Modulatory Effects of Dietary β-Carotene on Blood and Mammary Leukocyte Function in Periparturient Dairy Cows

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ABSTRACT

Beginning 4 wk prior to predicted calving, 14 Holstein cows per treatment were fed diets 1) unsupplemented (control) or supplemented daily with 2) 300 mg of β-carotene, 3) 600 mg of β-carotene, or 4) 120,000 IU of vitamin A. Blood was collected around calving on wk -4, -2, -1, 0 (within 24 h postcalving), 1, 2, and 4 for isolation of lymphocytes and neutrophils and for the analysis of plasma vitamins. Lacteal secretions were collected on wk 0, 1, 2, and 4 for the isolation of phagocytes. Cows supplemented with 600 mg of β-carotene had higher concentrations of plasma β-carotene and retinol than did unsupplemented cows. Supplemental vitamin A increased plasma retinol on wk 4 and decreased plasma β-carotene on wk -1 and 0. Treatment did not affect concentrations of plasma α-tocopherol. Blood lymphocyte proliferation in response to concanavalin A, phytohemagglutinin, and pokeweed mitogen during the peripartum period was higher in cows supplemented with β-carotene than in unsupplemented controls. Phagocytic activity of blood neutrophils was enhanced on wk 1 in cows fed 300 mg of β-carotene. Intracellular killing by blood neutrophils was enhanced in cows supplemented with β-carotene (wk 0) and vitamin A (wk 0 and 1). Iodine uptake and nitroblue tetrazolium reduction by blood neutrophils was stimulated in cows supplemented with β-carotene. Phagocytic activity, iodine uptake, and nitroblue tetrazolium reduction by mammary phagocytes from all cows generally were lower postpartum than on the day of calving. The incidence of retained placenta and metritis was higher for unsupplemented cows than for cows supplemented with β-carotene. Therefore, dietary β-carotene can elevate peripartum concentrations of blood β-carotene, enhance host defense mechanisms by potentiating lymphocyte and phagocyte function, and decrease the incidence of certain reproductive disorders.

(Key words: β-carotene, lymphocytes, phagocytes, health)

Abbreviation key: Con A = concanavalin A, EBSS = Earle's buffered salt solution, NBT = nitroblue tetrazolium, PHA = phytohemagglutinin, PMN = polymorphonuclear neutrophils, PWM = pokeweed mitogen, Th = helper T cell.
its immunoregulatory role. Indeed, β-carotene increased blood and milk phagocyte kill ability (9) as well as peripheral blood lymphocyte proliferation (10) in peripartum dairy cows in vitro. Similar in vivo studies in dairy cattle are not available. However, dietary supplementation of β-carotene increased the frequency of helper T (T_H) cells in human blood (1) and increased T-cell proliferation in rats (3). Prabhala et al. (20) reported similar increases in peripheral blood lymphocytes bearing certain surface activation markers, such as the IL-2 receptor, in human volunteers on diets supplemented with β-carotene or canthaxanthin. These results indicate that both in vitro and in vivo treatments with β-carotene enhanced host defense and may explain the protective effect of β-carotene during the peripartum period.

Under practical feeding conditions, dairy cows are generally fed lower quality forages during the dry period. This, together with lower feed intake and dramatic hormonal changes during the immediate prepartum period, could explain the rapid decrease in concentrations of β-carotene in blood of peripartum cows. Because new intramammary infections occur with high frequency during the peripartum period, most probably the result of impaired host defense mechanisms (19, 28), the objective of our study was to determine the effects of dietary β-carotene supplementation to dairy cows during the peripartum period on 1) peripheral blood lymphocyte proliferation and 2) blood and mammary phagocyte function.

MATERIALS AND METHODS

Beginning 4 wk prior to predicted calving, multiparous Holstein cows were fed an 80:20 mixture (DM basis) of old grass hay (devoid of β-carotene) and grass silage. In addition, each cow received 1.8 kg/d of a pelleted concentrate (Table 1) containing no preformed vitamin A. At 0700 h each day, 14 cows per treatment received one of four treatments top-dressed: 1) unsupplemented control, 2) 300 mg of β-carotene, 3) 600 mg of β-carotene (10% beadlet; Hoffmann La-Roche; Nutley, NJ), or 4) 120,000 IU of vitamin A as vitamin A palmitate. After parturition, all cows were fed a 54:46 mixture of forage:concentrate as a TMR. The forage component of the diet consisted of a 68:32 mixture of old grass hay and grass silage. Each group of 4 cows with the closest predicted calving dates were assigned randomly to each treatment to minimize seasonal variation.

Blood was collected from the jugular vein on wk −4 (immediately prior to supplementation), −2, −1, 0 (within 36 h postcalving), 1, 2, and 4 for isolation of plasma, lymphocytes, and polymorphonuclear neutrophils (PMN). Acid-citrate-dextrose was used as the anticoagulant. Milk was collected during the postpartum period (wk 0 through 4) for isolating mammary phagocytes. Sterile quarter mammary secretions were collected during the same periods, and bacterial isolates were identified at the veterinary disease diagnostic laboratory. Only cows with one or no infected quarters were used, and only uninfected quarters were used as a source of mammary phagocytes. Daily milk production and incidence of retained placenta, metritis, milk fever, and the number of AI per conception were recorded.

**Analysis of Plasma and Vitamins in Feed**

β-Carotene, retinol, retinyl acetate, and α-tocopherol in plasma and feed were analyzed by HPLC (Waters, Millipore Corp., Milford, MA) as described (7). All procedures were conducted in yellow light. Duplicate 200-μL
volumes of plasma were pipetted into extraction tubes, deproteinized with ethanol, and extracted with petroleum ether (J. T. Baker Inc., Phillipsburg, NJ). Exactly 800 µl of the petroleum ether layer were removed, filtered through a .22-µm membrane filter (Millipore, Bedford, MA), and dried under a stream of N2 in a 40°C water bath. The residue was reconstituted in 500 µl of mobile phase, and 50 µl were injected.

Feed samples were finely ground; 12.5 g were digested with 25 mg of pronase and 25 ml of water. The sample was extracted with 10 ml of ethanol and 25 ml of cyclohexane. An aliquot of the organic phase was removed, filtered, dried, and redissolved in mobile phase as described. The concentration of β-carotene in the feed averaged .2 mg of β-carotene/kg of feed DM.

Cell Isolation

Blood lymphocytes and PMN were obtained by density gradient centrifugation (29, 30). The purity of the lymphocyte preparation generally exceeded 95%, and cell viability was at least 95%. The PMN preparation was 85 to 90% pure, and cell viability exceeded 90%. Lymphocyte number was adjusted to 2.0 x 106 cells/ml in RPMI. The PMN concentration was adjusted to 2.5 x 106 PMN/ml in Hanks balanced salt solution (Sigma Chemical Co., St. Louis, MO) for use in phagocytosis and intracellular kill assays and to 2.5 x 107 PMN/ml in Earle's buffered salt solution (EBSS; Sigma Chemical Co.) for use in iodination and nitroblue tetrazolium (NBT) reduction assays (described later).

Mammary PMN were obtained after induction of leukocytosis by an intramammary infusion of 20 ml of sterile 1% oyster glycogen (wt/vol; Sigma Chemical Co.) in water (9). A different quarter was used at each sampling period. Mammary PMN were enumerated using a hemocytometer. Differential cell count and viability were determined as described for PMN. The final PMN concentrations then were adjusted as described for blood PMN. In a large number of cases, difficulties were encountered in obtaining sufficient numbers of mammary PMN for function assays. Therefore, no statistical comparison of treatment difference was possible. Data on mammary PMN function were analyzed collectively and reported to reflect changes in mammary PMN function during the postpartum period in all cows.

Lymphocyte Transformation Assay

Lymphocyte proliferation (30) was induced by the addition of concanavalin A (Con A; 5 and 10 µg/ml), phytohemagglutinin (PHA; 15 and 30 µg/ml), or pokeweed mitogen (PWM; 5 and 10 µg/ml) to 2 x 105 lymphocytes in 96-well round-bottom culture plates (Corning Glass Works, Corning, NY). Proliferation data are presented as stimulation indices (total uptake by mitogen-stimulated lymphocyte cultures divided by uptake by unstimulated lymphocyte cultures).

Phagocytosis and Intracellular Kill Assays

Staphylococcus aureus (Newbould 305; ATCC 29740) was prepared according to Daniel et al. (9). The bacteria then were opsonized with blood Ig obtained from calves immunized against the same strain of S. aureus. Just prior to the assay, the bacterial concentration was adjusted to 1.25 x 107 cfu/ml in EBSS. Actual bacteria count was reaffirmed by plating an aliquot on blood agar. The assays were conducted in triplicate in 96-well round-bottom culture plates (Corning Glass Works) according to Tjoelker et al. (29). Phagocytic and kill activity were expressed as phagocytic index or intracellular kill index (29).

Iodination Assay

This assay measures the activity of the myeloperoxidase-hydrogen peroxide-halide antimicrobial system of PMN. The method used was as previously described (22). All assays were conducted in triplicate.

Zymosan (Sigma Chemical Co.) was suspended in EBSS to a concentration of 10 mg/ml and homogenized for 1 min. Opsonized zymosan was prepared by the addition of fresh bovine serum to an equal volume of zymosan suspension and stirred for 1 h. The mixture was centrifuged at 250 x g for 10 min, and the supernatant was discarded. To reverse strong conglutination of the zymosan, the pellet was washed twice with 200 ml of .01 M Na-EDTA.
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(Sigma Chemical Co.) and stirred for 15 min between washes. The final pellet was resuspended in 100 ml of EBSS and frozen in small aliquots at -20°C. Opsonized zymosan was thawed and homogenized for 1 min just prior to use.

The reaction mixture contained .1 ml of 40 nmol NaI (Sigma Chemical Co.), .1 ml of 125I (.05 µCi), .05 ml of opsonized zymosan, and .3 ml of EBSS; the mixture was allowed to react in 12- x 75-mm polystyrene snap-cap tubes (Becton Dickinson and Co., Lincoln Park, NJ). The mixture was equilibrated in a 37°C water bath for 15 min after which time .1 ml of the blood PMN or milk PMN suspension (containing 2.5 x 10^7 cells/ml) was added to start the reaction. Opsonized zymosan was omitted in a similar set of tubes to determine iodine uptake by resting PMN. The mixture was incubated with tumbling end over end (20 times/min) for 20 min at 37°C, and the reaction was terminated by the addition of 2 ml of cold 10% TCA (J. T. Baker Inc., Phillipsburg, NJ). The tubes then were centrifuged for 5 min at 1000 x g at 4°C and washed twice with 2 ml of cold TCA. The supernatant was discarded, and radioactivity in the pellet was determined on a gamma counter (20120 Series; Iso-Data, Inc., Rolling Meadows, IL). The amount of NaI converted to a TCA precipitate was calculated as described (22), and values were expressed in nanomoles of NaI/10^7 PMN per h.

NBT Reduction Assay

This assay (22) measures the production of superoxides by PMN. The assay was conducted in triplicate. The NBT chloride (Sigma Chemical Co.) was suspended in EBSS to a concentration of 2 mg/ml and stirred in the dark for 1 h at room temperature (23°C). Insoluble NBT was removed by filtration through a .45-µm filter. The mixture was stored in darkness at 4°C until used.

The reaction mixture consisted of .1 ml of the NBT solution, .1 ml of opsonized zymosan, and .2 ml of EBSS. The mixture was allowed to react in siliconized 12- x 75-mm glass tubes. Reduction of NBT by resting cells was determined in a similar set of tubes in which opsonized zymosan was omitted. The mixture was equilibrated for 15 min in a 37°C oscillating water bath, and the reaction was initiated by the addition of .1 ml of the blood PMN or milk PMN suspension (2.5 x 10^7 cells/ml). After exactly 5 min, the reaction was stopped by the addition of 2.5 ml of cold 1 mM N-ethylmaleimide (Sigma Chemical Co.) dissolved in 9% saline. The mixture then was centrifuged for 10 min at 500 x g, and the pellet was resuspended in 2.5 ml of pyridine (J. T. Baker Inc.). The precipitate was sonicated for 5 s and then heated for 10 min in a boiling water bath; the mixture was clarified on a spectrophotometer using a pyridine blank. The results were reported as optical density/2.5 x 10^6 cells per 5 min in 2.5 ml of pyridine.

Statistical Analyses

Data were analyzed by the general linear models procedure of SAS (23) using a completely randomized split-plot ANOVA. The final statistical model used was

\[ Y_{ijk} = \mu + T_i + C_j(T_i) + W_k + (T_i \times W_k) + e_{ijk} \]

where

\[ Y_{ijk} = \text{concentration of plasma vitamins, lymphocyte proliferation, phagocytic index, kill index, iodine uptake, NBT reduction, feed intake, milk production, and composition;} \]

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

\[ T_i = \text{effect of the treatment i;} \]

\[ C_j(T_i) = \text{effect of the cow j within the treatment i (error A) used to test the effects of T_i;} \]

\[ W_k = \text{effect of the week k relative to calving;} \]

\[ T_i \times W_k = \text{interaction of the treatment i by week k;} \]

\[ e_{ijk} = \text{residual error.} \]

Each treatment within a sampling period was compared with the unsupplemented control by nonorthogonal contrasts. Treatment differences in the incidence of retained placenta, metritis, milk fever, and the number of AI per conception were compared using chi-square (26). Unless otherwise stated, treatment
RESULTS

Changes in Plasma Vitamins

β-Carotene. Mean plasma β-carotene in all cows was $4.3 \pm 0.1 \mu mol/L$ prior to supplementation, and concentrations were similar among treatments (Figure 1). Compared with concentrations at wk -4, concentrations of plasma β-carotene in unsupplemented cows decreased 45% by wk 1 and increased postpartum ($P < 0.001$). Cows supplemented with 600 mg of β-carotene had higher concentrations of plasma β-carotene than did unsupplemented cows from wk -2 through 4. In contrast, 300 mg of β-carotene failed to elevate plasma β-carotene above that of unsupplemented cows. Cows supplemented with 120,000 IU of vitamin A had lower plasma β-carotene on wk -1 and 0.

Retinol. Mean plasma retinol was $1.6 \pm 0.03 \mu mol/L$ prior to supplementation (Figure 2). Like plasma β-carotene, concentrations of plasma retinol decreased ($P < 0.01$) prepartum in all cows and generally were lowest at calving. Retinol increased ($P < 0.001$) postpartum and reached the highest concentrations on wk 4. Cows fed 600 mg of β-carotene had higher concentrations of plasma retinol than did unsupplemented cows from wk -1 through 4 ($P < 0.1$ to $< 0.05$). All cows supplemented with β-carotene or vitamin A had higher ($P < 0.1$) plasma retinol concentrations than controls on wk 4.

α-Tocopherol. Mean concentrations of plasma α-tocopherol were $7.0 \pm 0.5 \mu mol/L$ prior to supplementation (Figure 3). The large variation in concentrations of plasma α-tocopherol among treatments at the wk -4 is difficult to explain. As with β-carotene and retinol, plasma α-tocopherol in all cows decreased ($P < 0.1$ to $< 0.01$) during the prepartum period and generally reached the lowest concentrations on wk 0. Supplementation of diets with β-carotene or vitamin A did not significantly influence concentrations of plasma α-tocopherol during the peripartum period.

Blood Lymphocyte Proliferation

No treatment difference was significant for lymphocyte proliferation in unstimulated cultures. Mean spontaneous lymphocyte proliferation was $1 \times 10^3$ cpm throughout the peripartum period. Blood lymphocyte proliferation in response to all three mitogens remained relatively constant throughout the peripartum period in unsupplemented cows (Figures 4 to 6). In the presence of 5 and 10 μg/ml of Con A, lymphocytes from cows supplemented with
600 mg of β-carotene had significantly higher proliferative responses at wk -1 (P < .05 to .01) and 2 (P < .1 to .01) compared with those of unsupplemented cows (Figure 4). Lymphocyte proliferation increased approximately 65% between wk -2 and -1 cows on diets supplemented with β-carotene. A transient decrease in lymphocyte proliferation induced by Con A occurred at wk 0 and 1 for cows receiving the β-carotene supplement. In the presence of 10 μg of Con A/ml, lymphocyte proliferation in cows fed 300 mg of β-carotene generally was higher than in control cows and was significantly higher at wk 1. No treatment difference was significant with lymphocyte proliferation with cows supplemented with vitamin A (Figure 4).

Treatment effects on PHA-stimulated lymphocyte proliferation (Figure 5) generally were similar to those observed with Con A-induced stimulation. The proliferative response of blood lymphocytes in the presence of 15 and 30 μg of PHA/ml was higher at wk -1 (P < .05 to .01) and wk 2 (P < .1) in cows fed 600 mg of β-carotene compared with that in unsupplemented controls. A transient decrease in PHA-induced lymphocyte proliferation was observed immediately postpartum with the cows supplemented with 600 mg of β-carotene. Lymphocyte proliferative response in cows fed 300 mg of β-carotene also tended to be higher than that of control cultures between wk -1 and 4 and was significantly higher at wk 1 with 30 μg of PHA/ml. Cows supplemented with 120,000 IU of vitamin A also had higher lymphocyte proliferation in response to both concentrations of PHA at wk 1 (P < .1 to .05) (Figure 5).

Lymphocyte proliferation in response to PWM (Figure 6) generally was similar to those responses observed with Con A and PHA. Lymphocytes isolated from cows fed 300 or 600 mg of β-carotene showed a higher (P < .1 to .05) proliferative response when cells were stimulated with 5 and 10 μg of PWM/ml between wk -1 and 4 compared with that of unsupplemented cows. The proliferative response was significantly higher at wk -1 and 2 (P < .1) for cows fed 600 mg of β-carotene and at wk 1 for cows fed 300 mg of β-carotene. A transient decrease in PHA-induced lymphocyte proliferation was observed at wk 1 with cows fed 600 mg of β-carotene, but not with cows fed 300 mg of β-carotene. In contrast to that of
cows supplemented with β-carotene, lymphocyte proliferation in cows fed 120,000 IU of vitamin A was not significantly elevated over that of un-supplemented cows.

**Phagocytosis and Intracellular Kill**

The ability of blood PMN to phagocytize live *S. aureus* remained relatively constant during the periparturient period and generally was not affected by treatment (Figure 7). However, blood PMN from cows fed 300 mg of β-carotene exhibited enhanced phagocytosis on wk 1 compared with that of blood PMN from un-supplemented cows.

Intracellular killing of *S. aureus* by blood PMN isolated from un-supplemented cows was lowest on wk 0 and increased postpartum (Figure 7). In contrast, blood PMN from cows fed 600 mg of β-carotene showed increased \( (P < .1) \) killing ability on wk 0. Similarly, intracellular killing by PMN from cows fed 120,000 IU of vitamin A was higher on wk 0 and 1 \( (P < .1) \) than that of un-supplemented cows. The killing ability for PMN from cows fed 300 mg of β-carotene was similar to that of controls.

The ability of mammary PMN to phagocytize live *S. aureus* decreased by 33\% \( (P < .001) \) from wk 0 to 4 (Figure 8). Intracellular killing ability by mammary PMN increased approximately 45\% during the same period, but period difference was not significant because of large variations.

**Iodine Uptake**

Iodine uptake by resting blood PMN (Figure 9) remained constant throughout the study in un-supplemented cows and in cows fed 300 mg of β-carotene. However, iodine uptake by blood PMN from cows supplemented with 600 mg of β-carotene and vitamin A was significantly increased on wk 0 and 1 \( (P < .1) \) compared with that of un-supplemented cows. The killing ability for PMN from cows fed 300 mg of β-carotene was similar to that of controls.
mg of β-carotene or 120,000 IU of vitamin A generally increased \( (P < .1) \) with time. Uptake was higher on wk 0 and 4 \( (P < .1 \) and \( .05) \) for cows fed 600 mg of β-carotene and on wk 4 for cows fed 120,000 IU of vitamin A.

Iodine uptake by activated blood PMN from all cows tended to decrease prepartum (Figure 9). Cows fed 300 mg of β-carotene showed higher \( (P < .01) \) iodine uptake on wk 0 but lower uptake on wk 2. Iodine uptake by resting mammary PMN (Figure 10) was 63% lower \( (P < .01) \) on wk 2 and 56% lower on wk 4 compared with iodine uptake by resting mammary PMN on wk 0. In contrast, iodine uptake by activated mammary PMN (Figure 10) did not change from wk 0 to 4.

**NBT**

The NBT reduction by both resting and activated blood PMN was relatively constant throughout the periparturient period (Figure 11). A decrease \( (P < .1) \) in NBT reduction by resting blood PMN was observed on wk 2 for cows fed 600 mg of β-carotene. No other treatment differences were significant.

Reduction of NBT by resting mammary PMN (Figure 12) was 46% lower on wk 4 than on wk 0. Reduction of NBT by activated mammary PMN (Figure 12) was not different during the postpartum period.

**Feed Intake and Milk Production**

Mean total DMI (Figure 13) for all cows was 15 kg/d per cow at the beginning of the trial. Intake of all cows increased \( (P < .01 \) to \( .0001) \) postpartum and averaged 32 kg/d per cow on wk 4. No treatment difference in feed intake was observed.

Mean daily milk production throughout the experimental period was 34 ± 1 kg/d (Table 2). Interaction of treatment by week relative to

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**Figure 7.** Least squares means of phagocytic (overall SE = .012) and intracellular kill (overall SE = .028) indices of *Staphylococcus aureus* by blood polymorphonuclear leukocytes in cows supplemented with β-carotene and vitamin A from 4 wk prepartum through 4 wk postpartum. Significant difference from control between sampling period \( (P < .1); *P < .05) \).

**Figure 8.** Changes in phagocytosis and intracellular kill of *Staphylococcus aureus* by mammary phagocytes from cows during the postpartum period. Significant difference from wk 0 \( (**P < .001) \). Vertical bars indicate overall SEM.

calving was significant \( (P < .001) \). Daily milk production of unsupplemented cows increased \( (P < .001) \) 35% from 25.7 kg/d on wk 1 to 39.5 kg/d on wk 4. During the same period, the magnitude of increase in milk production was greater for cows fed 120,000 IU of vitamin A (56%; \( P < .001 \)), 300 mg of \( \beta \)-carotene (48%; \( P < .001 \)), or 600 mg of \( \beta \)-carotene (50%; \( P < .001 \)).

**Disorders**

Retained placenta occurred most frequently in unsupplemented cows (41%) and was lowest \( (P < .01) \) in cows supplemented with 600 mg of \( \beta \)-carotene (25%) (Table 3). Incidence of retained placenta was lower also in cows supplemented with either 300 mg of \( \beta \)-carotene (31%; \( P < .1 \)) or 120,000 IU of vitamin A (33%; \( P < .1 \)) compared with that of unsupplemented cows. The incidence of metritis was significantly lower in cows supplemented with either 300 or 600 mg of \( \beta \)-carotene and was positively correlated with the incidence of retained placenta. The incidence of milk fever was lower \( (P < .001) \) in cows supplemented with 120,000 IU of vitamin A. Mean number of AI services per conception was 2.5 for all cows and was not influenced by treatment.

No cases of clinical mastitis were observed in the study. Microbiological studies showed bacterial isolates in 53% of all quarters in cows on the study. Of these, none was identified as *S. aureus*, 3.7% were *Streptococcus agalactia*, 6.3% were *Escherichia coli*, 38% were *Staphylococcus* spp. other than *S. aureus*, and 6.3% were *Streptococcus* spp. other than *Strep. agalactiae*. Incidence of different isolates in treated animals were not significantly different from that of controls.

**DISCUSSION**

Lymphocyte proliferation was enhanced in peripartum dairy cows fed \( \beta \)-carotene (espe-
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Figure 11. Nitroblue tetrazolium (NBT) reduction (least squares means) by resting (overall SE = .001) and activated (overall SE = .006) blood polymorphonuclear leukocytes in cows supplemented with β-carotene and vitamin A from 4 wk prepartum through 4 wk postpartum. Significant difference from control between sampling period (*P < .1).

Figure 12. Changes in nitroblue tetrazolium (NBT) reduction (least squares means) by resting and activated milk polymorphonuclear leukocytes from cows during the postpartum period. Significant difference from wk 0 (***P < .05). Vertical bars indicate overall SE.

Figure 13. Daily DMI in cows supplemented with β-carotene and vitamin A from 4 wk prepartum through 4 wk postpartum. No treatment differences were significant any sampling period.

Specially 600 mg of β-carotene) compared with that of unsupplemented cows or cows supplemented with vitamin A. This result is in agreement with in vitro studies by Daniel et al. (10), who showed that β-carotene significantly enhanced bovine lymphocyte proliferation in response to Con A. Hoskinson et al. (14) similarly showed that weanling pigs injected with β-carotene had increased lymphocyte responses to PHA and Con A. Other studies have shown a modulatory role of β-carotene on lymphocyte function. Bendich and Shapiro (3) reported that rats fed very large doses (up to .2% of the diet) of β-carotene or canthaxanthin had significantly enhanced lymphocyte proliferation in response to Con A. Because canthaxanthin is a carotenoid, which cannot be converted to vitamin A, the increase in lymphocyte proliferation conceivably is directly attributable to the effects of carotenoids and not to its conversion to vitamin A. Seifer et al. (25) showed that mice, fed large doses of β-carotene and infected with Moloney murine sarcoma virus, had decreased tumor numbers and longer latent periods. Tumor regression rate was increased when previously infected,
TABLE 2. Daily milk production of cows fed β-carotene and vitamin A from 4 wk prepartum through 4 wk postpartum.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lactation (kg)</th>
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<tbody>
<tr>
<td></td>
<td>wk 1</td>
</tr>
<tr>
<td>Control</td>
<td>25.7</td>
</tr>
<tr>
<td>Vitamin A, 120,000 IU</td>
<td>17.5</td>
</tr>
<tr>
<td>β-Carotene 300 mg</td>
<td>23.8</td>
</tr>
<tr>
<td>β-Carotene 600 mg</td>
<td>20.6</td>
</tr>
</tbody>
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Overall SE = 1.2.

*Indicates significant difference (P < .05) from control.

Tumor-bearing mice were supplemented with β-carotene. In a separate study, Seifter et al. (24) showed that healthy mice, fed increasing doses of dietary β-carotene, had significant and progressive increases in thymic size. The number of small lymphocytes in the thymus and the number of peripheral blood lymphocytes increased in mice fed 90 mg of β-carotene/kg of diet. Additionally, Rettura et al. (21) found that mice supplemented with β-carotene had increased rejection of skin allografts. Graft rejection is a lymphocyte-mediated event, indicating a β-carotene effect on lymphocyte function.

The mitogens Con A and PHA primarily induce T-cell proliferation. The results of this study suggest that β-carotene primarily affects the T-lymphocyte population. Alexander et al. (1) showed that humans supplemented with 180 mg/d of β-carotene for 2 wk had significantly increased numbers of CD4+ (T_H cells) and CD3+ (all T cells). This increase in CD4+ cells continued for at least 6 wk after supplementation ended. In vitro studies on the effects of β-carotene on human lymphocytes showed that β-carotene dramatically increased the number of cells with NK cell markers and T_H cell markers (20). In contrast to Con A and PHA, PWM primarily stimulates the proliferation of B cells. Supplementation with β-carotene may have enhanced the activity of these cells, which is in accordance with work of Bendich and Shapiro (3), who showed that rats supplemented with β-carotene had increased B- and T-cell activities.

Supplementation with β-carotene and vitamin A during the peripartum period enhanced the nonspecific, cellular host defense system in the blood of dairy cows but did not significantly affect mammary host defense. The effect of β-carotene supplementation on phagocytic ability of blood PMN was inconclusive. Postpartum phagocytosis increased for cows supplemented with 300 mg of β-carotene but not for those supplemented with 600 mg of β-carotene. Daniel et al. (9) failed to show a significant in vitro effect of β-carotene on bovine blood PMN phagocytic ability during the peripartum period.

Although phagocytosis is the first step, and an important one, in the elimination of bacteria, killing of internalized bacteria is a better means of measuring the effectiveness of the nonspecific, cellular host defense system. Bacteria may be phagocytized by PMN but continue to multiply in the cell, as is true for S. aureus (16). In the present study, killing of S. aureus by bovine blood PMN was increased.

TABLE 3. Incidence of disorders in cows fed β-carotene and vitamin A from 4 wk prepartum through 4 wk postpartum.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disorder</th>
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<tbody>
<tr>
<td></td>
<td>Retained placenta</td>
</tr>
<tr>
<td>Control</td>
<td>41</td>
</tr>
<tr>
<td>Vitamin A, 120,000 IU</td>
<td>31**</td>
</tr>
<tr>
<td>β-Carotene 300 mg</td>
<td>33*</td>
</tr>
<tr>
<td>β-Carotene 600 mg</td>
<td>25**</td>
</tr>
</tbody>
</table>

*Indicates significant difference (P < .1; *P < .05; **P < .01; ***P < .001) from control.
by supplementation with both β-carotene and vitamin A. More importantly, these vitamins enhanced PMN killing ability at calving and immediately postpartum when PMN function is normally lowest. Daniel et al. (9) also observed increased killing of *S. aureus* by blood PMN from cows during the periparturient period when PMN were incubated with β-carotene. Killing of bacteria by PMN is accomplished by two separate pathways. The oxygen-independent pathway relies upon the release of enzymes (such as myeloperoxidase, lysozyme, and acid hydrolases) from intracellular granules, and the oxygen-dependent pathway utilizes reactive oxygen species generated during the respiratory burst. Supplementation of β-carotene appears to enhance the effectiveness of the myeloperoxidase-hydrogen peroxide-halide system of bovine blood PMN. Iodine uptake was greater in resting cells from both cows treated with vitamin A and with β-carotene. These results suggest that these β-carotene and vitamin A may "prime" the myeloperoxidase-hydrogen peroxide-halide system and thereby enhance overall killing of bacteria. Supplemental β-carotene and vitamin A did not enhance superoxide production, especially by activated blood PMN. Therefore, supplementation with β-carotene may be decreasing the production of superoxide anions. Similarly, repletion with β-carotene of previously carotenoid-depleted, healthy male adult humans did not affect superoxide production by PMN (17). These results suggest that β-carotene acts to quench reactive oxygen metabolites. Indeed, studies have shown β-carotene and canthaxanthin (15), as well as other carotenoids (11), to be extremely effective antioxidants and potent quenchers of free radical species. The free radical quenching ability of carotenoids is important because superoxides and free radicals produced in biological systems cause sister chromatid exchanges, chromosomal aberrations (premalignant indicators of DNA damage), and cell membrane destruction (4).

Phagocytosis and intracellular kill abilities of mammary phagocytes decreased during early lactation. The effectiveness of the myeloperoxidase-hydrogen peroxide-halide killing system is apparently decreased more than is the respiratory burst of mammary PMN. Lack of a sufficient energy source, in discriminate phagocytosis of milk constituents, and low oxygen tension in the mammary gland are several factors that may contribute to decreased mammary PMN function during early lactation (19, 28).

The incidence of retained placenta was greater in unsupplemented cows than in cows supplemented with vitamin A and, especially, β-carotene. Although such factors as environment, genetic makeup, physiological effects, and hormonal status have been associated with increased rates of retained placenta, β-carotene and vitamin A nutriture seem to be other important factors (6). Results of the present study suggest that adequate β-carotene or vitamin A intake, or both, are required for timely release of fetal membranes. Plasma concentrations of retinol and β-carotene decreased dramatically during the prepartum period. Sutton et al. (27) reported sharp decreases in plasma β-carotene (69%) and vitamin A (87%) from 4 wk prepartum to parturition in cows that freshened during the winter months and were fed good quality mixed alfalfa hay. Those authors observed a sharp decline in circulating concentrations of β-carotene and vitamin A in plasma during times of rapid udder filling and suggested that the decrease in these vitamins may be due, at least in part, to rapid transfer to colostrum. However, no correlation existed between concentrations of β-carotene and vitamin A in plasma and the output of these vitamins in the colostrum (27). A similar decrease in plasma carotenoids and vitamin A occurred in a mammanectomized cow that gave birth to a premature calf (31). Therefore, the decrease in plasma concentrations of β-carotene and vitamin A would seem to be influenced by hormonal status (12). In the present study, incidence of retained placenta appears to be related to concentrations of plasma retinol during the prepartum period. Unsupplemented cows had the lowest concentrations of retinol in plasma prior to calving, and cows supplemented with 600 mg of β-carotene had the highest concentrations of plasma retinol and the lowest incidence of retained placenta. Vitamin A is necessary for maintenance of epithelial integrity. The low concentration of plasma retinol in unsupplemented cows during the prepartum period may have been conducive to epithelial metaplasia and keratinization of placentomes, thereby increasing the incidence.
of retained placenta. It is difficult to conclude from the present study the possible direct role of β-carotene on retained placenta. However, supplementation with high levels of β-carotene appears to be beneficial. The significantly lower incidence of metritis of cows supplemented with β-carotene compared with that of unsupplemented cows suggests an important role of β-carotene in combating uterine infection.

Milk production was not affected by treatment. Why a decrease in daily milk production on wk 0 occurred in cows supplemented with vitamin A is unknown. However, supplementation with β-carotene or vitamin A resulted in greater increases in milk production during the postpartum period than was observed in unsupplemented cows.

α-Tocopherol also may affect the immune and reproductive systems. Studies showed that high dietary intakes of α-tocopherol increased phagocytic ability of human PMN (2) and rat alveolar macrophages (18). The PMN from rats deficient in vitamin E had decreased chemotactic and phagocytic abilities (13). Enhanced PMN function associated with the β-carotene and vitamin A supplementation in this study is unlikely due to changes in a-tocopherol status because cows supplemented with β-carotene and vitamin A tended to have lower concentrations of plasma α-tocopherol.

In summary, β-carotene enhanced lymphocyte proliferation induced by Con A, PHA, and PWM immediately prior to calving and after calving. The effects of β-carotene and vitamin A supplementation on phagocytic activity of bovine blood PMN during the peripartum period were inconclusive. However, the intracellular kill ability of blood PMN was increased by treatment. Furthermore, cows supplemented with β-carotene and vitamin A had decreased incidence of retained placenta and metritis. These results support our initial hypothesis that β-carotene enhances host defense mechanisms. Whether the observed effects of β-carotene on host defense translates into increased resistance of the mammary gland to new intramammary infections is unknown and warrants further research.

REFERENCES


MODULATORY EFFECTS OF DIETARY $\beta$-CAROTENE


