High Growth Rate Fails to Enhance Adaptive Immune Responses of Neonatal Calves and Is Associated with Reduced Lymphocyte Viability¹

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ABSTRACT

The objective of the study was to evaluate the effects of 3 targeted growth rates on adaptive (i.e., antigen-specific) immune responses of preruminant, milk replacer-fed calves. Calves (9.1 ± 2.4 d of age) were assigned randomly to one of 3 dietary treatments to achieve 3 targeted daily rates of gain [no growth (maintenance) = 0.0 kg/d, low growth = 0.55 kg/d, or high growth = 1.2 kg/d] over an 8-wk period. The NRC Nutrient Requirements of Dairy Cattle calf model computer program was used to estimate the milk replacer intakes needed to achieve target growth rates. All calves were fed a 30% crude protein, 20% fat, all-milk protein milk replacer reconstituted to 14% dry matter. Diets were formulated to ensure that protein would not be limiting. All calves were vaccinated 3 wk after initiation of dietary treatments with *Mycobacterium bovis*, strain bacillus Calmette-Guerin and ovalbumin. Growth rates for no-growth (0.11 kg/d), low-growth (0.58 kg/d), and high-growth (1.16 kg/d) calves differed throughout the experimental period. Blood glucose concentrations in high-growth calves increased with time and were higher than in low- and no-growth calves. Mononuclear and polymorphonuclear leukocyte percentages in peripheral blood were unaffected by growth rate but did change with advancing age. Percentages of CD4⁺ T cells increased with age in no-growth and low-growth calves, a characteristic of maturation, but failed to increase in high-growth calves. Growth rate did not affect the percentages of CD45RO⁺ (memory) CD4⁺ and CD8⁺ T cells, antigen (i.e., ovalbumin)-specific serum IgG concentrations, or antigen (i.e., purified protein deriva-

tive)-induced IFN- γ and nitric oxide secretion by mononuclear cell cultures. Antigen-elicited cutaneous delayed-type hypersensitivity responses of no-growth calves exceeded responses of low-growth, but not high-growth, calves. In resting- and antigen-stimulated cell cultures, viabilities of CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ T cells from high-growth calves were lower than those of the same T cell subsets from no-growth and low-growth calves. Alternatively, resting cultures of mononuclear leukocytes from high-growth calves produced more nitric oxide than those from no-growth and low-growth calves. In conclusion, adaptive immune responses were affected minimally by growth rate. The results suggest that protein-energy malnutrition in the absence of weight loss is not detrimental to antigen-specific responses of neonatal vaccinated calves and that a high growth rate does not enhance these responses. The negative effect of a high growth rate on the viability of circulating T cell populations may influence infectious disease resistance of the calf.

Key words: calf nutrition, calf growth, neonatal vaccination, nutritional immunology

INTRODUCTION

Neonatal animals are highly susceptible to bacterial and viral pathogens. Traditional calf-rearing programs limit nutrient intake from milk or milk replacer (MR) during the first few weeks of life to promote dry feed (i.e., starter) intake and allow early weaning. Dramatic improvements in the growth performance and feed efficiency resulting from feeding greater amounts of MR with higher protein concentrations have led to interest in intensified or accelerated feeding programs (Diaz et al., 2001; Tikofsky et al., 2001; Blome et al., 2003). Intensified or accelerated feeding programs may increase the plane of nutrition to more “natural” levels and provide more “biologically appropriate” early growth (Drackley, 2005). Despite limited supportive information, improving the plane of nutrition also may benefit the immune system of the calf, decreasing mor-

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bidity and mortality associated with infectious diseases.

Protein-energy malnutrition (**PEM**) is the major cause of immunodeficiency worldwide (Delafuente, 1991) and is manifested as acute (wasting) and chronic (stunting) forms, resulting in altered body composition and reduced linear growth. Although wasting PEM, but not stunting PEM, negatively influences adaptive (i.e., antigen-specific) immunity, there is evidence that stunting increases the risk of infection-related mortality in humans (Pelletier et al., 1995; Chandra and Sarchielli, 1996). Malnutrition results in high rates of fatal tuberculosis in malnourished children, despite vaccination with *Mycobacterium bovis*, strain bacillus Calmette-Guerin (**BCG**; Udani, 1994). A low CD4⁺:CD8⁺ T cell ratio in the blood has commonly been associated with PEM in humans and weanling mice and is considered a key indicator of depressed T cell-dependent immunity (Chandra, 1991). Imbalances within, rather than between, the 2 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 when compared with CD45⁺ effector and memory phenotypes (Woodward et al., 1999; ten Bruggencate et al., 2001). In addition, IFN- γ and IL-2 production and IL-2 receptor mRNA expression by splenic mononuclear cells are markedly reduced during severe protein deficiency (Mengheri et al., 1992).

Restricting dietary protein and energy enhances immune function in rodents. Mice fed a low-protein (6%) diet have more vigorous antibody and cell-mediated immune (**CMI**) responses after immunization than mice fed a normal-protein (22%) diet (Fernandes et al., 1976). A low-protein diet also abrogates age-related decreases in the responsiveness of lymphocytes to mitogenic stimulation. Similarly, lifelong caloric restriction prevents age-associated reductions in T cell proliferation (Grossmann et al., 1990). Interleukin-2 receptor expression on mitogen-stimulated lymphocytes from rats fed ad libitum is less than on cells from rats fed a 40% food-restricted diet (Iwai and Fernandes, 1989). An ad libitum diet also decreases mitogen-induced proliferation of splenic cells when compared with feed-restricted rats (Fernandes et al., 1997). Feed-restricted rats also have increased levels of IL-2 and lower levels of IL-6 and tumor necrosis factor- α , indicative of a more robust CMI response.

Effects of increased nutrition, in the form of an intensified MR, on the composition and functional capacities of peripheral blood mononuclear cell (**PBMC**) populations in MR-fed calves have been described (Nonnecke et al., 2003; Foote et al., 2005a). Mitogen-stimulated

PBMC from calves fed an intensified MR produce less IFN- γ and more inducible nitric oxide (**NO**) than PBMC from calves fed a standard MR diet. These results indicate that the plane of nutrition affects functional capacities of immune cells that are essential for the development of a CMI response. Feeding an intensified MR also decreases mitogen-induced proliferative responses of CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells; decreases IL-2 receptor expression by CD4⁺ and CD8⁺ cells; and decreases CD44 expression by CD8⁺ cells. Results from a recent study also suggest that the increased nutritional status associated with feeding an intensified MR does not enhance antigen-specific recall responses in calves vaccinated with BCG (Foote et al., 2005b).

The objective of the current study was to investigate the effects of growth rate on antigen-specific immune responses of vaccinated MR-fed calves. Three targeted daily rates of gain (no growth = 0.0 kg/d, low growth = 0.55 kg/d, or high growth = 1.2 kg/d) were evaluated during the neonatal period. Unlike previous studies (Nonnecke et al., 2003; Foote et al., 2005a,b), calves in the present study were vaccinated 3 wk after initiation of dietary treatments and their daily rate of gain was regulated only by the amount of MR fed rather than by both the composition and amount of MR fed.

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 were given 3.9 L of colostrum within 6 h of birth. At birth, navels were dipped in iodine and an *Escherichia coli* vaccine (Gene-col-99; Schering Plough Animal Health, Union, NJ) was administered orally. Calves were transported to the National Animal Disease Center where they were housed individually in elevated pens (1.52 m long $\times$ 0.91 m wide $\times$ 0.91 m high) in a temperature-controlled (18°C) barn. Each calf was given 2 mL of iron dextran (100 mg/mL; AmTech, Phoenix Scientific, Inc., St. Joseph, MO) intramuscularly, 2.5 mL of BoSe (2.19 mg/mL of sodium selenite and 50 mg of *RRR*- α -tocopherol/mL; Schering-Plough Animal Health) intramuscularly, and 2 mL of a vitamin B complex (Phoenix Scientific, Inc.) subcutaneously. Calf health was monitored and recorded daily.

Dietary Treatments

Before the trial, calves were fed twice daily 0.3 kg of a 20% CP, 20% fat MR (Instant Nursing Formula: Dairy

Table 1. Composition of milk replacer¹ fed to calves during the experimental period

Component	Analysis
CP	30.0% DM
Fat	20.0% DM
Lactose	30.0% DM
Crude fiber	≤0.15% DM
Calcium	1.0% DM
Phosphorus	≥0.70% DM
Vitamin A	≥20,000 IU/lb
Vitamin D ₃	≥5,000 IU/lb
Vitamin E	≥100 IU/lb

¹Manufactured by Land O'Lakes Animal Milk Products Co. (Shoreview, MN).

Herd & Beef Calf Milk Replacer; Land O' Lakes, Inc., Shoreview, MN) reconstituted to 15% DM. On the first Monday after arrival (average age 9.1 ± 2.4 d; wk 0 of the experiment), calves were weighed and assigned randomly to 1 of 3 treatment groups (8 calves per treatment) designed to achieve 3 targeted daily rates of gain (no growth = 0.0 kg/d, low growth = 0.55 kg/d, or high growth = 1.2 kg/d) in BW over an 8-wk period. The NRC calf model computer program (NRC, 2001) was used to estimate the MR intakes needed to achieve target growth rates. All calves were fed a 30% CP, 20% fat, all-milk protein MR (Land O' Lakes, Inc.) reconstituted to 14% DM. The diet was formulated to ensure that protein would not be a limiting nutrient. The MR composition is provided in Table 1. Calves were weighed weekly and the amount of MR fed was adjusted at these times to allow for changes in BW. Because vitamin concentrations in the MR were based on the DM intake of high-growth calves, no-growth and low-growth calves were supplemented once weekly to compensate for reduced MR consumption. Supplements were calculated to ensure that all calves received similar amounts of vitamins A, D, and E. Calves were bucket-fed twice a day (0700 and 1800 h) and offered water ad libitum. No starter grain was offered. The amounts of MR offered and refused were recorded at each feeding.

Measurement of Blood Glucose and NEFA

Serum was collected by jugular venipuncture weekly from wk 0 through wk 7 of the study. Samples were collected in the morning following 12 h of fasting. Serum NEFA concentrations were determined colorimetrically as described previously (Johnson and Peters, 1993). Serum glucose concentrations were determined colorimetrically using a commercially available kit for measuring glucose in biological samples (BioAssay Systems, Hayward, CA). In both assays, samples from calves in each treatment group were processed together so that interassay effects would be balanced across treatments.

The intraassay coefficient of variance in both assays was <3%.

Preparation and Administration of Vaccines

The BCG (Pasteur strain) was grown in Middlebrook's 7H9 medium supplemented with 10% oleic acid–albumin–dextrose complex (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) as described previously (Nonnecke et al., 2005). All calves were vaccinated subcutaneously in the right midcervical region with 10^7 cfu of *M. bovis* BCG at wk 3 of the experiment.

Crystallized ovalbumin (OVA, Grade V; Sigma) was dissolved in sterile PBS (2 mg/mL), diluted 1:1 (vol/vol) in incomplete Freund's adjuvant (MP Biomedicals, Inc., Aurora, OH) and emulsified. The OVA–adjuvant emulsion (4 mL) was administered to all calves ($n = 24$) subcutaneously in the left midcervical region at wk 3 and 5 of the experimental period.

Enrichment of PBMC Populations

Peripheral blood was collected by jugular venipuncture at wk 0 (before the initiation of dietary treatments), wk 3 (time of primary sensitization), wk 5 (time of OVA secondary vaccination), and wk 6 and 7 of the experimental period. Blood was collected into 10% (vol/vol) 2× acid–citrate–dextrose [sodium citrate (77 μ M), citric acid (38 μ M), and dextrose 122 μ M] and also into Vacutainers with potassium EDTA (Becton Dickinson). Serum was collected weekly during the study for quantification of antibody concentrations.

The PBMC, isolated as described previously (Nonnecke et al., 1991), were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) with 25 mM HEPES buffer, L-glutamine (2 mM; Sigma), antibiotics (100 U/mL of penicillin G and 100 μ g/mL of streptomycin sulfate; Sigma), 2-mercaptoethanol (50 μ M, Sigma), 1% nonessential AA (Sigma), and heat-inactivated fetal bovine serum (FBS, 10% vol/vol; Hyclone Laboratories, Inc., Logan, UT).

Compositional Analysis of Blood Leukocyte Populations

Flow cytometry was used to evaluate the composition of blood leukocyte populations (Nonnecke et al., 2003). Approximately 5×10^5 cells in 100 μ L of anticoagulated blood were added to each of 8 wells of 96-well, round-bottomed microtiter plates (Costar, Cambridge, MA). Individual wells were preloaded with monoclonal antibody (50 μ L; Table 2) diluted in PBS containing NaN₃.

Table 2. Primary and secondary antibodies used in the flow cytometric analysis of peripheral blood mononuclear cell populations

Primary antibody specificity ¹	Clone	Isotype	Working concentration, $\mu\text{g/mL}$	Secondary antibody ²
CD4 T cell	GC50A1	IgM	14	$\alpha\text{IgM-FITC}$, $\alpha\text{IgM-APC}$
CD8 T cell	CACT80C	IgG_1	14	$\alpha\text{IgG}_1\text{-FITC}$
CD8 T cell	BAQ111A	IgM	14	$\alpha\text{IgM-APC}$
$\gamma\delta\text{TCR}^+$ T cell	CACT61A	IgM	14	$\alpha\text{IgM-PE}$, $\alpha\text{IgM-FITC}$
B cell	BAQ155A	IgG_1	3.5	$\alpha\text{IgG}_1\text{-FITC}$
CD45RO	GC44A	IgG_3	20	$\alpha\text{IgG}_3\text{-FITC}$

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

²Phycoerythrin (PE)-conjugated secondary antibodies were from Southern Biotechnology Associates (Birmingham, AL) and were used at a concentration of 1 $\mu\text{g/mL}$. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Southern Biotechnology Associates and were used at a concentration of 5 $\mu\text{g/mL}$. Allophycocyanin (APC)-conjugated secondary antibodies were from Pharmingen (San Diego, CA) and used at a concentration of 2 $\mu\text{g/mL}$.

(0.02%) and FBS (1% vol/vol). Cells were incubated for 15 min at room temperature and then centrifuged ($400 \times g$ for 2 min at room temperature). The supernatant was decanted, contaminating erythrocytes were removed by hypotonic lysis, and plates were centrifuged. Cells were resuspended in 100 μL of each of 2 isotype-specific secondary antibodies conjugated to fluorochromes (Table 2) diluted in PBS with NaN_3 and FBS. Cells were incubated in the dark for 15 min at 20°C , centrifuged, and then resuspended in FACS Lyse buffer (200 μL ; Becton Dickinson, San Jose, CA). Plates were stored in the dark at 4°C until analysis by flow cytometry. Five thousand cells exhibiting light-scattering properties of bovine PBMC were analyzed. Data were acquired using a BDLSR flow cytometer (Becton Dickinson) and analyzed using FlowJo (Tree Star Inc., San Carlos, CA) and CellQuest software (Becton Dickinson). The percentages of cells staining positive for each marker were recorded.

Measurement of OVA-Specific Serum Antibody

Ovalbumin-specific IgG_1 and IgG_2 concentrations in serum samples were determined by a capture ELISA. Microtiter plates (96-well, Immulon II; Dynatech Laboratories Inc., Guernsey Channel Islands, UK) were coated with OVA (1.56 $\mu\text{g/mL}$ of PBS, 100 $\mu\text{L}/\text{well}$). Plates, including control wells containing PBS alone, were incubated for 15 h at 4°C . Plates were then washed 3 times with PBS with 0.05% Tween 20 (PBST; 200 $\mu\text{L}/\text{well}$) and blocked with a commercial milk diluent-blocking solution (200 $\mu\text{L}/\text{well}$; Kirkegaard and Perry Laboratories, Gaithersburg, MD). Plates were incubated (1 h at 37°C) and washed in PBST, and test sera (100 $\mu\text{L}/\text{well}$) were added to the wells. Test and control sera were diluted 1:100 in PBS containing 0.1% gelatin. After incubation (20 h at 4°C), wells were washed with

PBST and incubated (1 h at 37°C) with horseradish peroxidase-conjugated, antbovine IgG heavy and light chains (Kirkegaard and Perry Laboratories) in PBS plus 0.1% gelatin. Wells were washed with PBST and then incubated for 4.5 min at room temperature with 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories). The reaction was stopped by addition of sulfuric acid (0.18 M). Optical densities (450 nm) of individual wells were measured by using an ELISA plate reader (Dynatech MR7000, Dynatech Laboratories Inc.). The change in optical density was calculated by subtracting optical densities of wells containing PBS from optical densities of wells containing test samples.

In Vitro Production and Measurement of IFN- γ

Individual wells of 96-well round-bottomed microtiter plates were seeded with 4×10^5 cells in 200 μL of supplemented RPMI 1640 medium. Cultures were nonstimulated (medium alone), stimulated with *M. bovis*-derived purified protein derivative (PPDb, 10 $\mu\text{g/mL}$ of pyrogen-free PBS; Pfizer, Kalamazoo, MI) or OVA (10 $\mu\text{g/mL}$), and then incubated at 39°C in a humidified atmosphere of 5% CO_2 for 72 h. Each culture was prepared in triplicate. Plates were centrifuged ($400 \times g$, 5 min, 22°C) and supernatants were harvested and stored at -80°C . Interferon- γ (ng/mL) in supernatants was measured by IFN- γ capture ELISA as described previously (Ametaj et al., 1996). The IFN- γ concentrations in nonstimulated cultures were subtracted from IFN- γ concentrations in stimulated cultures.

In Vitro Production and Measurement of Inducible NO

Production of nitrite, the stable oxidation product of NO, by resting and antigen-stimulated cultures of

PBMC was evaluated in 96-well round-bottomed tissue culture plates. Wells were seeded with 4×10^5 cells/200 μ L of supplemented RPMI 1640 medium. Cultures prepared in triplicate were nonstimulated, stimulated with OVA (10 μ g/mL), or stimulated with PPDb (10 μ g/mL). N^G -Monomethyl-L-arginine, a competitive inhibitor of inducible NO synthase, was used as a negative control and was added to parallel nonstimulated or stimulated cultures to verify that nitrite production was due to inducible nitric oxide synthase. Plates were incubated at 39°C in a humidified atmosphere of 5% CO₂ for 48 h. Nitrite in culture supernatants was measured as described previously (Rajaraman et al., 1998).

Cutaneous Delayed-Type Hypersensitivity

In vivo delayed-type hypersensitivity (DTH) resulting from BCG vaccination was evaluated 5 wk after BCG vaccination. All calves were injected intradermally in the midcervical region with 100 μ L of PPDb (1 mg/mL). Skinfold thickness (mm) was measured immediately before and 72 h after administration of antigen using a vernier caliper. Responses were expressed as differences between these values.

Viability of Antigen-Stimulated Lymphocytes

Viabilities of nonstimulated and antigen-stimulated PBMC were evaluated 7 wk after initiation of dietary treatments. Blood was collected from a subset ($n = 4$ per treatment) of calves. Wells of 96-well round-bottomed plates were seeded with 4×10^5 PKH67-stained cells in a total culture volume of 200 μ L (2×10^6 cells/mL) with 10 replicates per stimulation. Cultures were nonstimulated, stimulated with OVA (10 μ g/mL), or stimulated with PPDb (10 μ g/mL) and incubated for 3 d at 39°C in a humidified atmosphere with 5% CO₂. Treatment replicates were pooled after the incubation period. Approximately 2×10^5 pooled cells in 150 μ L of culture medium were added to individual wells of 96-well round-bottomed microtiter plates. The primary and secondary antibodies used in the analyses are listed in Table 2. Subsets of T cells were labeled with anti-CD4, anti-CD8, or anti- $\gamma\delta$ TCR primary antibodies diluted in PBS containing 0.02% NaN₃ and 1% inactivated FBS. Cells were then incubated for 15 min at room temperature, centrifuged ($400 \times g$ for 2 min at room temperature), decanted, and then labeled with secondary antibodies (Table 2) diluted in PBS with NaN₃ and FBS. Cells were incubated for 15 min at room temperature in the dark and then centrifuged as described above. Cells were then resuspended in PBS (200 μ L) and 7-amino actinomycin D (7-AAD, 1 μ g; Molecular Probes, Eugene, OR), incubated for 15 min at room tempera-

ture, centrifuged, washed in PBS (200 μ L), centrifuged again, and then resuspended in FacsLyse buffer (200 μ L). Cells were stored in the dark at 4°C until analysis. Ten thousand cells per treatment exhibiting light-scattering properties indicative of bovine PBMC were analyzed. Data were acquired using a BDLSR flow cytometer and analyzed using FlowJo software. Cells without 7-AAD staining were considered viable, whereas apoptotic and dead cells showed low and high 7-AAD staining, respectively (Philpott et al., 1996).

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, metabolites, composition of PBMC 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 fixed effects of growth rate (no, low, or high), time (week of experiment), and treatment $\times$ time interaction, and calf was included in the model as the random effect. Average daily gain, cell viability, and DTH were analyzed as a split-plot with factorial ANOVA. Fisher's protected least significant differences test was applied when effects ($P < 0.05$) or trends ($P < 0.10$) were detected.

RESULTS

Growth and Health Performance

The BW of calves were not different before initiation of dietary treatments and averaged 45.0 ± 0.7 , 46.0 ± 2.1 , and 46.1 ± 1.3 kg for no-, low-, and high-growth treatments, respectively (Figure 1). Mean BW of calves from the 3 treatment groups differed ($P < 0.05$) by wk 1 and remained so for the duration of the study. Growth rates for no- (0.11 kg/d), low- (0.58 kg/d), and high-growth (1.16 kg/d) calves differed ($P < 0.0001$) throughout the experimental period. Growth rates during the period before vaccination (wk 0 to 3) differed ($P < 0.05$) and averaged 0.06 (no growth), 0.53 (low growth), and 1.16 kg/d (high growth).

Several calves required treatment throughout the experimental period for scours, respiratory illness, or both. Five of 8 high-growth calves and 2 of 8 no-growth calves required treatment for scours, whereas no low-growth calves experienced scours. Three of 8 high-growth calves were treated for respiratory illness, whereas no calves from the low- or no-growth groups showed clinical signs of respiratory illness.

Figure 1. Body weights (mean $\pm$ SEM) of calves fed milk replacer to achieve no, low, and high growth rates (8 calves per treatment). Dietary treatments were initiated at wk 0 and all calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5.

Concentrations of Glucose and NEFA in Blood

Growth rate affected blood glucose and NEFA concentrations in blood (Figure 2). Glucose concentrations increased ($P < 0.05$) with time in high-growth calves, decreased ($P < 0.05$) with time in no-growth calves, and did not change ($P > 0.05$) with time in low-growth calves (Figure 2A). Treatment $\times$ time interactions for glucose and NEFA data were also significant (glucose: $P = 0.0002$; NEFA: $P = 0.05$). Glucose concentrations in high-growth calves were higher ($P < 0.05$) compared with low-growth and no-growth calves at wk 2, 3, 4, and 7. Glucose concentrations in no-growth calves were lower ($P < 0.05$) than in low-growth calves at all time points after wk 1. Concentrations of NEFA decreased ($P < 0.05$) with time in high-growth calves but did not change ($P > 0.05$) in low-growth or no-growth calves. In addition, NEFA concentrations at wk 6 and 7 were lower ($P < 0.05$) in high-growth calves than in no-growth calves.

Figure 2. Effect of growth rate on glucose (A) and NEFA (B) concentrations (mean $\pm$ SEM) in the peripheral blood of milk replacer-fed calves. Dietary treatments were initiated at wk 0 and all calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5. ^{a-c}Treatment means with different superscripts differ at specific times. *Means within a treatment group differ from the wk 0 value, $P < 0.05$.

Composition of Blood Leukocyte Populations

Effects of growth rate, time, and their interaction on the composition of blood leukocyte populations from calves are summarized in Table 3. The relative contri-

Table 3. Results (*P*-values) of repeated-measures ANOVA for phenotype variables describing blood cell populations

Cell phenotype	Treatment effect	Time effect	Interaction
Polymorphonuclear leukocytes, %	<0.05	<0.0001	NS ¹ (0.55)
Mononuclear leukocytes, %	=0.05	<0.0001	NS (0.55)
Contribution to mononuclear cell population, %			
$\gamma\delta$ TCR ⁺ cells	NS (0.09)	<0.01	NS (0.06)
CD4 ⁺ T cells	NS (0.78)	<0.0001	NS (0.11)
CD8 ⁺ T cells	NS (0.51)	<0.0001	NS (0.72)
CD4:CD8 ratio	NS (0.18)	<0.0001	NS (0.78)
B cells	NS (0.48)	<0.001	NS (0.96)
CD45RO ⁺ CD4 ⁺ T cells	NS (0.70)	<0.0001	NS (0.80)
CD45RO ⁺ CD8 ⁺ T cells	NS (0.74)	<0.0001	NS (0.22)

¹NS = Not significant.

butions of polymorphonuclear leukocytes (PMNL) and mononuclear cells to the total blood cell population are shown in Figure 3. Although PMNL and mononuclear cell percentages were unaffected by treatment, PMNL percentages within the no-growth and high-growth groups decreased ($P < 0.05$) with time. Percentages in the low-growth calves did not change with time. In addition, mononuclear cell percentages increased ($P < 0.05$) with time in no-growth and high-growth calves, but not low-growth calves.

Percentages of $\gamma\delta$ TCR and B cells in the PBMC population are shown in Figure 4. Percentages of $\gamma\delta$ TCR⁺ cells increased ($P < 0.05$) with time in no-growth calves, but not in low- or high-growth calves. Growth rate did not affect ($P > 0.05$) B cell percentages, but B cell percentages did increase ($P < 0.05$) with age. The CD4⁺:CD8⁺ T cell ratio was not affected by growth rate ($P > 0.05$) but did change ($P < 0.05$) with time.

Although CD4⁺ cell percentages were not affected ($P > 0.05$) by growth rate across time (Figure 5A), they did increase ($P < 0.05$) with age in no-growth and low-growth calves. Percentages of CD8⁺ cells were not affected ($P > 0.05$) by growth rate, but did fluctuate ($P < 0.05$) with age (Figure 5B). Percentages of CD4⁺ and CD8⁺ cells expressing a memory phenotype (i.e., CD45RO⁺) were not affected ($P > 0.05$) by growth rate (Figure 5C and 5D, respectively) but did increase with time following vaccination.

Serum Antibody Response to OVA Vaccination

Relative concentrations of OVA-specific IgG₁ and IgG₂ increased (IgG₁: $P < 0.001$; IgG₂: $P = 0.002$) after vaccination (Figure 6A and 6B, respectively). Growth rate did not affect OVA-specific IgG₁ ($P = 0.38$) or IgG₂ ($P = 0.54$) concentrations (Figures 6A and 6B). The treatment $\times$ time interaction was not significant for either IgG₁ or IgG₂ (IgG₁: $P = 0.52$; IgG₂: $P = 0.88$).

Antigen-Induced IFN- γ and NO Secretion

Interferon- γ secretion by OVA-stimulated cells from no- and high-growth calves increased ($P < 0.01$) with time following vaccination, whereas OVA-induced responses of cells from low-growth calves did not change ($P > 0.05$; Figure 7A). Antigen (i.e., PPD_b)-induced IFN- γ secretion was not affected ($P > 0.05$) by growth rate but did increase ($P < 0.01$) with time following vaccination (Figure 7B). The treatment $\times$ time interaction was significant for both OVA ($P < 0.0001$) and PPD_b ($P < 0.05$) induced IFN- γ responses (OVA: $P < 0.0001$; PPD_b: $P < 0.05$).

Ovalbumin-stimulated PBMC from calves did not produce NO (data not shown). Antigen- (i.e., PPD_b)-induced NO responses of PBMC were not affected by the growth rate; however, these responses increased with time following vaccination (data not shown). In nonstimulated cultures, NO responses of cells from high-growth calves (20.89 ± 2.3) exceeded ($P < 0.05$) the responses of cells from low- (13.90 ± 1.7) and no-growth (12.34 ± 1.8) calves.

DTH Responses to PPD_b

Antigen-elicited DTH responses were evaluated 5 wk after vaccination with BCG (Figure 8). Responses of no-growth calves exceeded ($P < 0.05$) those of low-growth calves but were not different ($P > 0.05$) from responses of high-growth calves.

Viability of Nonstimulated and Antigen-Stimulated PBMC

Growth rate affected ($P < 0.05$) T cell viability in both nonstimulated and antigen-stimulated cell cultures (Figure 9). The viability of PBMC, CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells in nonstimulated and PPD_b-stimulated cultures from high-growth calves were markedly lower

Figure 3. Effect of growth rate on percentages (means $\pm$ SEM) of mononuclear cells (A) and polymorphonuclear leukocytes (B) in peripheral blood (8 calves per treatment). All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5.

($P < 0.01$) than the viabilities of the same T cell populations from no- and low-growth calves.

DISCUSSION

The effects of stunting PEM (i.e., no growth), or alternatively elevated growth rates, on immune function in

Figure 4. Effect of growth rate on $\gamma\delta$ TCR⁺ cell (A) and B cell (B) percentages, and CD4:CD8 T cell ratios (C) in blood mononuclear cell populations (8 calves per treatment). All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5. Effects of time, split by treatment, on $\gamma\delta$ TCR⁺ cell percentages were significant for no-growth calves ($P < 0.01$), and for B cell percentages were significant for high-growth calves ($P < 0.01$). Effects of time, split by treatment, on CD4⁺:CD8⁺ ratios were significant for no-growth ($P < 0.05$) and high-growth ($P < 0.001$) calves.

Figure 5. Effect of growth rate on CD4⁺ (A) and CD8⁺ (B) T cell percentages, and percentages of CD4⁺ (C) and CD8⁺ (D) cells expressing CD45RO (memory phenotype) in blood mononuclear cell populations (8 calves per treatment). All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5. Effects of time, split by treatment, on CD4⁺ cell percentages were significant for no-growth ($P < 0.0001$) and low-growth ($P < 0.001$) calves, and for CD8⁺ cell percentages were significant ($P < 0.01$) for all treatment groups. Effect of time, split by treatment, on percentages of CD4⁺ cells and CD8⁺ cells expressing CD45RO were also significant ($P < 0.01$) for treatment groups.

the neonatal calf are poorly characterized. This study describes the immunological consequences of feeding the preruminant calf at different rates to achieve 3 targeted growth rates. Growth rate affected the composition of PBMC populations. Mononuclear cell and CD4⁺ cell percentages increased with age in calves fed at rates to achieve no growth, characteristic of normal maturational changes in the composition of PBMC populations from healthy calves (Foote et al., 2005b; Non-

necke et al., 2005). The percentages of $\gamma\delta$ TCR⁺ cells in no-growth calves did not decrease with time, as would be expected in the maturing calf (Hein and Mackay, 1991), but actually increased. Although the functional role of the $\gamma\delta$ TCR⁺ cell is not well described, it has been suggested that this T cell subset is important in the development of a protective response during tuberculosis (Pollock et al., 1996; Waters et al., 2003). Wasting PEM is also associated with a reduction in the number

Figure 6. Effects of growth rate on ovalbumin (OVA)-specific IgG₁ (A) and IgG₂ (B) levels in serum from OVA-vaccinated calves (8 calves per treatment). Data represent the difference (mean $\pm$ SEM) between test and background absorbance values. All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with OVA at wk 3 and 5. Effects of time on OVA-specific IgG₁ and IgG₂ were significant ($P < 0.01$); however, neither IgG₁ or IgG₂ concentrations were affected by the growth rate.

Figure 7. Effect of growth rate on (A) ovalbumin (OVA)- and (B) purified protein derivative (PPDb)-induced IFN- γ secretion by blood mononuclear cells (8 calves per treatment). Values represent mean ($\pm$ SEM) responses by stimulated cell cultures minus responses by nonstimulated cell cultures. All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with OVA at wk 3 and 5. Effect of time and treatment $\times$ time interactions for OVA- and PPD-induced IFN- γ response data were significant ($P < 0.05$).

Figure 8. Effects of growth rate on cutaneous delayed-type hypersensitivity reactions (8 calves per treatment) evaluated 7 wk after initiation of dietary treatments. Values are changes in skinfold thickness (means $\pm$ SEM) 72 h after intradermal administration of *Mycobacterium bovis* purified protein derivative. All calves were vaccinated with *M. bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5. Values with different superscripts are different ($P < 0.05$).

of $\gamma\delta\text{TCR}^+$ cells in the circulation of *M. bovis*-infected cattle (Doherty et al., 1996). Results from the present study indicate that stunting PEM, represented by the no-growth calves, alters specifically age-related changes in a T cell subset (i.e., $\gamma\delta\text{TCR}^+$) important in adaptive immunity. Surprisingly, other T cell subsets (i.e., CD4^+ and CD8^+ cells) were not affected in these calves. These results and the failure of CD4^+ cell percentages in high-growth calves to increase, as would be expected in healthy calves, suggests that extremes in nutritional status can alter normal age-related changes in the composition of T cell populations in the neonatal calf.

Alterations in the composition of CD4^+ and CD8^+ T cell subsets may contribute to PEM-induced immunosuppression (Woodward et al., 1999; ten Bruggencate et al., 2001). Wasting PEM in mice is associated with an overabundance of T cell populations [i.e., $\text{CD4}^+\text{CD45RA}^+$ T cells (CD4^+ naïve phenotype) and $\text{CD8}^+\text{CD45RA}^+\text{CD62L}^+$ T cells (CD8^+ naïve phenotype)]

Figure 9. Effect of growth rate on viabilities of nonstimulated (A) and purified protein derivative-stimulated (B) blood mononuclear cells (4 calves per treatment) obtained 7 wk after initiation of dietary treatments. Data represent percentages (mean $\pm$ SEM) of viable mononuclear, CD4^+ , CD8^+ , and $\gamma\delta\text{TCR}^+$ cells in 3-d cultures. All calves were vaccinated with *Mycobacterium bovis*, bacillus Calmette-Guerin at wk 3 and with ovalbumin at wk 3 and 5. Values within a cell phenotype with different superscripts are different ($P < 0.05$). PBMC = Peripheral blood mononuclear cells.

that are relatively quiescent when compared with CD45⁺ effector and memory phenotypes of the same T cell populations. Results from the present study demonstrated that feeding calves to achieve 3 different growth rates did not affect the CD4:CD8 ratio or proportions of CD4⁺ and CD8⁺ cells expressing a memory phenotype (i.e., CD45RO). The proportions of memory CD4⁺ and CD8⁺ T cells did, however, increase with time, a possible consequence of immune maturation, vaccination, and natural exposure to antigens. These results suggest that stunting PEM in the neonatal calf does not affect relative proportions of memory CD4⁺ and CD8⁺ T cells. Weight loss (i.e., wasting PEM) may therefore be necessary to alter CD4⁺ and CD8⁺ T cell subsets in young calves.

Based on research indicating that OVA-vaccinated calves develop vigorous antibody responses (Husband and Lascelles, 1975), antigen-specific IgG₁ and IgG₂ responses to OVA vaccination were used to monitor the effects of growth rate on adaptive, humoral immune responses of neonatal calves. In the current experiment, OVA-specific IgG concentrations increased with time but were not affected by growth rate. These results suggest that feeding at rates producing no growth or high growth rates does not compromise the neonatal calf's capacity to generate serum antibody responses.

Neonatal calves vaccinated with BCG exhibit strong CMI responses in vitro and in vivo to recall antigen, PPDb (Nonnecke et al., 2005), and these responses may be associated with protective responses to subsequent challenge with virulent *M. bovis* (Buddle et al., 2003). In the present study, a BCG vaccination and PPDb challenge model was used to evaluate effects of growth rate on CMI responses of no-, low-, and high-growth calves. Calves were vaccinated 3 wk after initiation of dietary treatment to evaluate the effects of preexisting nutritional status on immune responsiveness. With this model, in vitro IFN- γ and NO production by resting and antigen-stimulated PBMC from calves were evaluated 4 wk after BCG vaccination. Growth rate did not affect IFN- γ or NO responses of PPDb-stimulated PBMC. Calves fed a maintenance (i.e., no growth) diet, however, had greater PPDb-induced DTH responses than low-growth calves, suggesting that maintenance-fed calves can produce competent in vivo CMI responses.

Neonatal calves fed at 50% maintenance from 2 to 28 d of age lose weight and have delayed primary humoral responses to K99 antigen compared with calves fed to achieve a growth rate of 1 kg/wk (Griebel et al., 1987). Decreased serum antibody and CMI responses also have been observed in preruminant calves fed a high plane of nutrition (Pollock et al., 1993, 1994). Feeding cattle to lose (-0.52 kg/d), maintain (+0.18 kg/d), or gain (1.07 kg/d) BW does not affect humoral (i.e., antibody)

or CMI responses (Fiske and Adams, 1985). In addition, wasting PEM (a 17% loss of weight over 133 d) in *M. bovis*-infected adult cattle does not affect DTH responses or antigen-induced IFN- γ production and lymphocyte proliferation in vitro (Doherty et al., 1996). These diverse observations characterizing the effects of nutritional status and growth rate on adaptive immunity likely reflect differences in the levels of nutrition investigated (i.e., some diets induced weight loss or wasting, whereas the low plane of nutrition in others met maintenance requirements). Taken together, these data indicate that dietary extremes causing weight loss or high rates of weight gain can influence adaptive immunity in cattle.

In the current experiment, PBMC from high-growth calves produced elevated amounts of NO (nonstimulated cultures only) and exhibited reduced viability (nonstimulated and antigen-stimulated cultures) when compared with the respective populations from no-growth and low-growth calves. These results are similar to those from previous studies (Nonnecke et al., 2003; Foote et al., 2005b) indicating that intensified nutrition resulting in accelerated growth augments NO production by PBMC from neonatal calves. Although NO produced by activated macrophages is an important mediator of bactericidal activities of the macrophages (Rockett et al., 1998), excess NO production can cause immune-mediated tissue damage. In addition to promoting NO secretion, inducible NO synthase activity can increase production of O₂⁻ and peroxynitrite, a highly reactive metabolite capable of nitrating tyrosines, inducing lymphocyte apoptosis, and damaging DNA directly (Bronte et al., 2003). These latter effects may have contributed to the observed increase in apoptosis in both resting and antigen-stimulated cultures of PBMC from high-growth calves. Previous research indicates that feeding an intensified MR is associated with decreased proliferation of mitogen-stimulated CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells and decreased IL-2r expression by mitogen-stimulated CD4⁺ and CD8⁺ cells (Foote et al., 2005a). It has been shown that NO acts at the level of IL-2r signaling, blocking phosphorylation and activation of several signaling molecules (Bingisser et al., 1998; Mazzoni et al., 2002). These results suggest that the effects of excess neonatal nutrition and increased growth rate on proliferative responses, functional capacities, and viability of calf PBMC populations may be related to excess production of NO.

Why an increased growth rate augments NO production by PBMC from neonatal calves is unknown, but it may be associated with effects of excess nutrition on circulating metabolites. In the current experiment, blood glucose concentrations were higher in high-

growth calves than in low-growth and no-growth calves. Hyperglycemia promotes production of reactive oxygen species and reactive nitrogen intermediates that can lead to oxidative and nitrosative stress-induced apoptosis in many cell types (Allen et al., 2005). Taken together, these data suggest that excess neonatal nutrition influences metabolism in a manner that may be detrimental to the stability of certain cell types.

CONCLUSIONS

Effects of a maintenance diet, resulting in negligible growth, on antigen-specific recall responses in vaccinated neonatal calves were minimal. These results suggest that PEM in the absence of weight loss does not influence vaccine-induced adaptive immune responses of the neonatal calf and that weight loss during the neonatal period may be required to depress these responses. In contrast, a plane of nutrition producing a high growth rate was associated with decreased viability of T cell populations that play pivotal roles in the development and regulation of antigen-specific immune responses. Consequences of this effect on immune response capacity of the neonatal calf are unknown. New research is needed to determine whether high rates of growth induce metabolic or oxidative stress that negatively influences cells of the immune system.

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