukocyte (MNL) populations from calves fed milk replacer at three levels of intake to achieve three targeted daily rates of gain.
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FIGURE 5. Relative amounts of ovalbumin-specific IgG1 (panel a) and IgG2 (panel b) in serum from calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates.
FIGURE 6. In vitro interferon (IFN)-γ secretion by blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates.
FIGURE 7. In vitro nitric oxide (NO) secretion by blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no \((n = 8)\), low \((n = 8)\), or high \((n = 8)\) growth rates.
FIGURE 8. *In vivo* delayed-type hypersensitivity reactions of calves fed milk replacer at three levels of intake to achieve no (n = 8), low (n = 8), or high (n = 8) growth rates.
FIGURE 9. Viability of blood mononuclear cells (MNL) from calves fed milk replacer at three levels of intake to achieve no (n = 4), low (n = 4), or high (n = 4) growth rates for 7 wk.
CHAPTER 5. VACCINATED NEONATAL CALVES MOUNT VIGOROUS B-CELL RESPONSES TO NON-NATURALLY OCCURRING BUT NOT TO NATURALLY OCCURRING ANTIGEN

A paper to be submitted to Veterinary Immunology and Immunopathology

M. R. Foote,1 B. J. Nonnecke,2 W. R. Waters, D. C. Beitz,
1Primary author, 2Corresponding author

Abstract

The objectives of this study were to characterize the adaptive immune response in young calves to ovalbumin (OVA), an antigen not present in the natural environment of dairy cattle, and secondly to compare B cell responses to OVA and Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccination in young calves. Six Holstein bull calves were vaccinated with OVA (4 mg) at 3 and 5 wk of age. Three of the six calves also were vaccinated with M. bovis BCG at 3 wk of age. Vaccination of 3-wk-old calves to OVA elicited antigen-specific IgG1 and IgG2 antibody responses that were amplified with a secondary vaccination 2 wk following primary sensitization, resulting in a strong anamnestic response. Blood mononuclear cells isolated from calves failed to secrete interferon-γ in response to OVA following primary and secondary vaccination. Secretion of OVA-induced interferon-γ by MNL from BCG-vaccinated calves was higher compared with OVA-only vaccinated calves, suggesting BCG vaccination influences immune responses to OVA. In a later experiment, 6 calves were vaccinated with OVA at 3- and 5-wk of age in addition to M. bovis BCG at 3 wk of age. In vitro stimulation of lymph node cells with OVA was associated with decreased expression of CD5, CD21, and CD40 and increased expression of B-B2, CD25, and CD80 on B cells. In vitro stimulation of lymph node cells with purified protein derivative (PPD) increased expression of CD25 and CD80 on B cells, but did not affect expression of any other activation molecule
investigated. Expression patterns of activation molecules on OVA- and PPD-stimulated CD5+ B cells were generally similar to those on all B cells, with the exception that antigen-stimulation increased MHC class II expression on CD5+ B cells but not on all B cells. These results suggest that calves can generate antigen-specific B cells to OVA and M. bovis BCG following vaccination. Vaccination with a naturally occurring antigen, however, resulted in poor in vivo and in vitro B cell responsiveness, despite generation of antigen-specific B cells. Non-responsiveness of B cells to in vitro PPD stimulation was characterized by no change in CD5, B-B2, CD21, and CD40 expression.

(Key words: calf vaccination, adaptive immunity, Mycobacterium bovis BCG, calf)

1. Introduction

Vaccination of the neonatal calf is frequently characterized by a lack of a demonstrable antibody response. Young calves fail to develop humoral responses to Salmonella pullorum, Klebsiella pneumoniae polysaccharide, Trichomonas foetus, bovine viral diarrhea virus (BVD), or Brucella abortus (Endsley et al., 2003; Husband and Lascelles, 1975; Ingram and Smith, 1965; Kerr and Robertson, 1954; Nonnecke et al., 2005). In addition, calves fail to produce endogenous antibody in response to Mycobacterium bovis bacillus Calmette-Guerin (BCG) vaccination despite strong humoral responses in adults (Nonnecke et al., 2005). Although the importance of colostral immunoglobulin in promoting calf health is well-established, maternal antibody may block humoral responses of the calf to vaccination (Aldridge et al., 1998; Husband and Lascelles, 1975). Neonatal calves fail to develop antibody responses to B. abortus if antigen-specific maternal immunoglobulin is present at the time of vaccination (Husband and Lascelles, 1975). Colostrum-deprived calves, however, do mount relatively strong humoral responses to B. abortus. In addition, neonatal calves sensitized to ovalbumin (OVA) at birth mount humoral responses similar to older cattle (120 d of age), regardless of colostral status (Husband and Lascelles, 1975).
Although neonatal calves vaccinated with *M. bovis* bacillus Calmette-Guerin (BCG) fail to demonstrate antigen-specific humoral responses, antigen-induced T cell subset proliferation and secretion of IFN-γ, NO, and TNF-α by PBMC are comparable to or greater than responses of vaccinated adults (Nonnecke et al., 2005). In addition, the magnitude of DTH reactions to purified protein derivative (PPD) are similar in vaccinated calves and adults, demonstrating the capacity of the bovine neonate to develop vigorous cell-mediated immune response *in vivo* (Nonnecke et al., 2005). In addition, T cell subsets from calves vaccinated with BVDV develop antigen-specific recall responses despite blocking of primary humoral responses by maternal antibody (Endsley et al., 2004; Endsley et al., 2003). Memory T and B cells also are generated by vaccination of young calves to BVDV, despite presence of maternal antibody (Endsley et al., 2004; Endsley et al., 2003). These data suggest that although maternal antibody may block endogenous Ig production, B cells differentiate into memory B cells.

Colostral-antibody may inhibit endogenous immunoglobulin production by reducing the immunoglobulin positive cells in lymphoid tissues of neonatal calves (Aldridge et al., 1998). The reduction of Ig-positive cells, however, does not explain the similar humoral responses of colostrum-deprived and colostrum-fed calves to OVA sensitization (Husband and Lascelles, 1975). The uptake, internalization, and processing of maternal antibody:antigen complexes by APC also contributes to the negligible antibody response of the neonate to vaccines and could result in observed inhibition of B cell but not T cell responses (Siegrist, 2003). In addition, evidence suggests that epitope-specific masking of immunodominant antigen epitopes by maternal antibody prevents recognition by corresponding neonatal B cells (Siegrist, 2003).

The predominant B cell population during human fetal life is the CD5⁺ B cell and the proportion of this B cell subset decreases with age (Berland and Wortis, 2002; Dono et al., 2004). CD5⁺ B cells are thought to produce antibodies that have low affinity and broad specificity (Berland and Wortis, 2002; Dono et al., 2004). CD5⁺ expression on B cells increases with lipopolysaccharide
treatment and decreases with IL-4, suggesting the CD5 molecule may play a role in cell signaling (Jyonouchi et al., 1990). In addition, bovine CD5+ B cells can be induced to express CD5 upon B cell receptor (BCR) crosslinking with anti-bovine IgM (Haas and Estes, 2000). Signaling through CD40, however, prevents the BCR crosslinking-induced expression of CD5 (Haas and Estes, 2000).

B-B2 is a B cell-restricted molecule with no apparent human orthologue. B-B2+ ileal intraepithelial lymphocytes from calves co-express slgM and CD21, but rarely co-express CD5 (Wyatt et al., 1999). Expression of CD21, the C3d receptor, forms a complex with CD19 and CD81 to form the B cell co-receptor. Coligation of the coreceptor complex with BCR reduces the amount of antigen required for B cell activation by 10-100 fold (Carroll and Prodeus, 1998). CD5+ B cell expansion and self-replenishment requires CD21, but CD21 expression can be decreased via signaling through CD70 (Arens et al., 2004). Expression of CD25 (α-chain of IL-2r) on B cells increases upon activation and is higher on memory than on naïve B cells (Arens et al., 2004; Tangye et al., 2003). CD40 is constitutively expressed on B cells and binding with its ligand (CD40L) induces isotype switching, increased CD25 expression, and enhanced MHC class II and CD80 expression (Clatza et al., 2003; McHeyzer-Williams and McHeyzer-Williams, 2005).

The objectives of these experiments were to describe antigen-specific responses of the young colostrum-fed calf sensitized to OVA, and secondly, compare the responses of calves to a naturally occurring antigen with those to a non-naturally occurring antigen. We hypothesized that immune responses to the non-naturally occurring OVA would be different than to M. bovis BCG vaccination.

2. Materials and Methods

2.1 Preparation of OVA and M. bovis BCG

Crystallized ovalbumin (Sigma Chemical Co., St. Louis, MO) was dissolved 2:1 (mg/mL) in sterile PBS and then diluted 1:1 (vol/vol) in incomplete Freund’s adjuvant (MP Biomedical, Inc., Aurora, Ohio). The mixture (OVA/IFA) was emulsified and administered to calves within an hour of
preparation. Calves were vaccinated subcutaneously (subQ) with 4 mL of OVA/IFA containing 4 mg of OVA.

*M. bovis* BCG (Pasteur strain) was grown in Middlebrook's 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma) as described for virulent *M. bovis* (Bolin et al., 1997). Bacilli were enumerated by serial dilution plate counting on Middlebrook’s 7H11 selective media (Becton Dickinson, Cockeysville, MD). Calves were vaccinated subcutaneously with $10^7$ cfu of *M. bovis* BCG in 2ml of PBS.

### 2.2 Vaccination Schedule for Experiment 1

Holstein bull calves were acquired at 1-4 d of age from a single herd and housed at the National Animal Disease Center. Calves received 3.9 L each of colostrum shortly after birth. At 3 wk of age, calves ($n = 6$) were vaccinated with OVA/IFA in the mid-cervical region on the left side of the neck. On the same day, a subset of calves ($n = 3$), was also vaccinated with *M. bovis* BCG subcutaneously in the mid-cervical region on the right side of the neck. At 5 wk of age, all calves ($n = 6$) were revaccinated with OVA/IFA.

### 2.3 Vaccination Schedule for Experiment 2

Holstein bull calves were acquired at 1-5 d of age from a single herd and housed at the National Animal Disease Center, ARS, USDA. All calves received 3.9 L each of colostrum shortly after birth. At 3 wk of age, calves ($n = 24$) were vaccinated with OVA/IFA in the mid-cervical region on the left side of the neck and also vaccinated with BCG in the mid-cervical region on the right side of the neck. At 5 wk of age, all calves ($n = 24$) were revaccinated with OVA/IFA. At 7 wk of age, a subset of calves ($n = 6$) was euthanized and superficial cervical lymph nodes from both sides of the neck were harvested.
2.4 Blood collection and mononuclear cell recovery and enrichment

Peripheral blood was collected from calves in both experiments immediately before vaccination (3-wk of age) and at 5, 6, and 7 wk of age. Sixty milliliters of blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose [sodium citrate (77 μmol/L), citric acid (38 μmol/L), and dextrose (122 μmol/L)] by jugular venipuncture. Smaller blood samples for analysis of antigen-specific Ig concentrations in serum were collected weekly into 10-mL vacutainers containing no additive (Becton Dickinson, Franklin Lakes, NJ).

Cells used in IFN-γ and in vitro Ig production assays were isolated from peripheral blood and enriched by density gradient centrifugation as described previously (Nonnecke et al., 1991). The mononuclear leukocyte (MNL)-enriched populations were resuspended in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine (Sigma, St. Louis, MO) antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin, Sigma), 50 μM 2-mercaptoethanol (Sigma), 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 10% (v/v) heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT).

2.5 Recall and capture antigens

Recall antigens used were ovalbumin (Sigma) and M. bovis-derived PPD (Pfizer, Kalamazoo, MI). A proteinase K-digested whole cell sonicate of M. bovis BCG (WCS-PK), used as capture antigen in ELISA, was prepared from cultures of M. bovis BCG as described previously (Nonnecke et al., 2005). Protein concentration of the WCS-PK antigen was determined using a kit provided by Bio-Rad (Hercules, CA). The WCS-PK antigen was stored at -20°C.

2.6 Measurement of antibody in serum and in culture supernatants
Ovalbumin- and WCS-PK-specific immunoglobulin (Ig) levels in supernatant from non-stimulated and antigen-stimulated blood MNL cultures were evaluated. Cells were from blood samples collected 2, 3, and 4 wk after primary vaccination. Triplicate cultures in 96-well tissue culture plates were seeded with $2 \times 10^5$ cells and were non-stimulated, stimulated with OVA (10 $\mu$g/mL), or stimulated with PPD (10 $\mu$g/mL). Cultures were incubated for 8 d at 39°C in a humidified atmosphere with 5% CO$_2$. Supernatants from centrifuged (400 x g, 5 min) plates were stored at -80°C until analyzed.

The relative amounts of OVA and WCS-PK specific Ig in serum and in culture supernatants were determined by capture ELISA as described previously for determination of WCS-PK specific Ig (Nonnecke et al., 2005). The concentration of OVA used in the ELISA was 1.56 $\mu$g/mL of PBS. The concentration of WCS-PK used in the ELISA was 40 $\mu$g/mL of carbonate/bicarbonate coating buffer (pH 9.6). Microtiter plates (96-well; Immulon II, Dynatech) were coated with either OVA or WCS-PK (100 $\mu$L/well). Plates containing controls (i.e., coating buffer or PBS alone) were incubated for 15 h at 4°C. Plates were then washed 3x with 0.05% Tween 20 in PBS (PBST; 200 $\mu$L/well), and blocked with a commercial milk diluent/blocking solution (200 $\mu$L/well; Kirkegaard and Perry Laboratories, Gaithersburg, MD). After incubating for 1 h at 37°C in the blocking solution, wells were washed with PBST and test sera were added to wells (100 $\mu$L/well). Test and control sera were diluted in PBS with 0.1% gelatin. Optimal dilutions of test sera were determined by evaluation of the reactivity of 2-fold serial dilutions ranging from 1:6 to 1:800. Culture supernatants were not diluted. After incubating for 20 h at 4°C, wells were washed with PBST and incubated for 1 h at 37°C with horseradish peroxidase-conjugated, anti-bovine IgG heavy and light chains (Kirkegaard and Perry Laboratories) in PBS plus 0.1% gelatin. Wells were then washed with PBST and incubated for 4.5 min at room temperature with substrate (3,3'5,5'-tetramethylbenzidine; Kirkegaard and Perry Laboratories). The reaction was stopped by addition of sulfuric acid (0.18 $M$). The absorbency (@ 450 nm) of individual wells was measured (ELISA plate reader, Molecular Devices, Menlo Park,
Optical densities of test samples were calculated by subtracting the mean optical densities for wells with coating buffer or PBS alone from the mean optical densities for antigen-coated wells containing test samples.

2.7 In vitro production and measurement of IFN-γ

Interferon-γ secretion by blood MNL was evaluated in 96-well, round-bottom tissue culture plates. Wells were seeded with $2 \times 10^5$ cells in a total culture volume of 200 µL (final concentration of $2 \times 10^6$ cells/mL in culture). Culture medium consisted of supplemented RPMI 1640 (Gibco Laboratories) as described for the isolation and enrichment of MNL. Cultures were non-stimulated, stimulated with OVA (1, 5, or 10 µg/mL, Sigma) or stimulated with M. bovis PPD. Triplicate wells were prepared for each in vitro treatment. Plates were incubated at 39°C in a humidified atmosphere of 5% CO₂ for 72 h. Culture supernatants were harvested by centrifugation (400 x g for 2 min at room temperature) and stored at −80°C until analysis. The IFN-γ (ng/mL) in supernatants was quantified using an IFN-γ capture ELISA (Ametaj et al., 1996).

2.8 Expression of activation molecules on B cells isolated from lymph nodes

Mononuclear cells were isolated from superficial cervical lymph nodes by straining through a wire-mesh screen. Cultures (six replicates) in microtiter plates were seeded with $2 \times 10^5$ cells. Cells were non-stimulated (media only), stimulated with OVA (10 µg/mL) or stimulated with PPD (10 µg/mL). Cultures were incubated for 3 d at 39°C in a humidified atmosphere with 5% CO₂.

Primary and secondary antibodies used for flow cytometric-staining are listed in Table 1. Monoclonal primary antibody diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS was added to individual wells in 50 µL aliquots. Cells were incubated for 15 min at room temperature and centrifuged (400 x g at room temperature for 2 min). Supernatant was decanted and cells were
labeled with 100 μL of a cocktail containing four secondary isotype-specific antibodies conjugated to fluorochromes (listed in Table 1). Secondary antibodies were diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were incubated for 15 min at room temperature in the dark and then centrifuged as described above. Cells were resuspended in 200 μL of FacsLyse buffer (Becton Dickinson, San Jose, CA) and stored in the dark at 4°C until examined on the flow cytometer. Ten thousand cells exhibiting light scattering properties consistent with bovine MNL were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson, San Jose, California). Data were acquired using CellQuest (Becton Dickinson, San Jose, California) software and were analyzed using FlowJo (Tree Star Inc., San Carlos, CA) software.

2.9 Statistical analysis

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. Serum antibody concentration was analyzed as a split-plot with repeated measures ANOVA. The model included the fixed effects of treatment (OVA vaccination or the combination of OVA and BCG vaccinations), time (wk after vaccination), and the treatment × time interaction, and calf was included in the model as the random effect. Fisher's protected-LSD test was applied when significant effects ($P < 0.05$) and trends ($P < 0.10$) were detected by the model. Secretion of IFN-γ and expression of activation molecules were analyzed as a split-plot with factorial ANOVA. Stimulation (i.e., non-, OVA-, or PPD-stimulated), treatment (OVA only or BCG with OVA vaccination), and location were included in the model as fixed effects, and calf was included in the model as the random effect. Fisher's protected-LSD test was applied when significant effects ($P < 0.05$) or trends ($P < 0.10$) were detected by the model.
3. Results

3.1 Antibody responses to OVA and M. bovis vaccination

Vaccination of 3-wk-old calves with OVA/IFA elicited detectable OVA-specific IgG_1 and IgG_2 responses 2 wk after vaccination. These responses were enhanced 1 wk after revaccination with OVA/IFA (Fig. 1a and 1b). Antibody to WCS-PK was detected in sera from all calves (Fig. 1c and 1d). Vaccination with BCG did not affect (P > 0.1) antibody responses to OVA (Fig. 1a and 1b) or induce an antibody response to WCS-PK (Fig. 1c and 1d). WCS-PK-specific IgG_1 and IgG_2 decreased following BCG vaccination. Blood MNL stimulated with OVA in vitro produced (P < 0.05) OVA-specific antibody but when stimulated with PPD failed to produce (P > 0.1) WCS-PK-specific antibody (data not shown), confirming in vivo results.

3.2 IFN-γ responses to OVA and PPD stimulation

Isolated MNL from BCG-vaccinated calves produced IFN-γ in response to PPD (Fig. 2), as reported previously (Nonnecke et al., 2005). Cells from vaccinated calves failed to secrete (P > 0.1) IFN-γ in response to stimulation with OVA. Cells from calves vaccinated with both OVA and BCG produced more (P < 0.05) IFN-γ in response to both OVA and PPD than cells from calves vaccinated with OVA only (Fig. 2).

3.3 Expression of B cell activation molecules in response to OVA and PPD

Expression of activation molecules on antigen-stimulated B cell populations (i.e., sIgM^+ cells) and the CD5^+ B cell subset (i.e., CD5^+ sIgM^+ cells) is shown in Tables 2-4. Percentages of B cells were similar (P > 0.05) in non-stimulated and antigen-stimulated MNL populations (data not shown).

Stimulation of MNL originating from the left superficial cervical lymph nodes with OVA affected activation molecule expression on the B cell population and CD5^+ B cell subset (Table 2). Percentages of CD5^+ B cells as well as the MFI of CD5 were lower (P < 0.05) in OVA-stimulated
than in non-stimulated cultures. Percentages of B cells expressing B-B2 and the MFI of B-B2, CD25, and CD80 were higher \( (P < 0.05) \) in OVA-stimulated cultures compared with non-stimulated cultures. The MFI of CD21 and CD40 on B cell populations was decreased \( (P < 0.05) \) by OVA-stimulation. Expression of CD21 was lower on CD25\(^+\) B cells compared with CD25\(^-\) B cells (data not shown). Expression of MHC class II and CD11a/18 on B cells was not affected \( (P > 0.05) \) by stimulation with OVA. In OVA-stimulated cultures, expression patterns of activation molecules on CD5\(^+\) B cell subset were similar to the larger, B cell population. Expression of MHC class II on CD5\(^-\) B cells was higher \( (P < 0.05) \) in OVA-stimulated than in non-stimulated cultures. Expression of CD5, B-B2, CD21, CD25, MHC class II, CD11a/18, CD80, or CD40 on B cell populations of cells from the left superficial cervical lymph nodes was not affected \( (P > 0.05) \) by stimulation with PPD.

Stimulation of MNL from right superficial cervical lymph nodes with PPD, however, increased CD25 and CD80 MFI on the B cell population and CD5\(^+\) B cell subset (Table 3). Expression of MHC class II was higher \( (P < 0.05) \) on PPD-stimulated than non-stimulated CD5\(^+\) B cells. Expression of CD5, B-B2, CD21, CD11a/18 and CD40 on non-stimulated and PPD-stimulated B cells was similar \( (P > 0.05) \). Stimulation with OVA did not affect \( (P > 0.05) \) expression of CD5, B-B2, CD21, CD25, MHC class II, CD11a/18, CD80, or CD40 on B cell populations isolated from right superficial cervical lymph nodes.

Location of the lymphoid MNL (i.e., left vs right superficial cervical lymph node) did not influence the expression of activation molecules on B cells in non-stimulated cultures (data not shown). Location, however, did affect expression on OVA-stimulated B cells (Table 4). Expression of B-B2 and CD25 on B cells was higher \( (P < 0.05) \) on OVA-stimulated B cells from the left lymph node than from B cells from the right lymph node. Conversely, expression of CD5 and CD21 was lower \( (P < 0.05) \) on OVA-stimulated B cells from the left lymph node than from the right lymph node. Expression of CD5, B-B2, CD21, CD25, MHC CLASS II, CD11a/18, CD80, or CD40 on B cells in PPD-stimulated cultures was not affected \( (P > 0.05) \) by the location of the lymph node.
4. Discussion

Results from the present study indicate that the neonatal calf can elicit a robust antibody response to early vaccination and that this primary response can be amplified by revaccination 2-wk after primary vaccination. These results confirm previous research (Husband and Lascelles, 1975) indicating that the very young calf can generate strong antibody responses to an antigen not present in the natural environment of dairy cattle. Blood MNL populations from OVA-vaccinated calves, however, failed to secrete demonstrable amounts of IFN-γ when stimulated with OVA in vitro, suggesting that young calves are incapable of mounting Th1 responses characterized by robust IFN-γ responses to recall antigen in the absence of conditions (i.e., complete Freund's adjuvant) that promote a Th1 response.

Vaccination with BCG increased the OVA specific IFN-γ responses of MNL, suggesting that the Th1-promoting effects of BCG are systemic. BCG vaccination enhances protection against respiratory syncytial virus and can ameliorate symptoms of Th2-mediated diseases (i.e., allergy) suggesting that BCG is capable of promoting Th1 responses as well as down-regulating Th2 responses to various antigens (Choi and Koh, 2002; Marks et al., 2003; Vekemans et al., 2001). In the present study, vaccination with BCG did not affect antibody responses to an unrelated antigen (i.e., OVA).

In the present study, stimulation of MNL from superficial cervical lymph nodes to OVA, an antigen not present in the natural environment of cattle and to which no maternal antibody would be expected in colostrum, affected proportions of certain B cell subsets. In vitro stimulation with OVA resulted in decreased proportions of CD5⁺ B cells and increased proportions of B-B2⁺ B cells. There is controversy concerning the origin and function of CD5⁺ B cells within and across species (Berland and Wortis, 2002; Dono et al., 2004). The CD5⁺ B cell may originate in fetal life and produce polyreactive antibodies with low affinity and broad specificity (Berland and Wortis, 2002; Dono et al., 2004). The prevalence of CD5⁺ B cells in the peripheral immune system is much higher in cattle
and sheep than in mice and humans (Berland and Wortis, 2002). The functional role of CD5+B cells in cattle has not been characterized extensively. If CD5+B cells are of fetal origin, non-clonal, and producers of polyclonal antibodies, it would be expected that this subset would decrease in antigen-stimulated cultures of MNL from vaccinated calves. Our data suggest that the expression patterns of B-B2, CD21, CD25, CD40, and CD80 on the CD5+B cell subset are similar to the general B cell population. Expression of MHC class II increased on CD5+B cells but not all B cells in response to antigenic stimulation suggesting that antigen-presenting functions of the CD5+B cell subset differ from other B cell subsets.

B-B2 is a B cell-restricted molecule in cattle that has no known human orthologue. Functional aspects of B-B2+B cells have not been described well (Wyatt et al., 1999). In the present study, stimulation of lymph node-derived MNL populations with OVA increased the proportion of B-B2+B cells. Stimulation of the same MNL populations with PPD was not associated with changes in the percentage of this B cell subset. Because only OVA sensitization was associated with a measurable antibody response, it is conceivable that the B-B2+B cell subset plays a role in antibody production in vaccinated cattle.

Expression of CD25, MHC class II, and CD80 on B cells in OVA- and PPD-stimulated cultures was similar. Although CD21 and CD40 expression on B cells decreased in response to OVA, expression of these molecules was unaffected by PPD. These results may indicate that colostrum-derived antibody to environmental mycobacteria inhibited the down-regulation of these activation molecules on antigen-stimulated B cells. The interaction of the constitutively expressed CD40 on B cells with CD40L on Thelper cells is critical for the development of CD4+i.e., helper) T cell effector functions, including support for B-cell differentiation and class switching (Grewal and Flavell, 1996). The significance of CD40 and CD21 down-regulation on B cells in OVA-stimulated cultures is not known, but may indicate B cell maturation. In addition, CD21 expression was lower on CD25+B cells than on CD25−B cells. Although this would imply that the proportion of CD25+B
cells in OVA-stimulated MNL populations would be greater than in PPD-stimulated MNL populations, this was not the case (data not shown).

Stimulation with PPD increased expression of CD25 and CD80 on B cells. These results suggest that antigen-specific B cells were generated in response to *M. bovis* BCG vaccination. Although antigen-specific B cells may have been generated, they failed to elicit an antibody response. In addition, PPD-stimulated B cells were relatively unresponsive compared with OVA-stimulated B cells. It has been shown previously that memory B cells are generated by vaccination of young calves to BVDV, despite blocking of primary responses by presence of maternal antibody (Endsley et al., 2004; Endsley et al., 2003). This suggests that although maternal antibody may block endogenous Ig production, B cells differentiate into memory B cells. The uptake, internalization, and processing of maternal antibody:antigen complexes by APC is thought to contribute to the negligible antibody response of the neonate to vaccines (Siegrist, 2003). These processes, however, do not account for the generation of antigen-specific B cells and antigen-specific memory B cells.

Although the Th1-promoting effects of BCG vaccination appeared to be systemic, effects of OVA and BCG vaccination on B cells appeared to be localized within the lymph node draining the vaccination site. The B cells isolated from superficial cervical lymph nodes from the opposite side of the neck from the vaccination site generally were unresponsive to antigenic stimulation.

In conclusion, vaccination of 3-wk-old calves to OVA elicited measurable antibody responses that were amplified by revaccination within 2 wk after primary sensitization. Calves vaccinated with OVA alone or with OVA and BCG, however, had negligible IFN-γ (i.e., CMI) responses to OVA. The expression of CD5, B-B2, CD21, and CD40 on B cell populations from vaccinated calves were either increased or decreased in response to stimulation with OVA but not PPD in the post-vaccination period. These results suggest that the presence of maternal antibody may interfere with expression of these molecules. In addition, expression patterns of B-B2, CD21, CD25, CD40, and
CD80, but not MHC class II, on the CD5+ B cell subset were similar to the general B cell population, suggesting antigen-presenting functions may differ between B cell subsets.

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References


Table 1. Primary and secondary antibodies used in the flow cytometric analysis of cells isolated superficial cervical lymph nodes from vaccinated calves.

<table>
<thead>
<tr>
<th>Primary antibody specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>slgM</td>
<td>Big73A</td>
<td>IgG1</td>
<td>VMRD</td>
<td>αIgG1-PerCP</td>
</tr>
<tr>
<td>slgM</td>
<td>PIG45A</td>
<td>IgG2b</td>
<td>VMRD</td>
<td>αIgG2b-Cy5</td>
</tr>
<tr>
<td>CD5</td>
<td>B29A</td>
<td>IgG2a</td>
<td>VMRD</td>
<td>αIgG2a-PE</td>
</tr>
<tr>
<td>B-B2</td>
<td>BAQ44a</td>
<td>IgM</td>
<td>VMRD</td>
<td>αIgM-FITC</td>
</tr>
<tr>
<td>CD25</td>
<td>LCTB2A</td>
<td>IgG3</td>
<td>VMRD</td>
<td>αIgG3-FITC</td>
</tr>
<tr>
<td>CD21</td>
<td>GB25A</td>
<td>IgG1</td>
<td>VMRD</td>
<td>αIgG1-PerCP</td>
</tr>
<tr>
<td>CD11a/18</td>
<td>BAT75A</td>
<td>IgG1</td>
<td>VMRD</td>
<td>αIgG1-PerCP</td>
</tr>
<tr>
<td>CD40</td>
<td>D. Mark Estes</td>
<td>D. Mark Estes</td>
<td>D. Mark Estes</td>
<td>αIgG1-PerCP</td>
</tr>
<tr>
<td>CD80</td>
<td>BBQ</td>
<td>IgM</td>
<td>Pharmingen</td>
<td>αIgM-FITC</td>
</tr>
</tbody>
</table>

1 VMRD, Pullman, WA
2 anti-CD40 monoclonal antibody generously donated by D. Mark Estes, University of Texas Medical Branch, Galveston.
3 Peridinin chlorophyll protein (PerCP)-conjugated secondary antibody was from Becton Dickinson; Phycoerythrin (PE)-conjugated secondary antibody was from Southern Biotechnology Associates, Birmingham, AL; Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Southern Biotechnology Associates, Birmingham, AL; Cyanine5 (Cy5)-conjugated secondary antibody was from Caltag Laboratories, Burlingame, CA.
Table 2. Expression of activation molecules on the B cell population and the CD5⁺ B cell subset from the left superficial cervical lymph nodes.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>NS</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OVA</td>
<td>PPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cell population</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD5 positive</td>
<td>66.20 ± 2.63a</td>
<td>49.36 ± 4.30b</td>
</tr>
<tr>
<td>CD5 MFI</td>
<td>67.84 ± 10.89a</td>
<td>25.32 ± 6.20b</td>
</tr>
<tr>
<td>% B-B2 positive</td>
<td>37.56 ± 2.97a</td>
<td>54.02 ± 3.21b</td>
</tr>
<tr>
<td>B-B2 MFI</td>
<td>7.53 ± 0.68a</td>
<td>12.05 ± 1.28b</td>
</tr>
<tr>
<td>CD21 MFI</td>
<td>4.93 ± 0.15a</td>
<td>4.15 ± 0.23b</td>
</tr>
<tr>
<td>CD25 MFI</td>
<td>3.95 ± 0.30a</td>
<td>7.05 ± 0.81b</td>
</tr>
<tr>
<td>MHC class II MFI</td>
<td>5.56 ± 0.70</td>
<td>9.22 ± 1.74</td>
</tr>
<tr>
<td>CD11a/18 MFI</td>
<td>11.06 ± 0.91</td>
<td>8.60 ± 0.93</td>
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<td>CD80 MFI</td>
<td>4.53 ± 0.50a</td>
<td>6.82 ± 0.54b</td>
</tr>
<tr>
<td>CD40 MFI</td>
<td>5.80 ± 0.36a</td>
<td>4.46 ± 0.34b</td>
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<td>CD5⁺ B cell subset</td>
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<td></td>
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<tr>
<td>% B-B2 positive</td>
<td>17.20 ± 1.96a</td>
<td>30.54 ± 2.48b</td>
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<tr>
<td>B-B2 MFI</td>
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<td>4.76 ± 0.22b</td>
</tr>
<tr>
<td>CD21 MFI</td>
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<td>3.95 ± 0.23b</td>
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<tr>
<td>CD25 MFI</td>
<td>2.95 ± 0.15a</td>
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<td>MHC class II MFI</td>
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<td>4.09 ± 0.51b</td>
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<td>CD11a/18 MFI</td>
<td>14.12 ± 0.89</td>
<td>12.40 ± 1.24</td>
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<tr>
<td>CD80 MFI</td>
<td>3.27 ± 0.25b</td>
<td>4.10 ± 0.19b</td>
</tr>
<tr>
<td>CD40 MFI</td>
<td>5.17 ± 0.28a</td>
<td>4.18 ± 0.26b</td>
</tr>
</tbody>
</table>

1 Mononuclear cells were from left superficial cervical lymph nodes from calves vaccinated with ovalbumin (OVA) on the left side of the neck at 3- and 5-wk of age in addition to M. bovis bacillus Calmette Guerin (BCG) on the right side of the neck at 3 wk of age. Lymph nodes were from calves (n = 6) euthanized at 7-wk of age. Cells from lymph nodes were non-stimulated (NS), stimulated with OVA (10 µg/mL) or stimulated with PPD (10 µg/mL) for 6 d. Mean fluorescence intensities (MFI; mean ± SEM) and percentages of B cells expressing several activation molecules are shown.

ab Means within a row with different superscripts are different (P < 0.05).
Table 3. Expression of activation molecules on the B cell population and CD5+ B cell subset from the right superficial cervical lymph nodes.

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>NS</th>
<th>OVA</th>
<th>PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B cell population</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>% CD5 positive</td>
<td>66.28 ± 3.73</td>
<td>66.15 ± 2.83</td>
<td>59.98 ± 3.55</td>
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<tr>
<td>CD5 MFI</td>
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<td>39.42 ± 7.42</td>
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<tr>
<td>% B-B2 positive</td>
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<td>MHC class II MFI</td>
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<td>CD11a/18 MFI</td>
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<td><strong>CD5+ B cell subset</strong></td>
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<tr>
<td>% B-B2 positive</td>
<td>20.53 ± 3.27</td>
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<td>B-B2 MFI</td>
<td>4.03 ± 0.26</td>
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<td>CD21 MFI</td>
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<td>3.53 ± 0.20a</td>
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<tr>
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<td>CD11a/18 MFI</td>
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<td>4.08 ± 0.23ab</td>
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<tr>
<td>CD40 MFI</td>
<td>5.01 ± 0.21</td>
<td>4.78 ± 0.24</td>
<td>4.72 ± 0.28</td>
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</tbody>
</table>

1 Mononuclear cells were from the left superficial cervical lymph nodes from calves vaccinated with ovalbumin (OVA) on the left side of the neck at 3- and 5-wk of age in addition to M. bovis bacillus Calmette Guerin (BCG) on the right side of the neck at 3 wk of age. Lymph nodes were from calves (n = 6) euthanized at 7-wk of age. Cells from lymph nodes were non-stimulated (NS), stimulated with OVA (10 µg/mL) or stimulated with PPD (10 µg/mL) for 6 d. Mean fluorescence intensities (MFI; mean ± SEM) and percentages of B cells expressing several activation molecules are shown.

ab Means within a row with different superscripts are different (P < 0.05).
Table 4. Effects of location (left vs right superficial cervical lymph node) of vaccination site on expression of activation molecules on B (sIgM⁺) cells.

<table>
<thead>
<tr>
<th></th>
<th>Location</th>
<th>Left</th>
<th>Right</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OVA-stimulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD5 positive</td>
<td>49.36 ± 4.30</td>
<td>66.15 ± 2.83*</td>
<td></td>
</tr>
<tr>
<td>CD5 MFI</td>
<td>25.32 ± 6.20</td>
<td>54.68 ± 8.49*</td>
<td></td>
</tr>
<tr>
<td>% B-B2 positive</td>
<td>54.02 ± 3.21</td>
<td>43.3 ± 2.84*</td>
<td></td>
</tr>
<tr>
<td>B-B2 MFI</td>
<td>12.05 ± 1.28</td>
<td>8.26 ± 0.59*</td>
<td></td>
</tr>
<tr>
<td>CD21 MFI</td>
<td>4.15 ± 0.23</td>
<td>4.86 ± 0.22†</td>
<td></td>
</tr>
<tr>
<td>CD25 MFI</td>
<td>7.05 ± 0.81</td>
<td>4.58 ± 0.27*</td>
<td></td>
</tr>
<tr>
<td>MHC class II MFI</td>
<td>9.22 ± 1.74</td>
<td>6.34 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>CD11a/18 MFI</td>
<td>8.60 ± 0.93</td>
<td>9.90 ± 0.55</td>
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</tr>
<tr>
<td>CD80 MFI</td>
<td>6.82 ± 0.54</td>
<td>5.40 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>CD40 MFI</td>
<td>4.46 ± 0.34</td>
<td>5.02 ± 0.16</td>
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<tr>
<td><strong>PPD-stimulated</strong></td>
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</tr>
<tr>
<td>% CD5 positive</td>
<td>61.74 ± 2.15</td>
<td>59.98 ± 3.55</td>
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</tr>
<tr>
<td>CD5 MFI</td>
<td>45.30 ± 6.43</td>
<td>39.42 ± 7.42</td>
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</tr>
<tr>
<td>% B-B2 positive</td>
<td>42.04 ± 2.04</td>
<td>45.28 ± 1.96</td>
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</tr>
<tr>
<td>B-B2 MFI</td>
<td>8.50 ± 0.61</td>
<td>9.56 ± 0.92</td>
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<tr>
<td>CD21 MFI</td>
<td>4.69 ± 0.21</td>
<td>4.77 ± 0.23</td>
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<tr>
<td>CD25 MFI</td>
<td>4.86 ± 0.26</td>
<td>6.12 ± 0.53</td>
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<tr>
<td>MHC class II MFI</td>
<td>7.03 ± 0.99</td>
<td>8.92 ± 1.83</td>
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<tr>
<td>CD11a/18 MFI</td>
<td>10.54 ± 1.02</td>
<td>11.04 ± 1.35</td>
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<tr>
<td>CD80 MFI</td>
<td>5.32 ± 0.40</td>
<td>6.32 ± 0.53</td>
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</tr>
<tr>
<td>CD40 MFI</td>
<td>5.13 ± 0.34</td>
<td>5.07 ± 0.21</td>
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</tr>
</tbody>
</table>

1 Mononuclear cells were from superficial cervical lymph nodes from calves vaccinated with ovalbumin (OVA) at 3- and 5-wk of age and M. bovis bacillus Calmette Guerin (BCG) at 3 wk of age. Calves (n = 6) were euthanized at 7-wk of age and lymph nodes were harvested. Cells were non-stimulated (NS), stimulated with OVA (10 μg/mL) or stimulated with PPD (10 μg/mL) for 6 d.

Mean fluorescence intensities (MFI; mean ± SEM) and percentages of B cells positive for indicated activation molecules are shown.

* Means within a row are different (P < 0.05)

† Means within a row are different (P = 0.05)
Fig. 1. Relative amounts of ovalbumin (OVA)- and a proteinase K-digested whole cells sonicate of *M. bovis* bacillus Calmette-Guerin (WCS-PK)-specific IgG1 and IgG2 in sera from calves vaccinated with ovalbumin (OVA) alone at 3 and 5 wk of age (OVA; n = 3) or vaccinated with *M. bovis* bacillus Calmette Guerin (BCG; at wk 3 only) in addition to OVA at 3 and 5 wk of age (OVA+BCG; n = 3). Serum was collected before vaccination (1 and 3 wk of age) and after vaccination (wk 5, 6, 7, and 8 of life). Absorbencies (mean ± SEM) are shown.

Fig. 2. Interferon (IFN)-γ secretion by blood mononuclear cells from calves vaccinated with ovalbumin (OVA) alone at 3 and 5 wk of age (OVA; n = 3) or vaccinated with *M. bovis* bacillus Calmette Guerin (BCG; at wk 3 only) in addition to OVA at 3 and 5 wk of age (OVA+BCG; n = 3). Cells were isolated from calves at 5, 6, and 7 wk of age and stimulated with OVA (10 μg/mL) or PPD (10 μg/mL) for 48 hr. Responses (mean ± SEM) of stimulated cells from OVA vaccinates only and OVA+BCG vaccinates during the post-vaccination period (wk 5, 6, and 7) are shown. Asterisk indicates responses of OVA vaccinates differed from those of OVA + BCG vaccinates (P < 0.05).
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CHAPTER 6. GENERAL CONCLUSIONS

Chapter 6. General Conclusions

Neonates are highly susceptible to bacterial and viral pathogens. This susceptibility increases incidence of morbidity and mortality. The National Animal Health Monitoring Service (NAHMS) reported mortality of dairy heifers in the United States from birth to weaning to be 8.4% in 1991, 11% in 1995, and a range of 7.7% to 9.4% in 2002 (NAHMS, 2002). The NAHMS also has reported that 34% of calves are reported as sickly or morbid, suggesting that over 40% of calves were considered to be ill prior to weaning. Traditional calf-rearing programs limit nutrient intake from milk or milk replacer during the first few weeks of life in order to promote dry feed (i.e., starter) intake and early weaning. Reports of the dramatic improvements in growth performance and feed efficiency by feeding greater amounts of milk replacer with higher protein concentrations has led to interest in intensified or accelerated feeding programs (Bartlett, 2001, Blome et al., 2003, Diaz et al., 2001, Tikofsky et al., 2001). It is believed that intensified or accelerated feeding programs increase the plane of nutrition to more “natural” levels and provide more “biologically appropriate” early growth (Drackley, 2005). The series of experiments presented investigated the effects of age and plane of nutrition on immune responses of the neonatal calf.

Effects of plane of nutrition and age on mitogen-induced proliferation and activation of lymphocyte subsets from calves were investigated in the first experiment (Chapter 2). T-cell subsets from 1-wk-old calves showed decreased proliferative activity, a delayed increase in CD25 expression and no demonstrable increase in CD44 expression or decrease in CD62L expression when compared to the responsiveness of cells from steers. These results suggest that within the first wk of life neonatal lymphocytes may exhibit defects in activation and homing mechanisms. Mitogen-induced proliferation and expression of activation antigens by T cells from 8-wk-old, standard-fed calves, however, were similar to responses of cells from steers indicating T cell function during the neonatal period matures rapidly.
Results did not demonstrate that increasing plane of nutrition above industry-standard recommendations or maintenance requirements benefits immune responses of the neonatal calf. Enhanced plane of nutrition was associated with decreased immune responses to mitogen stimulation, including decreased proliferation of CD4⁺, CD8⁺, and γδ TCR⁺ cells; decreased CD25 expression by CD4⁺ and CD8⁺ cells; and decreased CD44 expression by CD8⁺ cells (Chapter 2). Effects of plane of nutrition on adaptive immune responses of calves vaccinated with *M. bovis* BCG were investigated in the second experiment (Chapter 3). Antigen-specific recall responses to PPD were minimally affected by increased nutrition. Peripheral blood mononuclear cells from intensified-diet calves, however, produced less IFN-γ and more NO in response to mitogen than did cells from standard-diet calves. These results suggest that neonatal nutrition influences functional activities of T lymphocyte subsets essential in the development of cell-mediated immunity. In these two experiments (Chapters 2 and 3), it was determined that the low plane of nutrition diet was not sufficient to induce PEM. In the later study, calves were vaccinated prior to initiation of dietary treatments.

A third experiment (Chapter 4) was designed to investigate the effects of different feeding rates achieving three targeted growth rates (No Growth, Low Growth, and High Growth) on adaptive immune responses of neonatal calves vaccinated with *M. bovis* BCG and OVA 3 wks after initiation of dietary treatments. Humoral responses to OVA and CMI responses to *M. bovis* BCG vaccination were affected minimally by growth rate. These results suggest PEM in the absence of weight loss does not affect negatively adaptive immune responses of calves. In addition, these results suggest also that increasing growth rate or plane of nutrition above maintenance requirements does not benefit adaptive immune responses. These results imply that nutrients are partitioned to the metabolically consumptive immune system in sufficient quantities to support adaptive immune responses in the neonatal calf fed at maintenance requirements. In addition, feeding calves to achieve high growth rates may not affect nutrient partitioning to the immune system in a manner which benefits immunity.
Investigation of the effects of neonatal nutrition or growth rate on immune responses and/or susceptibility to experimental infection would provide further insight.

High growth rate in calves was associated with increased NO production and decreased immune cell viability. Excess NO production and decreased lymphocyte viability may have deleterious effects on disease susceptibility. Further investigation is required to determine if high rates of growth induce metabolic/oxidative stress on immune cells. In addition to the data presented in this dissertation, effects of growth rate on metabolic (glucose, cholesterol, non-esterified fatty acids) and endocrine (glucagon, insulin, ghrelin, growth hormone, leptin) variables that are capable of influencing immunity and metabolic stress are being assessed.

Vaccination of the neonatal calf is frequently characterized by a lack of a demonstrable antibody response to antigen. Objectives of the last studies (Chapter 5) included characterization of adaptive immune responses in young calves to OVA and comparison of B cell responses to OVA (a non-naturally occurring antigen) and *M. bovis* BCG (a naturally occurring antigen) vaccination in neonatal calves. Calves mounted vigorous humoral immune response to OVA but not to *M. bovis* BCG vaccination. Results suggest these differences may be a result of differential expression of CD5, B-B2, CD21 and CD40 on B cells. B cell activity and function in the calf, however, need to be investigated further. In addition, mechanisms of maternal-antibody blocking of humoral responses need to be delineated.

In conclusion, these results suggest that age and neonatal nutrition influence mitogen-induced functional activities of T lymphocyte subsets essential in the development of cell-mediated immunity. In vivo responses to vaccination and in vitro antigen-specific recall responses of MNL from vaccinated calves, however, were affected minimally by neonatal nutrition or growth rate. Results suggest that increasing plane of nutrition above industry-standard recommendations or maintenance requirements does not benefit immune responses of the neonatal calf.
References Cited


Figure 1. Effects of feeding neonatal calves milk replacer at three levels of intake to achieve no (0.11 kg/d), low (0.58 kg/d), or high (1.16 kg/d) growth rates for 7 wk on plasma ghrelin concentrations. Repeated measures ANOVA results (P values) for the effects of growth rate, time, and their interaction on ghrelin concentrations were <0.0001, <0.0001, and 0.0008, respectively.
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