Bovine Vitamin A and β-Carotene Intake and Lactational Status. 1. Responsiveness of Peripheral Blood Polymorphonuclear Leukocytes to Vitamin A and β-Carotene Challenge In Vitro

L. W. TJOELKER, B. P. CHEW, T. S. TANAKA, and L. R. DANIEL
Department of Animal Sciences
Washington State University
Pullman 99164-6320

ABSTRACT

Dietary vitamin A and β-carotene were assessed on their interaction with lactational status to influence neutrophil function in vitro. Cows were fed 1) 53,000 IU or 2) 213,000 IU vitamin A, or 3) 53,000 IU vitamin A plus 400 mg β-carotene/cow per d from 6 wk before to 2 wk after dry off. Blood neutrophils were isolated the day of dry off and 2 wk after dry off and incubated with retinol, retinoic acid, or β-carotene. Phagocytosis and kill of Staphylococcus aureus were measured. Across all treatments, kill was higher after dry off than before dry off. Phagocytosis tended to be lower after dry off than before in cows fed vitamin A only. In vitro, $10^{-6}$ M β-carotene stimulated phagocytosis after dry off and kill before dry off in cows fed vitamin A only. In general, retinol and retinoic acid suppressed phagocytosis but did not affect kill. Neutrophils from cows fed high amounts of vitamin A were more susceptible to in vitro suppression than those from cows fed adequate amounts of vitamin A. Therefore, vitamin A and β-carotene supplementation interacts with lactational status to influence the responsiveness of bovine neutrophils to vitamin challenge in vitro.

INTRODUCTION

The importance of vitamin A and β-carotene in maintaining health is well-documented. Scrimshaw et al. (18) concluded that vitamin A deficiency facilitates bacterial, viral, parasitic, and rickettsial infections. Others [(5, 7) for review] have described the protective effects of vitamin A and β-carotene against numerous infectious organisms, including mastitis pathogens.

The exact mechanisms by which vitamin A and β-carotene exert their protective effects have not been defined. However, a number of studies suggest that the protective function of vitamin A may be mediated by enhanced polymorphonuclear neutrophil (PMN) function. Ongsakul et al. (16) reported decreased phagocytic capacity of blood PMN from vitamin A-deficient rats. Others (2, 9, 12) found certain retinoids (retinol, retinoic acid, all-trans-retinal) stimulated superoxide production in vitro by human and guinea pig PMN. In agreement with these studies, our laboratory has recently reported that retinol and retinoic acid stimulate in vitro phagocytosis and intracellular kill of live Staphylococcus aureus by bovine milk PMN (21). The PMN used in the latter study were obtained from cows in mid to late lactation but of unknown vitamin A and β-carotene status. However, subsequent experiments using PMN from cows in all stages of lactation and also of unknown vitamin A and β-carotene status showed variable effects of the vitamins on in vitro PMN function (B. P. Chew, unpublished data). Therefore, some undefined factor(s) associated with the PMN donor may be the source of this variability.

A dairy cow's physiological and nutritional status changes throughout lactation. For example, the stress of high milk production and weight loss in early lactation is followed by

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lower production and increased weight gain in late lactation. Changes in the nutritional needs of the cow parallel these physiological changes. Therefore, we hypothesize that a cow's physiological state and vitamin A and β-carotene status influence the in vitro responsiveness of PMN to vitamin A and β-carotene challenge. The objectives of the present study are to evaluate the effects of 1) different vitamin A and β-carotene status and 2) different physiological states (lactating versus nonlactating) on the in vitro responsiveness of blood PMN to vitamin A and β-carotene challenge.

**MATERIALS AND METHODS**

Thirty Holstein cows were used. The study was conducted at Washington State University Knott Dairy Center from May 1986 through February 1987. Cows were selected on the basis of lactation number and mammary health. Prior to inclusion in the study, cows in their third or earlier lactation were screened for mammary infection. Quarter milk samples were collected aseptically three separate times within a 3-wk period (14). Only cows with one or no quarters infected on at least two out of three samples were used.

Six weeks before drying off, cows were assigned to one of three dietary treatments: 1) 53,000 IU vitamin A palmitate/cow per d (adequate vitamin A; AVA), which represents 100% of NRC (15) recommendation; 2) 213,000 IU vitamin A palmitate/cow per d (high vitamin A; HVA); or 3) 53,000 IU vitamin A palmitate + 400 mg β-carotene/cow per d (vitamin A plus β-carotene; VA + BC). Vitamin A palmitate and β-carotene were supplied by Hoffman-La Roche, Inc. Vitamin A palmitate and β-carotene were supplied by Hoffman-La Roche Inc. (Nutley, NJ). Cows fed VA + BC received an equivalent of 213,000 IU vitamin A based on a conversion ratio of 1 mg β-carotene = 400 IU vitamin A (15). This allows a meaningful assessment of vitamin A and β-carotene effects to be made by comparing treatments HVA and VA + BC. All cows were fed a 75:25 mixture of an alfalfa-grass silage and low quality grass hay (containing no detectable β-carotene). Concentrates fed during lactation were formulated to be free of vitamin A but were otherwise balanced for all other essential nutrients. Dietary supplements were top-dressed daily at 0700 h from 6 wk before through 2 wk after dry off.

Cows were blocked in groups of three, and the cows within each block were randomly assigned to treatments. Blood was collected on wk -6, 0 (day of dry off), and 2 for analysis of concentrations of serum vitamin A and β-carotene (6). At wk 0 (before dry off) and 2 (after dry off), blood for isolating PMN was collected aseptically from the coccygeal vein into tubes containing 10% sodium citrate (wt/vol; 5 ml/10 ml blood).

**Isolation of Polymorphonuclear Neutrophils**

Polymorphonuclear neutrophils were isolated by gradient centrifugation using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). Contaminating erythrocytes were lysed for 30 s using distilled water. The cells were then resuspended in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered RPMI 1640 (Rosewell Park Memorial Institute) supplemented with .5% (wt/vol) bovine serum albumin (RPMI + BSA; Sigma). Total cells were enumerated using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, FL) and percent PMN determined using a Wright-stained smear. Cell viability was determined by Trypan blue dye exclusion. Percent PMN generally exceeded 85%; cell viability exceeded 90%. Final cell concentration was adjusted to 6.25 × 10⁶ viable PMN/ml.

**Preparation of Vitamins**

Retinol (Ro 1-4955, lot 11375-30-1) and retinoic acid (Ro 1-5488/000, lot 102098) were dissolved in absolute ethanol while β-carotene (Ro 1-8300/000, lot 264055) (all from Hoffman-La Roche, Nutley, NJ) was dissolved in chloroform. Appropriate volumes were then resuspended in RPMI + BSA and the mixture thoroughly vortexed. Vitamins resuspend well in medium containing at least .5% BSA. The vitamin treatments were as follows (final concentrations): 1) controls containing RPMI + BSA alone or with .1% ethanol (control for retinol and retinoic acid) or .05% chloroform (control for β-carotene); 2) retinol at 0, 10⁻⁶, and 10⁻⁷ M; 3) retinoic acid at 0, 10⁻⁶, and 10⁻⁸ M, and 4) β-carotene at 0, 10⁻⁵, and 10⁻⁶ M. The concentrations of the vitamin A and β-carotene are within physiological concentrations found in bovine blood (10).
Phagocytosis and Intracellular Kill Assays

The assays of phagocytosis and intracellular kill involve coincubation of PMN with live *Staphylococcus aureus* (ATCC 29740). Bacteria were prepared by transferring a pure culture into 5 ml of brain-heart infusion broth (Difco Labs, Detroit, MI) and incubated for 18 h at 37°C. An aliquot (150 µl) was transferred into 10 ml of fresh broth and incubated an additional 4 h. The resultant culture was centrifuged (10 min, 13,000 × g, 4°C), washed twice in phosphate-buffered saline (PBS), and then resuspended in PBS to a concentration of 3 × 10⁶/ml by determining the optical density. Subsequently, the bacteria were diluted to 5 × 10⁷/ml in RPMI. Immediately prior to the assay, the bacteria were preopsonized by adding 5% of sterile, pooled cow serum and incubating the mixture for 20 min at 37°C in a Dubnoff metabolic shaking incubator (Precision Scientific Group, Chicago, IL). An aliquot was diluted and plated on blood agar to determine the concentration of bacteria used in the assay.

The assay was conducted in 96-well round bottom microtiter plates using the methods of Rajkovic and Williams (17) with slight modifications. The incubation mixture contained one of the following (n = 6 each): 1) 100 µl of PMN alone, 2) 100 µl of bacteria alone, or 3) 50 µl of PMN plus 50 µl of bacteria. The final concentrations of PMN and the bacteria were 2.5 × 10⁶ and 2.5 × 10⁷/ml. Plates were incubated in an oscillating water bath (Lab-Line Instruments, Inc., Melrose Park, IL) for 30 min at 37°C. Following incubation, three out of each set of six wells received 50 µl of Triton X-100 (.8% in sterile distilled water; Sigma) plus 50 µl deoxyribonuclease (1 mg/ml in RPMI; Type IV from bovine pancreas; Sigma). The former treatment results in PMN lysis and subsequent release of viable intracellular bacteria and thus provides a basis for the estimation of intracellular kill. The remaining three wells received 100 µl of a 1:1 mixture of RPMI and sterile distilled water. Here, the PMN remained intact for estimation of phagocytosis. The contents of the wells were harvested on glass microfiber filter paper (934-AH; Whatman, Clifton, NJ) using a 12-channel cell harvester (Model M12V, Brandel, Rockville, MD). The filters were dried and the radioactivity counted in a liquid scintillation counter.

Because the labelled uridine is incorporated into viable bacteria, the radioactivity reflects the number of viable bacteria following incubation in the presence or absence of PMN. Because incorporation of the radiolabel by PMN alone was always negligible, this uptake was not considered in the final calculation. Therefore, phagocytic (PI) and intracellular kill (KI) indices were calculated as follows:

\[
KI \text{ or } PI = \left[1 - \frac{(cpm \text{ of PMN + bacteria})}{(cpm \text{ of bacteria alone})}\right] \times \frac{PMN \cdot \text{bacteria}}{PMN + \text{bacteria}}
\]

The triplicate wells treated with Triton X-100 plus deoxyribonuclease were used for calculating KI while the corresponding untreated wells were used for calculating PI. The ratio of PMN: bacteria ranged from 10 to 20 throughout the study. Therefore, this factor was included in the calculation as a way to normalize the assays (17).

Statistical Analysis

Data were analyzed by the General Linear Models procedure of SAS 79 (3). Least squares analysis using a randomized complete block, split-split-plot design was used to test treatment, period of sampling, and in vitro vitamin challenge. Treatment differences with in vitro vitamin were compared using nonorthogonal contrast. Each vitamin treatment was compared to its appropriate control containing the same solvent. The statistical model used was:

\[
Y_{ijkl} = \mu + T_i + B_j + (T_i \cdot B_j) + P_k + (T_i \cdot P_k) + [P_k \cdot B_j(T_i)] + V_l + (T_i \cdot V_l) + [V_l \cdot B_j(T_i)] + (P_k \cdot V_l) + (T_i \cdot P_k \cdot V_l) + e_{ijkl}
\]

where:

\[
Y_{ijkl} = PI \text{ or } KI;
\]

\[
\mu = \text{overall mean};
\]

\[
T_i = \text{effect of the } i^{th} \text{ treatment, } i = 1 \text{ to } 3 \text{ where } 1 = \text{ group 'AVA}, 2 = \text{ group 'HVA}, \text{ and } 3 = \text{ group 'VA + BC};
\]
RESULTS

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

Prior to vitamin supplementation, concentrations of vitamin A were similar in all treatment groups (Figure 1). Subsequently, concentrations generally decreased for cows in groups AVA and HVA and tended to increase for cows in group VA + BC.

Concentrations of serum \( \beta \)-carotene were similar in all treatment groups at wk -6 (Figure 2). Concentrations remained unchanged throughout the sampling period for cows that did not receive supplemental \( \beta \)-carotene (groups AVA and HVA), but concentrations for cows in group VA + BC increased (\( P < .01 \)) about 50% by wk 0 and 2.

Phagocytosis

In groups AVA and HVA, the PI of PMN incubated in the absence of vitamin in vitro tended to decrease after dry off. However, \( 10^{-6} \) M \( \beta \)-carotene stimulated (\( P < .07 \)) PI after dry off in groups AVA and HVA (Figure 3). A similar trend was detected with cows in VA + BC.

These stimulatory effects were not observed with \( 10^{-5} \) M \( \beta \)-carotene for all treatment groups. Before dry off, in vitro \( \beta \)-carotene did not influence PI in any of the treatment groups.

In contrast, retinol and retinoic acid lacked the stimulatory activity of \( \beta \)-carotene. Retinol did not affect PI of cows in group AVA before or after dry off (Figure 4). In contrast, both

![Figure 1. Changes in serum vitamin A (least squares means ± SEM) for cows fed (per cow/d) adequate vitamin A (53,000 IU), high vitamin A (213,000 IU), or vitamin A (53,000 IU) plus \( \beta \)-carotene (400 mg).](image1)

![Figure 2. Changes in serum \( \beta \)-carotene (least squares means ± SEM) for cows fed (per cow/d) adequate vitamin A (53,000 IU), high vitamin A (213,000 IU), or vitamin A (53,000 IU) plus \( \beta \)-carotene (400 mg). Numbers above bars represent probability when compared to wk -6.](image2)
concentrations of retinol suppressed ($P<.1$ to $.002$) PI in group HVA at both time periods. In general, as with group AVA, retinol did not influence PI in group VA + BC (both groups received an equivalent amount of preformed vitamin A) except after dry off where $10^{-6}$ M retinol tended ($P<.09$) to be suppressive.

As with retinol, retinoic acid was the least suppressive on PI in cows from group AVA (Figure 5). In this group, only PMN treated with $10^{-7}$ M retinoic acid before dry off exhibited decreased PI ($P<.06$). In contrast, PMN from groups HVA and VA + BC demonstrated suppressed ($P<.01$) PI when challenged with $10^{-7}$ M retinoic acid both before and after dry off. In addition, $10^{-8}$ M retinoic acid was suppressive to PI after dry off for cows in group VA + BC.

**Intracellular Kill**

In all treatment groups, KI of PMN cultured in the absence of vitamin was greater ($P<.07$) after dry off than before (Figures 6 to 8). As with PI, in vitro β-carotene tended to reverse this trend (Figure 6). At $10^{-6}$ and $10^{-5}$ M, β-carotene stimulated ($P<.03$) KI in group AVA before dry off. Similarly, $10^{-5}$ M β-carotene increased ($P<.03$) and $10^{-6}$ M tended to increase KI in group HVA. β-Carotene also tended to promote KI with cows in group VA + BC before dry off. However, β-carotene had no effect on KI after dry off except with cows in group HVA where the provitamin tended ($P = .1$) to suppress KI.

In contrast to β-carotene, in vitro retinoid supplementation had no effect on KI (Figures 7 and 8).
DISCUSSION

This experiment was designed to evaluate how the physiological state may interact with the vitamin A and ß-carotene status of a cow to influence the responsiveness of her blood PMN to in vitro vitamin A or ß-carotene challenge. In vitro, ß-carotene stimulated both PI and KI, especially in cows not supplemented with ß-carotene (groups AVA and HVA). Even though concentrations of ß-carotene were high both before and after dry off, the stimulatory influence on PI occurred only after dry off, whereas KI was stimulated only before dry off. These two periods correlate well with the observed trends in PI and KI around dry off, i.e., PI tends to be lower after dry off than before while KI is lower before than after dry off. Thus, in vitro ß-carotene appears to stimulate PI and KI at only those times when these PMN functions are suboptimum. These observations suggest that the protective effects of ß-carotene against disease may be mediated in part by the provitamin's modulation of PMN function. This is particularly relevant in the cow as well as the human since both species directly absorb ß-carotene from the intestine (4). Furthermore, these data suggest a direct role for ß-carotene in promoting PMN function that is independent of its prior conversion to vitamin A.

Unlike ß-carotene, retinol and retinoic acid had either no effect or an inhibitory effect on
PI in vitro, depending on treatment group and week relative to dry off. The PMN from cows in group HVA were more susceptible to inhibition of phagocytosis than cows in group AVA. This suggests that even though concentrations of serum vitamin A did not differ significantly between these two groups, the PMN were somehow sensitive to the higher dietary vitamin A intake. Concentrations of serum vitamin A is homeostatically controlled by the liver. In humans and rats that ingest excessive vitamin A, liver uptake and storage of retinol esters from the diet is reduced, resulting in elevated concentrations of serum retinyl esters (13, 19). A simultaneous reduction in serum retinol-binding protein was observed in hypervitaminotic rats (13). Retinol-binding protein is secreted by the liver and carries retinol to peripheral tissues. This protein is thought to prevent toxic effects of vitamin A on biological membranes (8). Based on these observations, a condition similar to hypervitaminosis may have developed for cows in group HVA, resulting in higher retinyl ester:retinol-binding protein ratios than cows in group AVA. Thus, PMN isolated from these cows would have been exposed to the hypervitaminosis-like condition prior to collection; in vitro supplementation may simply exacerbate the toxic effects, resulting in suppressed PI.

Alternatively, vitamin A treatment in vitro may indirectly inhibit phagocytosis. Several studies have shown that certain retinoids (retinoic acid, retinol, all-trans-retinal) stimulate superoxide production by PMN in vitro (2, 9, 12). Others have reported that superoxide and its metabolites inhibit PMN function (1,
Therefore, the suppression of PI observed in the presence of retinol and retinoic acid may be mediated by elevated superoxide rather than a direct effect of the vitamins. However, superoxide is an important antimicrobial product of activated PMN [(11) for review]. Therefore, if retinol or retinoic acid stimulate superoxide production by PMN in our assay system, a consequential rise in KI would be expected. This was not seen.

Supplementation of PMN with β-carotene in vitro resulted in elevated PI after dry off and KI before dry off to a greater degree with cows in AVA and HVA than in VA + BC. In contrast, retinol and retinoic acid had either null or negative effects on PMN function depending upon treatment group and time relative to dry off. These data indicate that 1) physiological state (lactating vs. nonlactating) and 2) vitamin A and β-carotene status influence the response of bovine blood PMN to in vitro treatment with vitamin A and β-carotene. This may explain the apparent disagreement between the current study and an earlier report (21), which reported apparent disagreement between the current study and an earlier report (21), which reported

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