Effects of dietary vitamin A content on antibody responses of feedlot calves inoculated intramuscularly with an inactivated bovine coronavirus vaccine

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Objective—To investigate effects of low dietary vitamin A content on antibody responses in feedlot calves inoculated with an inactivated bovine coronavirus (BCoV) vaccine.

Animals—40 feedlot calves.

Procedures—Calves were fed diets containing high (3,300 U/kg) or low (1,100 U/kg) amounts of vitamin A beginning on the day of arrival at a feedlot (day 0) and continuing daily until the end of the study (day 140). Serum retinol concentrations were evaluated in blood samples obtained throughout the study. Calves were inoculated IM with an inactivated BCoV vaccine on days 112 and 126. Blood samples obtained on days 112 and 140 were used for assessment of BCoV-specific serum IgG1, IgG2, IgM, and IgA titers via an ELISA.

Results—The low vitamin A diet reduced serum retinol concentrations between days 112 and 140. After the BCoV inoculation and booster injections, predominantly serum IgG1 antibodies were induced in calves fed the high vitamin A diet; however, IgG1 titers were compromised at day 140 in calves fed the low vitamin A diet. Other isotype antibodies specific for BCoV were not affected by the low vitamin A diet.

Conclusions and Clinical Relevance—Dietary vitamin A restriction increases marbling in feedlot cattle; however, its effect on antibody responses to vaccines is unknown. A low vitamin A diet compromised the serum IgG1 responses against inactivated BCoV vaccine, which suggested suppressed T-helper 2–associated antibody (IgG1) responses. Thus, low vitamin A diets may compromise the effectiveness of viral vaccines and render calves more susceptible to infectious disease. (*Am J Vet Res* 2013;74:1353–1362)

Vitamin A and its bioactive metabolites modulate mucosal epithelial cell proliferation and differentiation, apoptosis, permeability of the intestinal epithelium, and immune functions of the intestinal epithelium.[1–4] Vitamin A deficiency is one of the major risk factors for animals with enteritis and pneumonia caused by infectious agents.[5,6] Provision of supplemental vitamin A reduces morbidity or fatalities (or both) in children with measles,[7,8] diarrhea,[6,9] respiratory tract infections,[6,10,11] malaria,[12,13] and HIV infection.[14,15] In addition, effects of vitamin A on immunity to infectious agents have been identified in community- and hospital-based studies.[16–19] In most studies, the role of vitamin A in immune responses to infectious agents has been investigated clinically in humans with disease or experimentally in mice. However, the association between host vitamin A status in domestic animals and many infectious diseases or after administration of vaccines is poorly understood.

Abbreviations

BCoV	Bovine coronavirus
DM	Dry matter
HVA	High vitamin A
IFN	Interferon
IL	Interleukin
LVA	Low vitamin A
NRC	National Research Council
PFU	Plaque-forming unit
RT	Reverse transcriptase
Th	T-helper

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It is unclear whether dietary vitamin A restriction, which is used to increase intramuscular fat or marbling and results in higher quality beef production in terms of meat palatability, influences the prevalence or severity of disease in feedlot calves.[20–22] Moreover, it is unknown whether vitamin A restriction has detrimental effects on immune responses to pathogens or vaccines. In calves, effects of vitamin A on the immune system have been reported in terms of antibody responses against ovalbumin or keyhole limpet hemocyanin,[20,23] mononuclear leukocyte populations,[24] height of the villi in the ileum and size of follicles in Peyer's patches,[25] superoxide production, intracellular signaling in neutrophils,[26] and nitric oxide production by blood mononuclear leukocytes.[27] However, most of these studies found immune responses to or induction of effector immune function by nonmicrobial protein antigens, which may not reflect responses to pathogens or vaccines (including inactivated vaccines) under field conditions and may not detect vaccine- and disease-associated effects of vitamin A.[28]

Bovine coronavirus is a nonsegmented, positive-sense, single-stranded RNA virus in the family Coronaviridae and order Nidovirales. Bovine coronavirus has major tissue tropism for epithelium of the respiratory and intestinal tracts. Infection results in diarrhea in calves, winter dysentery in adult cattle, and nasolacrimal discharge associated with coughing and pneumonia in calves and adult cattle.[29–40] Infection with BCoV and BCoV-associated clinical signs are frequently detected in feedlot and dairy cattle[31–40]; fecal-oronasal infection is one of the major transmission routes for these cattle in field conditions.[32,34] The incidence of BCoV infections and clinical signs are associated with 2 major risk factors: environmental risks (ie, stressful conditions during transport and animal crowding[30,35–37]; a specific outbreak period from October to January[37–41]; herds with high animal density[37,42,43]; cattle housed in barns with tie stalls or stanchions; and the same equipment used to handle manure and feed[37]) and immunologic risks (ie, low serum IgG titers against BCoV in calves at arrival to feedlots[36,44] or in dairy cattle[38]). Other immunologic risks include low serum IgA titers against BCoV in dairy calves associated with diarrhea of prolonged duration[34] and nasal shedding of BCoV and low nasal or lacrimal IgA titers against BCoV in dairy calves.[45] However, little is known about nutritional effects on BCoV infections and immune responses against BCoV.

The objective of the study reported here was to evaluate the impact of intake of a diet low in vitamin A content on the immune responses of cattle to vaccination with BCoV. Our hypothesis was that a diet low in vitamin A would compromise antibody responses against the BCoV vaccine.

Materials and Methods

Animals—Forty Angus calves (mean age, 199 days; range, 163 to 220 days) were obtained from 2 experimental stations of the Ohio Agricultural Research and Development Center (located in Belle Valley, Ohio, and Coshocton, Ohio). They were transported to the feedlot of the The Ohio State University Beef Research Center in Wooster, Ohio. Calves received an intial vaccination and a booster vaccination for infectious bovine rhinotracheitis,[a] parainfluenza-3,[a] *Haemophilus somnus*,[b] *Pasteurella* spp,[b] and *Clostridium* spp[c] and were dewormed with a parasiticide[d] 6 weeks prior to arrival at the feedlot. Calves arrived at the feedlot in early October, and the study was concluded in late February. The calves in this study were part of another larger study[46] on the effects of vitamin A restriction on carcass quality in beef cattle. Sample collection protocols, animal management, and treatments and vaccinations were evaluated and approved by The Ohio State University Institutional Animal Care and Use Committee.

Vitamin A dietary regimens—On the day of arrival at the feedlot (day 0), calves were assigned by use of a completely randomized design to 2 groups (20 calves/group). High and low dietary vitamin A are defined by the NRC[47] as > 2,200 U/kg of dietary DM and < 2,200 U/kg of dietary DM, respectively. Calves in the LVA group were fed a diet that contained 1,100 U of vitamin A/kg of dietary DM, whereas calves in the HVA group were fed a diet that contained 3,300 U of vitamin A/kg of dietary DM. Each calf was housed separately in a pen and fed approximately 7.5 kg of feed/d during the experiment throughout the 140-day study period. The feed was composed of (DM basis) 5% corn silage, 80% whole shell corn, and 15% vitamin and mineral products. The vitamin and mineral products for the HVA group contained 0.06% vitamin A, whereas those for the LVA group contained no vitamin A.

BCoV vaccination—It was assumed that the vitamin A concentration in serum of calves in the LVA group would decrease at approximately 90 to 112 days after initiation of the LVA diet, which would result in a difference of serum vitamin A concentrations between the HVA and LVA groups.[20,48] To study the effect of altered serum retinol concentrations on antibody responses after administration of a defined vaccine antigen (BCoV), calves were administered a commercial vaccine[e] on day 112 and a booster immunization on day 126. As recommended by the manufacturer, 2 mL of the vaccine was administered IM into the brachiocephalicus muscle of each calf on each of those days. The vaccine contained inactivated BCoV (Mebus strain), rotavirus G6 and G10, *Escherichia coli* K99 bacterin, and *Clostridium perfringens* type C toxoid with a saponin adjuvant to enhance the immune response.

Sample collection—Nasal swab specimens, fecal samples, and blood samples were collected from calves on days 0, 4, 35, 112, and 140. Nasal swab specimens were diluted 1:5 in minimum essential medium[f] containing 1% antimicrobial-antimycotic and then centrifuged at 1,000 × *g* for 10 minutes at 4°C. Fecal samples were diluted 1:10 in minimum essential medium containing 1% antimicrobial-antimycotic and then centrifuged at 850 × *g* for 20 minutes at 4°C. Supernatants of nasal swab specimens and fecal samples were stored at –70°C. Blood samples (10 to 15 mL/sample) were collected via jugular venipuncture. Samples were centrifuged at 2,000 × *g* for 20 minutes, and serum then was harvested, heat-inactivated at 56°C for 30 minutes, and stored at –20°C. Processed nasal swab specimens and fecal samples were used for BCoV detection and quan-

tification with a real-time RT-PCR assay. An antibody ELISA was performed with serum and fecal samples to evaluate systemic and fecal antibody responses against BCoV, respectively.

Real-time RT-PCR assay—Extraction of total RNA and amplification of the target gene were performed by use of methods described elsewhere,[49] with a few modifications. Briefly, total RNA was extracted from fecal samples and nasal swab specimens with phenol and guanidine isothiocyanate reagent.[g] A 1-step RT-PCR assay was performed to detect and quantify BCoV in fecal samples and nasal swab specimens. Oligonucleotide primers (forward primer, 5′-GYKGTTWTTATKTTAARCC-3′; and reverse primer, 5′-CATTRGCDGAAACAGCTTG-3′) were designed to anneal to the open reading frame 1b sequence, which resulted in 99-bp amplicions. A probe (fluorescein amidite 5′-ACTAGTAGTGGTGATGCAACTACTGCTTTTGC-3′ black hole quencher 1), was also designed to anneal to open reading frame 1b between the forward and reverse primer. Total RNA extracted from 2 × 10^7 PFUs of BCoV Mebus strain/mL was diluted with 0.1% diethylpyrocarbonated buffer to generate 10-fold serially diluted standards that ranged from 10^1 to 10^7 PFUs/mL. In addition, total RNA extracts from minimum essential medium containing 1% antimicrobial-antimycotic were used as negative control samples. The detection limit for the quantitative real-time RT-PCR assay was 10^2 PFUs/mL. For BCoV detection with the real-time RT-PCR assay, PCR inhibitors in the samples were considered negligible on the basis of results obtained with 10-fold and 100-fold dilutions of RNA.

Antibody ELISA—An indirect antibody-capture ELISA was used to measure titers of serum IgG1, IgG2, IgA, and IgM and fecal IgA against BCoV. In the feces, only IgA was assayed as representative of intestinal antibody responses. The 96-well ELISA plates[h] were coated with monoclonal antibodies against bovine IgG1,[i] IgG2,[j] IgA,[k] and IgM.[l] After blocking with PBS solution–1% bovine serum albumin[m] to prevent nonspecific binding, 4-fold serial dilutions of the serum or fecal samples were added to the plates. Sera from gnotobiotic calves infected with the BCoV Mebus strain or naïve calves were used as positive and negative control samples, respectively. After incubation, 10^6 PFUs of BCoV Mebus strain/mL was added to detect serum and fecal antibodies against BCoV. Finally, diluted guinea pig anti-BCoV Mebus strain hyperimmune serum was added to react with the BCoV Mebus strain. For color development, goat anti–guinea pig IgG conjugated to horseradish peroxidase[n] and horseradish-peroxidase substrate[o] were added to each well. Optical density was measured at 405 nm with a microplate spectrophotometer.[p] Antibodies detected in serum and fecal samples were quantified via endpoint titration, which was defined as the reciprocal of the highest dilution with an optical density above the cutoff value (mean of negative control samples + [2.077 × SD of the negative control samples]).[50]

Serum retinol concentrations—Serum retinol concentrations were determined by use of methods described elsewhere[46]; results for these calves were previously reported for that study.[46] Briefly, 10 calves in each of the LVA and HVA groups were randomly selected and used for analysis of serum retinol concentrations. Blood samples (10 mL/sample) were obtained from the jugular vein at intervals of 28 days from days 0 to 140. Blood samples were immediately wrapped in aluminum foil to prevent light from damaging the retinol, and samples were stored on ice. Samples were centrifuged at 2,200 × *g* for 10 minutes at 4°C; serum was harvested and stored at –20°C until analysis of serum retinol concentration via high-performance liquid chromatography.[46,48] Serum samples were extracted with hexane and dried under nitrogen gas at 37°C, reconstituted with ethanol, and injected into a high-performance liquid chromatography apparatus equipped with a reverse-phase column.[q] The solvent initially was 75% water and 25% methanol (vol/vol), which was gradually changed to 100% methanol during a 2-minute period. Flow rate

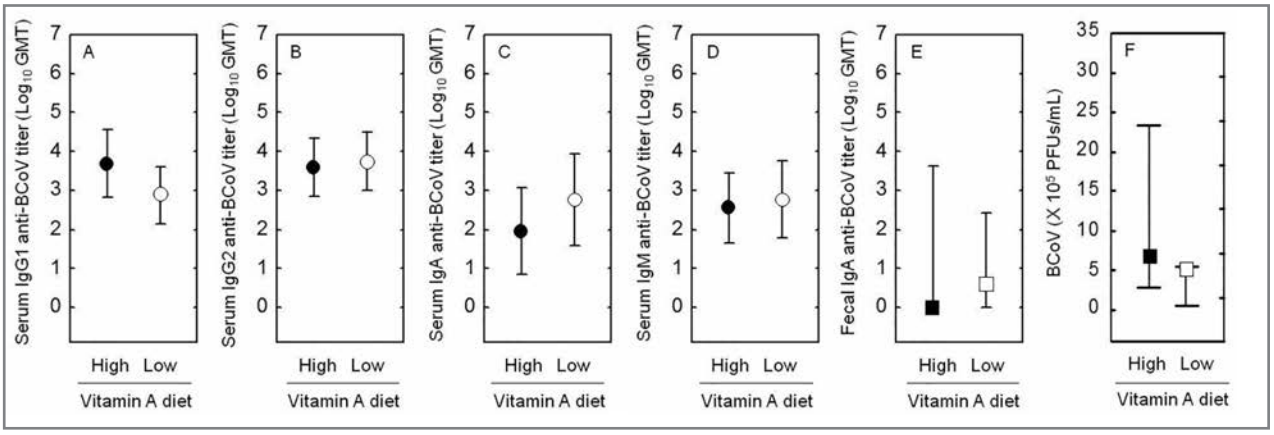

Figure 1—Plots of the geometric mean titer (GMT) for serum IgG1 (A), IgG2 (B), IgA (C), and IgM (D) titers; fecal IgA titers (E); and number of BCoV in fecal samples (F) obtained from calves in HVA (black symbols) and LVA (white symbols) groups at the time of arrival at a feedlot (day 0). Calves in the HVA and LVA groups were fed diets containing 3,300 U of vitamin A/kg of dietary DM and 1,100 U of vitamin A/kg of dietary DM, respectively, daily on days 0 through 140. Antibody GMTs are logarithmically (base 10) transformed for each group (n = 20 and 18 for the HVA and LVA groups, respectively). In panels A–D, the circles are the GMTs and the error bars represent 95% confidence intervals. For panels E and F, the squares are the median values and the error bars represent the upper and lower quartiles (± 1.5 × the interquartile range). For all variables, values did not differ significantly ($P < 0.05$) between groups.

was 1.8 mL/min. All procedures were performed in the dark to prevent light from damaging the retinol.

Statistical analysis—Values quantified with the antibody ELISA were transformed to logarithmic values (base 10) for statistical analysis. An analysis of homoscedasticity of dependent variables was conducted by means of the Cochran C and Hartley F_{max} tests. Although the normality assumption was violated, an ANOVA was used because of its robustness for balanced sample sizes in the comparision groups. The Mann-Whitney *U* test was used to determine significance when the normality assumption was violated and the comparison groups had different sample sizes. A repeated-measures ANOVA was used to determine differences among days of the study, which was followed by the Duncan multiple range test for multiple comparisons. Data were graphed with mean or median ± error bars All statistical analyses were performed with a commercial computer software program.[r] For all tests, results were considered significant at values of $P < 0.05$.

Results

Animals—Two calves in each group were euthanized on day 109 and used for nutritional evaluation of the carcass. One calf in the LVA group died of undefined causes before day 112.

Antibodies against BCoV and shedding on day 0—To verify that both groups (HVA and LVA) had similar natural exposure to BCoV at the time of arrival at the feedlot, BCoV shedding as well as antibody responses against BCoV were measured on day 0 (**Figure 1**). Serum IgG1, IgG2, IgA, and IgM and fecal IgA titers against BCoV as well as the amount of BCoV shedding were plotted for each group. Antibody titers and the amount of BCoV shedding did not differ significantly between the HVA and LVA groups, which indicated that all calves in the HVA and LVA groups had similar exposure to BCoV prior to the study.

BCoV shedding—The frequency and amount of BCoV shedding in naturally infected calves before vaccination was summarized (**Table 1**). Shedding of BCoV was analyzed via the number of BCoV-shedding calves, prevalence of calves shedding BCoV, and amount of BCoV shed. Fecal and nasal shedding of BCoV was defined as the number of fecal samples and nasal swab specimens, respectfully, with positive results for the real-time RT-PCR assay. Samples were available for only 38 calves on day 0 and 39 calves on day 4.

Shedding of BCoV was detected only at early time points (ie, days 0 and 4). On day 0, 8 (21%) calves (3 in the HVA group and 5 in the LVA group) shed BCoV in the feces; no nasal shedding was detected on day 0. On day 4, 16 (41%) calves (7 in the HVA group and 9 in the LVA group) shed BCoV; of these, 8 calves (4 in the HVA group and 4 in the LVA group) also shed BCoV in nasal secretions. Twenty calves (9 in the HVA group and 11 in the LVA group) shed BCoV in feces or nasal secretions at least once on day 0 or 4. The prevalence of shedding of BCoV increased from 8 of 38 (3/20 in the HVA group and 5/18 in the LVA group) on day 0 to 16 of 39 (7/19 in the HVA group and 9/20 in the LVA group) on day 4. However, BCoV shedding was not detected at the later times (days 35, 112, and 140). Generally, the detectable mean amount of BCoV shed in nasal swab specimens was lower than that in fecal samples. On day 4, the mean amount of BCoV shed in nasal swab specimens was 4×10^3 PFUs/mL (6×10^3 PFUs/mL for the HVA group and 3×10^3 PFUs/mL for the LVA group), compared with 7×10^6 PFUs/mL (11×10^6 PFUs/mL for the HVA group and 5×10^6 PFUs/mL for the LVA group) in fecal samples on day 0 and 14×10^6 PFUs/mL (15×10^6 PFUs/mL for the HVA group and 13×10^6 PFUs/mL for the LVA group) in fecal samples on day 4.

Effect of dietary vitamin A content on serum retinol concentration—The serum retinol concentration of the LVA group increased significantly from days 0 to 56 and decreased significantly from days 112 to 140 (**Figure 2**). The serum retinol concentration of the HVA group increased significantly from days 0 to 28 and maintained a plateau from days 28 to 140. Although consistently lower serum concentrations of retinol were detected for the LVA group after day 56, the concentrations did not differ significantly between the HVA and LVA groups from days 0 to 112. However, the serum retinol concentration for the LVA group was significantly lower on day 140 than that in the HVA group.

Effect of dietary vitamin A content on serum IgG1 response to the inactivated BCoV vaccine—To determine the IgG subclass responses against inactivat-

Table 1—Frequency and prevalence of BCoV shedding and amount of BCoV shed by 40 feedlot calves in samples assessed with a real-time RT-PCR assay.

Variable	Day 0	Day 4	Day 35
No. of calves*	38	39	40
No. of infected calves†‡	8 (21)	16 (41)	0 (0)
Fecal shedding‡	8 (21)	16 (41)	0 (0)
Nasal shedding‡	0 (0)	8 (21)	0 (0)
Fecal and nasal shedding‡	0 (0)	8 (21)	0 (0)
Mean amount of BCoV shed			
Fecal (X 10^6 PFUs/mL)	7	14§	0
Nasal (X 10^3 PFUs/mL)	ND	4‖	0

Day of arrival at the feedlot was designated as day 0.
*Samples were not available for 2 calves on day 0 and from 1 calf on day 4. †A calf was considered infected if it had at least 1 fecal sample or nasal swab specimen with a positive result when tested with the real-time RT-PCR assay. ‡Values reported are number (percentage). §,‖Value differs significantly (§$P < 0.05$; ‖$P < 0.001$) from the value for day 0.
ND = Not detected.

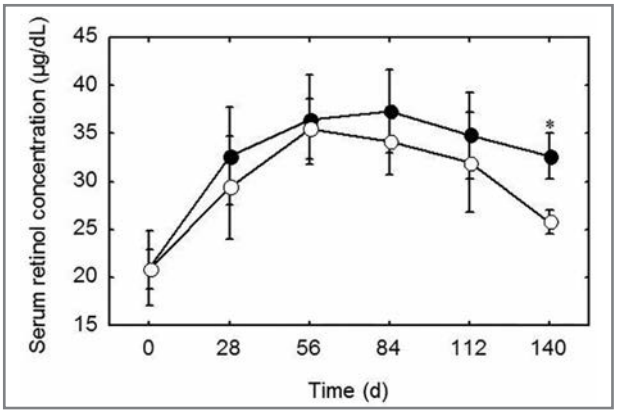

Figure 2—Serum retinol concentrations in the HVA (black circles) and LVA (white circles) groups from days 0 to 140. Results represent mean ± 95% confidence interval of serum retinol concentrations for 10 randomly selected calves in each group. *Within a day, value differs significantly ($P < 0.05$; repeated-measures ANOVA followed by the Duncan multiple range test) from the value for the LVA group. (Adapted from Pickworth CL, Loerch SC, Fluharty FL. Effects of timing and duration of dietary vitamin A reduction on carcass quality of finishing beef cattle. *J Anim Sci* 2012;90:2677–2691. Reprinted with permission.) *See* Figure 1 for remainder of key.

ed BCoV vaccine, serum IgG1 and IgG2 titers against BCoV and the ratios of IgG1 to IgG2 on days 112 (before vaccination) and 140 (after administration of 2 doses of vaccine) were analyzed on the basis of vitamin A groups (**Figure 3**). Serum IgG1 titers against BCoV for the HVA group were significantly ($P = 0.005$) higher on day 140 than on day 112, whereas those for the LVA group did not differ significantly between days 112 and 140. On day 140, serum IgG1 titers against BCoV were significantly higher for the HVA group than for the LVA group. Serum IgG2 titers against BCoV for the HVA and LVA groups typically were lower on day 140 than on day 112; however, the values for days 112 and 140 did not differ significantly, and there were no significant differences in serum IgG2 titers between the HVA and LVA groups on day 112 or 140. Other antibody titers (ie, serum IgM and IgA and fecal IgA) against BCoV were not significantly affected by the dietary vitamin A regimens (data not shown).

In addition, the ratios of IgG1 to IgG2 were used to estimate dominance of antibody immune responses (IgG1 or Th2) over cell-mediated immune responses (IgG2 or Th1) in cattle. The ratios of IgG1 to IgG2 for the HVA group were significantly ($P = 0.005$) higher on day 140 than on day 112, whereas those for the LVA group did not differ significantly between days 112 and 140 (Figure 3). On day 140, the ratios of IgG1 to IgG2 were significantly higher for the HVA group than for the LVA group.

Data on days 112 and 140 were important for understanding the effect of vitamin A on antibody responses to the inactivated BCoV vaccine because significantly reduced serum retinol concentrations were detected on day 140 for the LVA group, which resulted in significant differences in serum retinol concentrations between the HVA and LVA groups on day 140. Interestingly, feeding 1,100 U of vitamin A/kg of dietary DM during the 140-day study resulted in a serum retinol concentration of 25.77 µg/dL, which appeared to

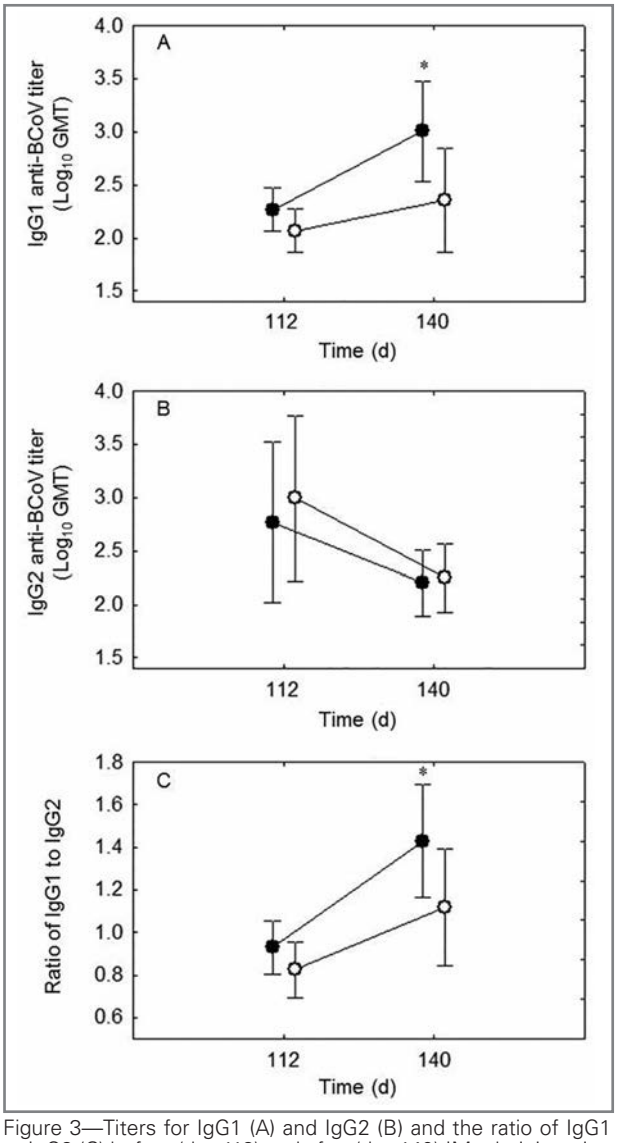

Figure 3—Titers for IgG1 (A) and IgG2 (B) and the ratio of IgG1 to IgG2 (C) before (day 112) and after (day 140) IM administration of 2 doses of an inactivated BCoV vaccine (administered on days 112 and 126) to calves in the HVA (black circles; n = 18) and LVA (white circles; 17) groups. Values reported are the GMT (panels A and B) and mean (panel C) ± 95% confidence interval (panels A–C). *Within a day for each variable, value differs significantly ($P < 0.05$; repeated-measures ANOVA followed by the Duncan multiple range test) between the HVA and LVA groups. *See* Figure 1 for remainder of key.

be low enough to compromise vaccine-induced IgG1 (Th2) responses in the LVA group.

Effects of dietary vitamin A content on serum IgG1 response to BCoV vaccination in calves previously infected with BCoV—The ratios of IgG subclasses for the HVA and LVA groups were assessed in calves naturally infected with BCoV on days 0, 35, and 112 (before vaccination) as well as day 140 (after administration of 2 doses of vaccine). As mentioned previously, 20 calves (9 calves in the HVA group and 11 calves in the LVA group) shed BCoV in the feces or nasal secretions at least once on days 0 or 4. For these 20 calves, the ratios of IgG1 to IgG2 were calculated for the HVA and LVA groups (**Figure 4**). In calves with natural BCoV

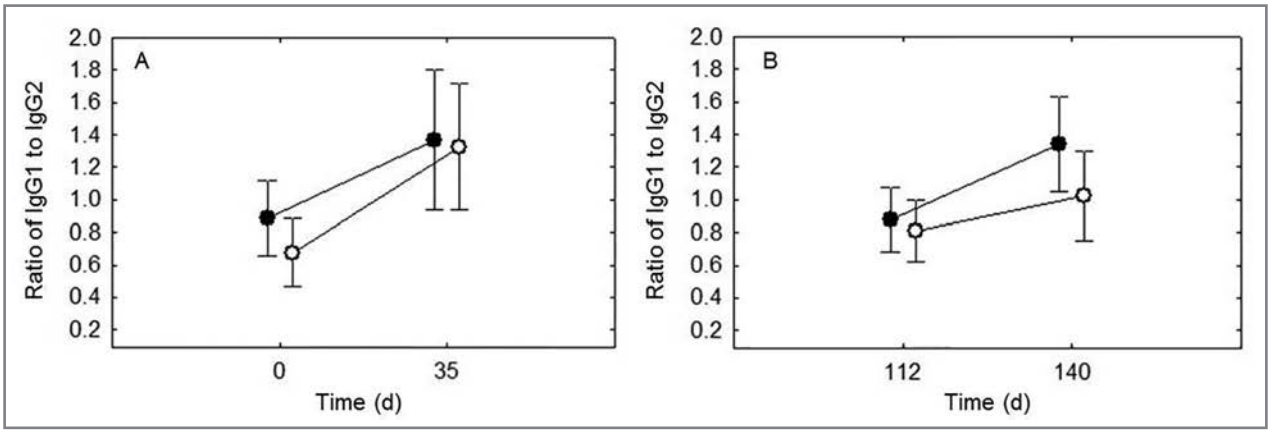

Figure 4—Ratio of IgG1 to IgG2 in response to natural BCoV infection (A) and to administration of an inactivated BCoV vaccine (B) for calves in the HVA (black circles) and LVA (white circles) groups. Data represent the mean ± 95% confidence interval for 20 calves (9 calves in the HVA group and 11 calves in the LVA group) that shed BCoV in the feces or nasal secretions at least once on days 0 or 4. In panel B, values on day 140 differ, but not significantly ($P = 0.06$; repeated-measures ANOVA followed by the Duncan multiple range test), between the HVA and LVA groups. *See* Figure 1 for remainder of key.

infection, the ratios of IgG1 to IgG2 were higher, but not significantly ($P = 0.088$) different, on day 35 than on day 0 in the HVA group and were significantly ($P = 0.025$) higher in the LVA group on day 35 than on day 0, which reflected the fact that natural BCoV infection induced an IgG1-biased response that was not compromised by the short-term period (days 0 to 35) of low dietary vitamin A. After vaccination, the ratios of IgG1 to IgG2 for the HVA group were significantly higher on day 140 than on day 112. In contrast, the ratios of IgG1 to IgG2 for the LVA group did not differ significantly between days 112 and 140. On day 140, the ratios of IgG1 to IgG2 for the HVA group were substantially higher, but did not differ significantly ($P = 0.06$), from those for the LVA group.

Discussion

In the study reported here, the effects of 2 vitamin A diets on the immune responses to BCoV vaccine administered via the parenteral route to feedlot calves were evaluated. We investigated BCoV shedding by means of a real-time RT-PCR assay and antibody responses to a BCoV vaccine via an isotype-specific antibody ELISA in calves fed diets that contained different amounts of vitamin A (HVA group vs LVA group). The number of calves naturally infected with BCoV doubled from day 0 to day 4. In addition, fecal shedding of BCoV was detected on days 0 and 4, whereas nasal shedding of BCoV was detected only on day 4. The BCoV shedding pattern observed in this study may reflect fecal-oral transmission of BCoV in which fecal shedding of BCoV precedes nasal shedding of BCoV. A similar transmission pattern has been found in other studies.[32,34] It has also been suggested that BCoV replication in the respiratory tract may result in subsequent BCoV-induced enteritis after the virus is swallowed and infects enterocytes of the small intestine, which suggests that calves may have BCoV-induced respiratory tract infections before or concurrent with BCoV-induced enteric tract infections.[36,44,45,51,52] However, this was not the case in the present study because fecal shedding preceded nasal shedding. Monitoring BCoV shedding before and after arrival of calves at feedlots may be required to elucidate mechanisms of initial replication and the transmission route of BCoV in feedlot calves. In addition, the effect of dietary vitamin A content on vaccine-mediated antibody responses that were observed could have been influenced by the transport duration, density of animal population, and environmental conditions in the feedlot. Further investigation and analysis would be required to determine effects of these variables on the responses in commercial feedlots.

It has been suggested that vitamin A deficiency in cattle over a wide range of serum retinol concentrations (eg, < 7 or 8 µg/dL or < 20 µg/dL) may be associated with clinical signs in growing calves.[53] By these definitions, vitamin A deficiency was not induced in the LVA group on day 112 or 140. However, the reduced serum retinol concentration induced by the low dietary vitamin A was in parallel with compromised IgG1 (Th2) responses in the LVA group between days 112 and 140 (Figures 2 and 3). In addition, the lowest serum retinol concentration in the present study was detected in calves at the time of arrival on day 0 (ie, 20.85 µg/dL for the HVA group and 20.97 µg/dL for the LVA) and was associated with BCoV shedding, which reflected that infectious agents or transport (or both) might reduce the availability of vitamin A.[54] This observation warrants further investigation with regard to enhancing immunity in calves prior to arrival at feedlots.

Storage and release of hepatic vitamin A are dependent on the serum retinol concentration. In cattle, hepatic vitamin A is released into the blood to maintain the serum retinol concentration at approximately 30 µg/dL.[55] However, once the hepatic vitamin A concentration reaches a certain minimum threshold, the serum retinol concentration begins to decrease, which results in failure to maintain retinol homeostasis.[53] In the present study, the amount of vitamin A for the HVA group (3,300 U/kg of dietary DM) maintained the serum retinol concentration between days 112 and 140; however, the amount of vitamin A for the LVA group (1,100 U/kg of dietary DM) failed to maintain homeostasis of serum retinol concentrations on day 140, which suggested that the minimum threshold concentration of hepatic vitamin A required to decrease the serum reti-

nol concentrations was already reached before day 140. In another study,[46] a 93% reduction in hepatic retinol concentration was detected on day 56 when a diet low in vitamin A content was fed.

Diets low in vitamin A content have frequently been used to increase the amount of intramuscularly deposited fat (ie, marbling), which is one of the criteria for production of higher quality beef in terms of meat palatability.[20–22] To our knowledge, the effect of LVA diets fed to feedlot calves on the incidence of infections or diseases, vaccine efficacy, or immune responses has not been reported. However, dairy calves fed a diet low in vitamin A content developed diarrhea and fever.[53] In that study,[53] male Holstein calves fed 2,300 U of vitamin A/kg of dietary DM (the NRC recommendation for dairy calves is 3,800 U of vitamin A/kg of dietary DM)[56] had lower serum vitamin A concentrations after 28 days. Characteristic signs of vitamin A deficiency were not observed in the calves; however, a higher incidence of high fecal scores (feces was not as formed and contained more liquid) and high rectal temperatures were evident. In particular, the incidence of febrile calves was approximately 3 times as high in calves fed 2,300 U of vitamin A/kg of dietary DM as in those fed vitamin A concentrations greater than NRC recommendations.[53] Thus, it is desirable that the concentration of dietary vitamin A should be optimized for higher quality dairy or beef production without detrimental effects on animal health.

In the present study, results of serologic assays revealed the possibility of reduced efficacy of the BCoV vaccine associated with low dietary vitamin A content. It has been suggested[57] that IgG is the major BCoV-specific virus-neutralizing and hemagglutination-inhibiting antibody in bovine serum. In previous studies[58,59] conducted by our research group, IgG1 titers against BCoV closely paralleled the virus-neutralizing antibody responses in serum. In addition, spike and hemagglutinin-esterase glycoproteins contain the virus antigenic-neutralizing epitopes.[30] In the present study, serum IgG1 responses to the inactivated BCoV vaccine were compromised on day 140 in calves fed the LVA diet (Figure 3). Thus, calves fed diets with low amounts of vitamin A could be more susceptible to infections attributable to BCoV or could fail to have an adequate antibody response to vaccination targeting BCoV.

The relationship between vitamin A and IgG responses has been reported for in vitro and in vivo studies. Retinoic acid, an active metabolite of vitamin A, at concentrations ranging from 10^{-12}M to 10^{-14}M augmented IgG synthesis from peripheral blood mononuclear cells stimulated with formalinized *Staphylococcus aureus*.[60] In addition, retinol binding protein–deficient mice had serum total IgG concentrations that were less than approximately 50% those of wild-type mice.[61] In the present study, the inactivated BCoV vaccine induced a relatively high serum IgG1 titer against BCoV in the HVA group but not in the LVA group. Thus, the prolonged feeding of a diet low in vitamin A content might reduce the immune responses to vaccine antigens as well as infectious agents.

The influence of vitamin A as a micronutrient for enhancing antibody responses is dependent on the nature of the antigen or pathogen. The antibody response to 5 bacterial antigens (polysaccharides from *Streptococcus pneumonia* and *Neisseria meningitides*, lipopolysaccharides from *Pseudomonas aeruginosa* and *Serratia marcescens*, and tetanus toxoid) was investigated in vitamin A–deficient rats.[62] Serum IgM concentrations in vitamin A–deficient rats immunized with polysaccharides from *S pneumonia* and *N meningitides* and tetanus toxoid were extremely low or negligible, compared with IgM concentrations in control rats. In contrast, almost normal IgM concentrations were detected in vitamin A–deficient rats immunized with lipopolysaccharides from *P aeruginosa* and *S marcescens*.[62] These results indicated that compromised antibody responses in rats with vitamin A deficiency are dependent on the type of antigen because polysaccharides from *S pneumonia* and *N meningitides* are classified as T-cell–independent type 2 antigens, lipopolysaccharides from *P aeruginosa* and *S marcescens* are classified as T-cell–independent type 1 antigens, and tetanus toxoid is classified as a T-cell–dependent antigen. Thus, antibody responses were compromised to T-cell–independent type 2 and T-cell–dependent antigen but were not compromised for T-cell–independent type 1 antigens in immunized vitamin A–deficient rats.[62] Other coronaviruses, such as transmissible gastroenteritis virus, are T-cell–dependent antigens that induce CD4+ T lymphocytes and B lymphocytes to synthesize neutralizing antibodies.[63,64] Thus, the characteristic of BCoV antigen, (ie, T-cell–dependent antigen) can be one of the reasons for compromised serum IgG1 responses in calves fed a diet low in vitamin A content.

The dominant IgG1 response in calves in the HVA group naturally infected with BCoV or inoculated with inactivated BCoV vaccine is consistent with that listed in previous reports.[65–68] The T lymphocytes from vitamin A–deficient mice overproduce INF-γ, which diminishes IgG1-secreting B cells and decreases IgG1 production. However, provision of supplemental retinoic acid decreases T-cell secretion of INF-γ and fully restores IgG1 production in mice.[65–67] In addition, retinoic acid positively regulates tetanus toxoid–specific serum IgG1 and IgG2b but negatively regulates IgG2a in primary and secondary responses to tetanus toxoid, which results in elevated ratios of IgG1 to IgG2a (an indicator of Th2-to-Th1 lymphocyte imbalance).[68] The ratios of IgG1 to IgG2 were used in the present study to assess dominance of antibody immune responses (IgG1 or Th2) over cell-mediated immune responses (IgG2 or Th1) in cattle.[69–71] Expression patterns for the IgG subclass in cattle are also related to Th1 or Th2 lymphocyte responses because IL-4 and IL-13 induce IgG1 preferentially over IgG2, whereas INF-γ and IL-12 strongly induce IgG2 over IgG1.[72–74] The relatively higher IgG1 titers against BCoV, compared with the IgG2 titers against BCoV, in the study reported here suggested that vitamin A treatment also modulated adaptive immunity to Th2-like (IgG1) responses in cattle. In cattle, most infectious agents stimulate both Th1 and Th2 lymphocytes, which express both IFN-γ and IL-4. The unpolarized CD4+ Th0 lymphocytes, which enhance production of both IgG1 and IgG2, coexpress IFN-γ and IL-4.[69,75,76] Interestingly, the inactivated BCoV vaccine did not induce increased serum IgM titers against

BCoV, which reflected the fact that the vaccine-mediated immunity was mostly recall-booster responses.[77] In addition, the vaccine did not enhance production of serum and fecal IgA, which may have been related to the parenteral route of vaccination or lack of a mucosal adjuvant (or both).[78] The isotype responses to inactivated BCoV for the 2 vitamin A diets were the focus in the present study. Because microenvironments induced by T cells are critically important for antibody-class switch in B cells, T-cell–cytokine profiles in animals fed restricted amounts of vitamin A should be examined in future studies. Concentrations of the Th2 signature cytokines (eg, IL-4) are expected to be lower in animals fed a diet low in vitamin A content because antigen-specific IgG1 (Th2) responses are compromised. Therefore, vitamin A (or its derivatives) is likely to be an important micronutrient to maintain Th2-mediated immune responses in cattle.

To the authors' knowledge, the study reported here is the first in which investigators have evaluated the impact of a micronutrient (ie, vitamin A) on antibody responses to a BCoV vaccine in feedlot calves. Vitamin A status in the feedlot calves of the present report affected the antibody responses to an inactivated BCoV vaccine. Serum IgG1 production against BCoV was induced in calves fed the HVA diet, whereas IgG1 production was compromised in calves fed the LVA diet, which suggested that the LVA diet suppressed Th2-associated antibody (IgG1) stimulation. In addition, whether restricted vitamin A diets will affect antibody responses to other vaccine antigens (rotavirus G6 and G10, *E coli* K99 bacterin, and *C perfringens*) contained in the multivalent vaccine or will affect systemic biological activities (eg, phagocytic or bactericidal activity of monocytes or neutrophils) remains to be investigated.

a. Quadraplex, Bio-Ceutic, St Joseph, Mo.
b. Somnugen 2P, Bio-Ceutic, St Joseph, Mo.
c. Dybelon, Bio-Ceutic, St Joseph, Mo.
d. Ivomec pour-on, Merck, Rahway, NJ.
e. ScourGuard 3 (K)C, Pfizer Animal Health, New York, NY.
f. Gibco Invitrogen, Grand Island, NY.
g. TRIzol LS reagent, Gibco Invitrogen, Grand Island, NY.
h. MaxiSorp high protein-binding capacity ELISA plates, Nunc Brand Products, San Diego, Calif.
i. Mouse anti-bovine IgG1, Serotec, Raleigh, NC.
j. Mouse anti-bovine IgG2, Serotec, Raleigh, NC.
k. Mouse anti-bovine IgA, Serotec, Raleigh, NC.
l. Monoclonal anti-bovine IgM antibody produced in mouse, Sigma-Aldrich, St Louis, Mo.
m. EMD Chemicals, Gibbstown, NJ.
n. Peroxidase-labeled affinity-purified antibody to guinea pig IgG (H+L), Kirkegaard & Perry Laboratories, Gaithersburg, Md.
o. ABTS peroxidase substrate system, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
p. SpectraMax 340PC^{384}, Molecular Devices, Sunnyvale, Calif.
q. Supelcosil LC-18 (25 cm × 4.6 mm), Supelco Inc, Bellefonte, Pa.
r. Statistica 8 software package, StatSoft Inc, Tulsa, Okla.

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