Bovine Vitamin A and \(\beta\)-Carotene Intake and Lactational Status. 2. Responsiveness of Mitogen-Stimulated Peripheral Blood Lymphocytes to Vitamin A and \(\beta\)-Carotene Challenge In Vitro

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ABSTRACT

The interaction of dietary vitamin A and \(\beta\)-carotene with lactational status on the in vitro proliferation of mitogen-induced peripheral blood lymphocytes was studied. Cows were fed (IU/cow per d) 1) 53,000 IU vitamin A, 2) 213,000 IU vitamin A, or 3) 53,000 IU vitamin A plus 400 mg \(\beta\)-carotene from 6 wk before to 2 wk after dry off. Lymphocytes were incubated with retinol, retinoic acid, or \(\beta\)-carotene. Concanavalin A-induced blastogenesis was inhibited by \(10^{-6}\) M retinol and \(10^{-8}\) M retinoic acid in cows fed 53,000 IU vitamin A before dry off. In contrast, \(10^{-7}\) M retinol and \(10^{-7}\) M retinoic acid stimulated Concanavalin A-induced blastogenesis for cows fed vitamin A plus \(\beta\)-carotene before dry off. After dry off, retinol and retinoic acid did not affect Concanavalin A-induced blastogenesis in all treatment groups. In vitro, \(10^{-5}\) M \(\beta\)-carotene inhibited Concanavalin A-induced blastogenesis before and after dry off in all treatment groups. Blastogenesis in the absence of mitogen stimulation or induced by lipopolysaccharide was inhibited by all vitamins before and after dry off in all treatment groups. These data indicate that vitamin A and \(\beta\)-carotene supplementation interact with lactational status to influence the responsiveness of bovine blood lymphocytes to vitamin challenge in vitro.

INTRODUCTION

Vitamin A and \(\beta\)-carotene play important roles in protecting animals against numerous infections including mastitis [(3, 5, 14) for review]. However, the mechanisms by which these vitamins afford the protection are unclear. Numerous studies indicate that the vitamins modulate lymphocyte function. In vivo experiments have shown vitamin A enhanced mitogen-induced lymphocyte proliferation in rats, mice, and humans [(3, 6,) for review]. However, others (9) have failed to show such effects in retinoic acid-supplemented mice. Similar disagreement is found with in vitro studies. Daniel et al. (5) reported that retinol, retinoic acid, and \(\beta\)-carotene stimulated the proliferation of bovine blood lymphocytes in the presence of Concanavalin A (Con A) but not lipopolysaccharide (LPS). Others (1) found retinoic acid to stimulate human peripheral blood lymphocyte proliferation in the presence of phytohemagglutinin or anti-thymocyte globulin but not Con A. Sidell et al. (16) confirmed the stimulatory effect of retinoic acid on phytohemagglutinin-induced lymphocyte proliferation using human thymocytes and tonsil lymphocytes but were unable to show such effects with peripheral blood lymphocytes.

The discrepancies encountered in vitamin A research have been attributed to differences in species, the type and amount of mitogen used, or the form of vitamin A used (3). However, possible effects of the physiological state or vitamin A and \(\beta\)-carotene status of the experimental animals have been largely overlooked. The dairy cow provides an excellent model in which to test possible interactions of these
effects with the in vitro influence of vitamin A and \(\beta\)-carotene on lymphocyte function. Physiologically, the lactating cow undergoes the stress of high milk production and weight loss during early to midlactation. In later lactation, production declines and the cow gains weight. At the time of dry off, milk production is abruptly halted. Changes in the nutritional needs of the cow parallel these physiological changes. Therefore, we hypothesize that such physiological and nutritional changes in the donor animal may influence the in vitro responsiveness of its lymphocytes to vitamin A or \(\beta\)-carotene challenge. The objectives were to determine the effects of 1) different physiological states (lactating vs. nonlactating) and 2) different dietary amounts of vitamin A and \(\beta\)-carotene on the in vitro responsiveness of blood lymphocytes to vitamin A and \(\beta\)-carotene challenge of cows at dry off.

**MATERIALS AND METHODS**

Details of the experimental procedure are described by Tjoelker et al. (18). Briefly, 30 Holstein cows were used. Six weeks before drying off, cows were assigned to be fed (IU/cow per d): 1) 53,000 IU vitamin A palmitate (adequate vitamin A; AVA), which represents 100% of NRC (11) recommendation, 2) 213,000 IU vitamin A palmitate (high vitamin A; HVA), or 3) 53,000 IU vitamin A palmitate plus 400 mg \(\beta\)-carotene (vitamin A plus \(\beta\)-carotene; VA + BC).

**Lymphocyte Isolation**

At wk 0 (before drying off) and wk 2 (after dry off), blood was collected aseptically from the coccygeal vein into tubes containing 10% sodium citrate (wt/vol). Lymphocytes were obtained by gradient centrifugation using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). Contaminating erythrocytes in the buffy layer were lysed by the addition of 20 ml of distilled, pyrogen-free water. Isotonicity was restored after 30 s by adding 10 ml of 2.7% NaCl (wt/vol). After washing, the lymphocytes were resuspended in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-buffered RPMI-1640 (Roswell Park Memorial Institute) (Sigma) supplemented with 100 units penicillin/ml, 10 \(\mu\)g streptomycin sulfate/ml, 10% heat-inactivated (56°C for 20 min) fetal calf serum (Gibco, Grand Island, NY), and \(5 \times 10^{-5}\) M 2-mercaptoethanol. Total cells were enumerated using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, FL) and percent lymphocytes determined using a Wright-stained smear. Cell viability was determined by trypan blue dye exclusion. Both percent lymphocytes and cell viability generally exceeded 95%. Final cell concentration was adjusted to \(2.5 \times 10^6\) viable lymphocytes/ml.

**Preparation of Vitamins**

Retinol (0, \(0^{-6}\), and \(10^{-7}\) M; final concentrations), retinoic acid (0, \(10^{-7}\), and \(10^{-8}\) M) and \(\beta\)-carotene (0, \(10^{-5}\), and \(10^{-6}\) M) were prepared as described by Tjoelker et al. (18).

**Lymphocyte Transformation Assay**

Concanavalin A (10 \(\mu\)g/ml), which induces T cell proliferation, and Escherichia coli LPS (100 \(\mu\)g/ml), which induces B cell proliferation, were the two mitogens (Sigma) used. The lymphocyte suspension (80 \(\mu\)l) was pipetted into triplicate 96-well round bottom microtiter plates (Corning Glass Works, Corning, NY). Subsequently, 100 \(\mu\)l of mitogen and 20 \(\mu\)l of the vitamins were added to each well. Plates were incubated 48 h at 37°C in a humidified 5% CO\_2, 95% air atmosphere. Following incubation, 1 \(\mu\)Ci (in 20 \(\mu\)l) of thymidine-(methyl-\(^3\)H) (NET027; specific activity 6.7 Ci/mmol; New England Nuclear) was added. Plates were incubated for another 24 h. The contents of the wells were harvested onto glass microfiber filters (Skatron Inc., Sterling, VA). The filters were dried and the radioactivity counted in a liquid scintillation spectrometer (Packard, Model Tri-Carb 460 C, Downers Grove, IL). Mean counts per minute of each triplicate was used for statistical analysis.

**Statistical Analysis**

Data were analyzed as described by Tjoelker et al. (18).

**RESULTS**

**Serum Vitamin A and \(\beta\)-Carotene**

Details on changes in the concentrations of serum vitamin A and \(\beta\)-carotene are reported by
Tjoelker et al. (18). Briefly, concentrations of serum vitamin A and β-carotene were similar in all treatment groups at the start of the experiment. Subsequently, concentrations of serum vitamin A generally decreased for cows in groups AVA and HVA while those in group VA + BC tended to increase. Concentrations of β-carotene remained unchanged throughout the sampling period for cows that did not receive supplemental β-carotene (groups AVA and HVA). However, concentrations for cows in group VA + BC increased (P<.01) about 50% by the day of dry off and 2 wk after dry off.

Lymphocyte Function

Concanavalin A. Both 10−7 M retinol (Figure 1) and 10−7 M retinoic acid (Figure 2) stimulated (P<.01 to .01) Con A-induced lymphocyte transformation before dry off in cows in group VA + BC. However, 10−6 M retinol and 10−5 M retinoic acid inhibited (P<.05) Con A-induced blastogenesis before dry off in group AVA. A similar trend was noted with group HVA. These retinoids had no effect in all treatment groups after dry off.

In contrast, 10−5 M β-carotene inhibited (P<.01) lymphocyte proliferation by about 40% in the presence of Con A before and after dry off in all three treatment groups (Figure 3). The lower concentration of β-carotene had no significant effect.

Lipopolysaccharide. Retinol, retinoic acid, and β-carotene suppressed (P<.007) LPS-induced lymphocyte proliferation before and after dry off at all concentrations tested in all treatment groups (Figures 4 to 6).

![Figure 1. Incorporation of [3H]thymidine (least squares means ± SEM) by Concanavalin A-stimulated lymphocytes incubated in vitro with retinol during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.](image)

![Figure 2. Incorporation of [3H]thymidine (least squares means ± SEM) by Concanavalin A-stimulated lymphocytes incubated in vitro with retinoic acid during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.](image)
**No Mitogen.** As with LPS-induced blastogenesis, lymphocyte proliferation in the absence of mitogen was generally inhibited (P<.09 to .0001) by retinol, retinoic acid, and β-carotene at all concentrations tested before and after dry off (Figures 7 to 9).

**DISCUSSION**

This experiment was designed to evaluate how stage of lactation interacts with the vitamin A and β-carotene status of a cow to influence the responsiveness of her blood lymphocytes to in vitro vitamin A or β-carotene challenge. Retinol and retinoic acid stimulated Con A-induced lymphocyte proliferation before dry off in cows fed VA + BC. This confirms an earlier report by Daniel et al. (5), which showed that the two retinoids stimulated Con A-induced peripheral blood lymphocyte blastogenesis in nonlactating pregnant heifers. Others (1) demonstrated similar stimulatory effects of retinoic acid on human peripheral blood lymphocytes induced by two other T cell mitogens, phytohemagglutinin, and anti-thymocyte globulin. However, they were unable to demonstrate a similar effect on blastogenesis induced by Con A or pokeweed mitogen. Sidell et al. (16) reported retinoic acid stimulated Con A-induced and phytohemagglutinin-induced lymphocyte transformation with thymocytes and tonsil lymphocytes but not with peripheral blood lymphocytes in humans. In contrast, Valone and Payan (19) reported that proliferation of human peripheral blood T lymphocytes was stimulated by retinoic acid in the presence of phytohemagglutinin. Therefore, even though

![Figure 3](image1.png)

![Figure 4](image2.png)

Figure 3. Incorporation of [3H] thymidine (least squares means ± SEM) by Concanavalin A-stimulated lymphocytes incubated in vitro with β-carotene during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.

Figure 4. Incorporation of [3H] thymidine (least squares means ± SEM) by lipopolysaccharide-stimulated lymphocytes incubated in vitro with retinol during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.
the literature disagrees on the source and appropriate mitogenic stimulant of retinoid-sensitive lymphocytes, under certain conditions, the retinoids clearly can stimulate T cell proliferation in vitro.

In contrast to their stimulatory effects for cows in group VA + BC, retinol and retinoic acid were inhibitory on lymphocyte proliferation for cows in groups AVA and HVA before dry off. This difference may reflect differences in concentrations of serum β-carotene. The higher endogenous β-carotene for cows in group VA + BC may exert a carry-over effect to reduce the inhibitory effects of retinol and retinoic acid in vitro. Similar interactions among the vitamins have been shown to influence steroidogenesis by bovine luteal cells (17) and human interferon action (12). Rhodes (12) showed that β-carotene can reverse the inhibitory effects of retinol and retinoic acid on the action of interferon on monocyte membrane function. Talavera and Chew (17) showed similar interactions of β-carotene with the retinoids on progesterone secretion by bovine luteal cells. Alternatively, differences in the effects of the retinoids on the three treatment groups may stem from variations in lymphocyte subpopulations. Alexander et al. (2) reported that humans fed β-carotene for 2 wk had an increase in the total number of T lymphocytes in the blood. This increase was attributed to a 30% increase in circulating helper/inducer T cells. It would be of interest to determine 1) whether a similar response occurs in the bovine, and 2) whether different bovine lymphocyte subpopulations respond differently to in vitro vitamin A challenge.

![Figure 5](image1.png)  
Figure 5. Incorporation of [3H] thymidine (least squares means ± SEM) by lipopolysaccharide-stimulated lymphocytes incubated in vitro with retinoic acid during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.

![Figure 6](image2.png)  
Figure 6. Incorporation of [3H] thymidine (least squares means ± SEM) by lipopolysaccharide-stimulated lymphocytes incubated in vitro with β-carotene during the period around dry off (before = day of last milking; after = 2 wk after dry off). Numbers above bars represent the probability when comparing each vitamin concentration to the control within each sampling period.
In contrast to the variable effects observed before dry off, retinol and retinoic acid did not influence Con A-induced lymphocyte proliferation after dry off. The difference between the periods before and after dry off may be attributed to active milk synthesis before dry off as compared to involution after dry off. Mammary involution is characterized by degenerating secretory tissue and an influx of blood cells, including lymphocytes (9, 10). The majority of the mammary lymphocytes are T cells (4, 8, 15, 20). More specifically, Richie et al. (13) demonstrated a higher suppressor:helper T lymphocyte ratio in human colostrum than in blood, suggesting a selective transfer of suppressor T cells into a mammary gland. If a similar mechanism was operational in the involuting bovine mammary gland, a transient shift in blood lymphocyte subpopulations would likely occur after dry off. This scenario further motivates interest in evaluating responses of individual T cell subsets to vitamin A and β-carotene challenge in vitro.

β-Carotene inhibited Con A-induced lymphocyte proliferation both before and after dry off in all three treatment groups. This is contrary to an earlier report (5), which showed that β-carotene at concentrations of 10⁻⁸ to 10⁻⁶ M stimulated Con A-induced blastogenesis of blood lymphocytes from nonlactating heifers. This discrepancy may be due to differences in the physiological state or vitamin A and β-carotene status of the experimental animals.

Retinol, retinoic acid, and β-carotene were highly inhibitory to lymphocyte proliferation in the presence of LPS and the absence of any
mitogen. Valone and Payan (19) also found retinoic acid to inhibit mitogen-induced B lymphocyte proliferation. In addition, Daniel et al. (5) reported either inhibitory or no effects of retinol, retinoic acid, and \( \beta \)-carotene on LPS-induced blastogenesis. However, Daniel et al. (5) also observed that in the absence of mitogen, \( \beta \)-carotene and retinol stimulated spontaneous lymphocyte proliferation as opposed to an inhibitory effect in the present study.

These data indicate that 1) physiological state (lactating vs. nonlactating) and 2) vitamin A and \( \beta \)-carotene status influence the response of bovine blood lymphocytes to in vitro treatment with vitamin A and \( \beta \)-carotene. This extends our earlier observations (18) that the responsiveness of bovine blood PMN to vitamin A and \( \beta \)-carotene challenge in vitro are also affected by these parameters. Therefore, interpretation of data on the in vitro effects of vitamin A and \( \beta \)-carotene on lymphocyte and PMN function must take into consideration the physiological state and vitamin A and \( \beta \)-carotene status of the experimental animal.

REFERENCES


