Reactivity and Phenotype of Mononuclear Leukocytes from Nongravid Heifers After In Vitro Exposure to 9,13-di-cis-Retinoic Acid

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ABSTRACT

The predominant isomer of retinoic acid in the plasma of dairy cows during the periparturient period is 9,13-di-cis-retinoic acid. Because retinoic acids influence the activity of cells in a variety of tissues, including the immune system, the potential for this isomer to modulate the bovine immune system during the periparturient period must be considered. The present study examined the in vitro effects of 9,13-di-cis-retinoic acid on the reactivity and phenotype of blood mononuclear leukocytes from nongravid Holstein heifers that were sensitized to antigens and that had naturally low plasma concentrations of 9,13-di-cis-retinoic acid. In this system, 9,13-di-cis-retinoic acid, approximating the highest plasma concentrations of 9,13-di-cis-retinoic acid occurring in vivo during the periparturient period, had no effect on DNA synthesis, secretion of interleukin-2 or interferon-γ, or secretion of immunoglobulin by unstimulated cultures or cultures stimulated by mitogen (pokeweed mitogen) or antigen (ovalbumin). The composition of unstimulated and stimulated mononuclear leukocyte populations, based on percentages of specific cell types, was unaffected by 9,13-di-cis-retinoic acid at the physiologic concentration of 10⁻⁸ M. 9,13-di-cis-Retinoic acid did not affect the actual number of cells in unstimulated cultures and cultures stimulated by antigen but did cause a moderate reduction in the number of cells, primarily CD4⁺ lymphocytes, in cultures stimulated by mitogen. Overall, these results suggest that the elevated concentration of 9,13-di-cis-retinoic acid in maternal plasma may have a negligible effect on the reactivity and phenotype of cells constituting the circulating mononuclear leukocyte population.

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Abbreviation key: FBS = fetal bovine serum, HBSS = Hanks balanced salt solution, IFN-γ = interferon-γ, IL = interleukin, OVA = ovalbumin, PBML = peripheral blood mononuclear leukocytes, PWM = pokeweed mitogen, RA = retinoic acid, RXR = retinoid X receptor.

INTRODUCTION

A previously unrecognized, naturally occurring isomer of retinoic acid (RA) has been identified in the plasma of the calf and adult dairy cow immediately postpartum (10, 11). The concentration of this isomer, characterized as 9,13-di-cis-RA, increases approximately 8-fold (to 6 ng·ml⁻¹ or 2.0 × 10⁻⁸ M) in maternal plasma within 24 h of parturition. During this period, 9,13-di-cis-RA is the predominant isomer of RA. A similar increase in the concentration of 9,13-di-cis-RA occurs in the plasma of the neonatal calf fed colostrum. The biological and, more specifically, immunological significance of the elevated concentrations of 9,13-di-cis-RA in maternal bovine plasma during the period immediately postpartum is unknown.

The dairy cow is at increased risk of infectious disease during the peripartum period (23, 27). Blood neutrophil functions (14) and mitogen-induced blastogenesis by blood lymphocytes are also impaired at this time (13, 15). Factors contributing to altered leukocyte function and increased susceptibility to infectious disease have not been fully elucidated, although dramatic physiological changes in the dairy cow during the periparturient period can modulate immune function, potentially compromising disease resistance (16). Because isomers of RA can affect the proliferation and function of bovine peripheral blood mononuclear leukocytes (PBML) in vitro (20, 22), the specific and profound increase in plasma concen-
trations of 9,13-di-cis-RA in the postpartum dairy cow was considered a potential modifier of immune cell function.

The objective of the present study was to evaluate the in vitro effects of 9,13-di-cis-RA on the proliferation, phenotype, and functional capacities of PBML from normal, nonpregnant heifers with significantly lower concentrations of this RA isomer in plasma than that in plasma of dams during the periparturient period.

MATERIALS AND METHODS

Cows and PBML Isolation

Four nonpregnant Holstein heifers that were matched in age were used in the study. Heifers were housed together at the USDA, ARS, National Animal Disease Center (Ames, IA) and were fed a pelleted diet containing ground corn cobs, wheat middlings, and corn, in addition to hay and pasture. This diet was sufficient to maintain requirements for energy and protein. Cows had free access to water and remained clinically normal throughout the experimental period. Experimental procedures were approved by the Institutional Animal Care and Use Committee. The 9,13-di-cis-RA was sufficient to maintain requirements for energy and protein. Cows had free access to water and remained clinically normal throughout the experimental period. Experimental procedures were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center).

Plasma concentrations of retinol and β-carotene were determined by reverse-phase HPLC and averaged 350 and 430 ng·ml⁻¹, respectively, throughout the experimental period. These values were within normal ranges for adult, nonlactating dairy cows (4, 8). Mean plasma RA concentrations measured by reverse-phase HPLC were 1.11 ng·ml⁻¹ (3.70 × 10⁻⁹ M) of all-trans-RA, 0.62 ng·ml⁻¹ (2.06 × 10⁻⁹ M) of 13-cis-RA, and 0.11 ng·ml⁻¹ (3.7 × 10⁻¹⁰ M) of 9,13-di-cis-RA; 9-cis-RA was undetectable.

Each heifer was sensitized to the T-dependent antigen ovalbumin (OVA; salt-free chicken egg albumin; Sigma Chemical Co., St. Louis, MO) prior to the initiation of the study as has been previously described (19). One sample of peripheral blood (–250 ml) provided enough cells to perform all in vitro analyses. The PBML were isolated, enriched by Percoll® gradient centrifugation (Pharmacia, Piscataway, NJ) as previously described (21), and resuspended in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, and antibiotics. The leucocyte population consisted of >95% mononuclear leucocytes with >95% viability.

Retinoids

The 9,13-di-cis-retinoic acid was prepared by oxidation of 9-cis-retinal with Tollens reagent as described previously (11); purity was confirmed by spectrophotometric and HPLC analyses. The RA was solubilized in 100% HPLC-grade ethanol and immediately diluted in fetal bovine serum (FBS; Hydene Labs, Logan, UT). Using this method, the concentration of ethanol in all cultures was <0.01% (vol/vol). Control cultures received an equivalent volume of ethanol. Fetal bovine serum contained 34 ng·ml⁻¹ of β-carotene, no detectable retinol, and 0.15 ng·ml⁻¹ (5 × 10⁻¹⁰ M) of 9,13-di-cis-RA. The FBS contributed <3.4 × 10⁻¹¹ M 9,13-di-cis-RA to each culture.

Lymphocyte DNA Synthesis Assay

Synthesis of DNA in PBML was determined by measuring the incorporation of [³H]thymidine into DNA of unstimulated PBML and PBML stimulated by pokeweed mitogen (PWM) and OVA (21). Tissue culture plates (96-well; Costar, Cambridge, MA) were inoculated with 1.0 × 10⁶ cells·ml⁻¹ in a final volume of 200 µl. The amount of PWM and OVA added to stimulated cultures is indicated in the figures. Cultures of PBML were supplemented by 0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷, and 10⁻⁶ M 9,13-di-cis-RA at the initiation of the incubation period. All cultures contained FBS at 5% (vol/vol). Individual cultures were prepared in duplicate. Cultures stimulated by PWM were incubated for 90 h, and the cultures stimulated by OVA were incubated at 39°C for 138 h in a humidified atmosphere containing 5% CO₂. Incubation times for lectin and antigen cultures were based on preliminary studies that evaluated the conditions supporting DNA synthesis. Cultures received 18.5 Bq of [methyl-³H]thymidine (Amersham, Arlington Heights, IL) in 50 µl of RPMI-1640 medium at 18 h before the conclusion of the incubation period. Cultures were then harvested onto glass fiber filters, and the retained radioactivity was counted by liquid scintillation spectrophotometry. Synthesis of DNA by PBML was expressed as counts per minute.

Cell Proliferation and Phenotype Analyses

Effects of 9,13-di-cis-RA on the proliferation and phenotype of cultured PBML were evaluated as previously described (20). Cultures were established in 24-well tissue culture plates (Costar). The PBML at 1.0 × 10⁶ cells·ml⁻¹ in 1.5-ml cultures were unstimulated (no mitogen or antigen), stimulated by OVA (0.5 µg·ml⁻¹) or PWM (0.08 µg·ml⁻¹), or costimulated by OVA and PWM (0.5 and 0.08 µg·ml⁻¹, respectively). The 9,13-di-cis-RA (0 and 10⁻⁸ M) was added to the cultures at the beginning of a 6-d incubation period. Cells were harvested from control and test cultures on d 6 by centrifuging (650 × g at 4°C for 10 min) the tissue culture plates, removing superna-
tants, and gently freeing cells with Hanks balanced salt solution (HBSS; pH 7.2; 0.015 M without Ca\(^{2+}\) or Mg\(^{2+}\)) supplemented with trypsin (0.05% (wt/vol)). Cells were washed twice by centrifugation (first wash at 250 \( \times \) g at 4°C for 20 min; second wash at 650 \( \times \) g at 4°C for 5 min). Cells that had been washed were then resuspended in cold HBSS with FBS [1% (vol/vol)] and NaN\(_3\) [0.01% (vol/vol)]. The total number of cells in individual suspensions was determined electronically (Celltrack 1; Nova Biomedical, Waltham, MA).

Individual suspensions corresponding to specific control and test conditions were subsequently labeled with monoclonal antibodies to bovine leukocyte surface antigens (Table 1). Flow cytometric analyses were performed on individual cell suspensions as described previously (20) using a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

The composition of the cell suspensions was based on percentages of specific cell types and the actual number of cells per milliliter in a culture expressing specific antigens.

### Quantification of Secreted Ig

Secretion of Ig by PBML was assayed in flat-bottomed, 24-well polystyrene tissue culture plates inoculated with 1.0 \( \times \) \( 10^6 \) cells·ml\(^{-1}\) in a total culture volume of 1.5 ml. Culture medium consisted of RPMI-1640 medium supplemented with FBS [6.7% (vol/vol)]. Secretion of polyclonal IgM was evaluated in cultures stimulated by PWM (0, 0.04, and 0.08 \( \mu \)g·ml\(^{-1}\)) and in cultures costimulated by OVA and PWM (5 and 1 \( \mu \)g·ml\(^{-1}\), respectively). The 9,13-di-cis-RA was added at the beginning of the incubation period to yield a concentration in the culture of 10\(^{-8}\) M. Cultures were incubated for 48 h at 39°C in a humidified atmosphere containing 5% CO\(_2\). Culture supernatants were harvested from centrifuged plates (400 \( \times \) g for 5 min at 18°C) and stored at -80°C until analysis.

Interleukin (IL)-2 was quantified by measuring the proliferation of the bovine T-cell line, 300B1, which is dependent on IL-2. Growth and preparation of the cells and the assay procedure have been described previously (30). The concentration of PWM or OVA in the culture supernatants did not influence the proliferation of 300B1 cells (data not shown). The assay was performed in 96-well polystyrene plates and consisted of 100 \( \mu \)l of test sample or IL-2 standards (recombinant human IL-2 at 0 to 1.0 U·ml\(^{-1}\) in culture; Genzyme Diagnostics, Kent, England); 25 \( \mu \)l of IL-1 receptor antagonist (50 ng·ml\(^{-1}\); R & D Systems, Minneapolis, MN), which inhibited recombinant bovine IL-1\(\beta\) activity; and 50 \( \mu \)l of 300B1 cells at 400,000 cells·ml\(^{-1}\). Cultures were incubated for 48 h at 37°C in 5% CO\(_2\) and then pulsed for 18 h with 18.5 Bq of [methyl-\(^3\)H]thymidine. The counts per minute were converted to units of IL-2 per milliliter by interpolation from the IL-2 standard curve. Results are presented in units per milliliter of IL-2.

Interferon-\(\gamma\) was measured using an IFN-\(\gamma\)-capture ELISA (1). Bovine recombinant IFN-\(\gamma\) and antibodies were provided by D. Godson (Veterinary Infectious Disease Organization, Saskatoon, SK, Canada). Assays were performed in Immunolon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA).

### Cytokine Production and Quantification

Secretions of IL-2 and interferon-\(\gamma\) (IFN-\(\gamma\)) were evaluated in PBML cultures established in flat-bottomed, 24-well polystyrene tissue culture plates inoculated with 2 \( \times \) \( 10^6 \) cells·ml\(^{-1}\) in a total volume of 1.5 ml of RPMI-1640 medium and FBS [6.7% (vol/vol)]. Cultures were unstimulated, stimulated by PWM (1 \( \mu \)g·ml\(^{-1}\)) or OVA (5 \( \mu \)g·ml\(^{-1}\)), or costimulated by OVA and PWM (5 and 1 \( \mu \)g·ml\(^{-1}\), respectively). The 9,13-di-cis-RA was added at the beginning of the incubation period to yield a concentration in the culture of 10\(^{-8}\) M. Cultures were incubated for 48 h at 39°C in a humidified atmosphere containing 5% CO\(_2\). Culture supernatants were harvested from centrifuged plates (400 \( \times \) g for 5 min at 18°C) and stored at -80°C until analysis.

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### Table 1. Monoclonal antibodies used in flow cytometric analysis of leucocyte surface expression of antigens.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Designation</th>
<th>Ig Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2 T Cell (pan T)</td>
<td>BAQ95A</td>
<td>G1</td>
</tr>
<tr>
<td>CD8 T Cell (suppressor and cytotoxic)</td>
<td>CACT80C</td>
<td>G1</td>
</tr>
<tr>
<td>CD4 T Cell (helper and inducer)</td>
<td>GC50A</td>
<td>M</td>
</tr>
<tr>
<td>B cell (B2)</td>
<td>BAQ44A</td>
<td>M</td>
</tr>
<tr>
<td>Null cells (N2)</td>
<td>BAQ4A</td>
<td>G1</td>
</tr>
<tr>
<td>Interleukin-2 receptor (ACT5)</td>
<td>CACT116A</td>
<td>G1</td>
</tr>
<tr>
<td>MHC2 Class II antigen</td>
<td>TH14b</td>
<td>G2</td>
</tr>
</tbody>
</table>

1Monoclonal antibodies from VMRD Inc. (Pullman, WA).
2Major histocompatibility complex.

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Reagents were capture antibody (mouse antirecombinant bovine IFN-γ, IgG fraction, lot TB-4-91), detection antibody (rabbit ant-bovine IFN-γ, IgG fraction, lot no. 90-81), recombinant bovine IFN-γ (lot TB-4-91), biotinylated goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, CA), horseradish peroxidase-conjugated streptavidin-biotinylated complex (Amersham), and substrate [2,2-azino-di-(3-ethyl-benzthiazoline sulfonate)-6] in citrate buffer and H2O2 at 0.1% (vol/vol)). Internal standards consisting of serially diluted recombinant bovine IFN-γ were prepared in PBS with Tween 80 [0.1% (vol/vol)] and gelatin [0.1% (vol/vol)]. Positive and negative control standards were also serially diluted in PBS with Tween 80 and gelatin. Capture antibody was diluted 1:4000 (vol/vol) in carbonate-coating buffer, and detection antibody was diluted 1:1000 (vol/vol) in PBS with Tween 80 and gelatin. Biotinylated goat anti-rabbit Ig was diluted 1:10,000 (vol/vol), and the horseradish peroxidase-conjugated streptavidin-biotinylated complex was diluted 1:2000 in PBS without gelatin. Absorbance of standards and test samples was read at 405 and 490 nm using an automated ELISA plate washer and reader (Dynatech, Guernsey, Channel Islands). The concentration of IFN-γ in test samples (culture supernatants) was determined by comparison of their absorbance with that of the standards within a linear curve fit. The IFN-γ concentration in culture supernatants was expressed in nanograms per milliliter.

**Analysis of RA Uptake by Bovine Mononuclear Leukocytes Stimulated by Mitogen**

Three 250-ml flasks of anticoagulated peripheral blood were collected from one cow. Each flask was centrifuged (1170 × g for 20 min at 22°C), and theuffy coat layers enriched with PBML were recovered, pooled, and resuspended in HBSS (0.015 M; pH 7.2; without Ca or Mg). Cell suspensions were subsequently washed by centrifugation (650 g for 10 min at 22°C), and suspended to 10 × 10^6 cells·ml⁻¹ in 200 ml of RPMI-1640 medium. This population consisted of approximately 25% granulocytes and 75% mononuclear leukocytes.

Smaller individual flasks of PBML were treated with 10⁻⁹ M [³H]-9,13-di-cis-RA, [³H]-all-trans-RA, or [³H]-9-cis-RA. All cultures were stimulated by PWM (10 µg·ml⁻¹) and were incubated for 8 or 24 h at 39°C in a humidified atmosphere of 5% CO₂. Cells were collected from 8- and 24-h cultures, extracted, and analyzed by HPLC (10). Separation of [³H]RA isomers was accomplished by using a Suplex pkb-100 column (5 µm; 25 × 4.6 mm) with a ratio of acetonitrile to methanol to water to chloroform to 6% acetic acid of 17:68:10:5:0.6 and a flow rate of 2 ml/min. Thirty-second fractions were collected, dried, and counted in a liquid scintillation counter. The RA isomer associated with cells was expressed in counts per minute.

**Statistical Analysis**

All data are presented as means (±SEM). Treatment means were analyzed by ANOVA, and, when statistical differences were detected, the Tukey-Kramer multiple comparison test was applied. Differences were considered significant at P < 0.05.

**RESULTS**

**Effects of 9,13-di-cis-RA on DNA Synthesis**

Supplemental RA did not affect DNA synthesis in 4- and 6-d unstimulated cultures (P > 0.05; data not shown). In control cultures, DNA synthesis in 4-d cultures stimulated by OVA was less than that in 4-d cultures stimulated by PWM (Figure 1). The 9,13-di-cis-RA at 10⁻⁶ M inhibited (P < 0.05) DNA synthesis in cultures stimulated by OVA (Figure 1a). Synthesis of DNA in maximally inhibited cultures stimulated by OVA was still greater (P ≥ 0.05) than that in unstimulated control cultures.

Effects of 9,13-di-cis-RA on DNA synthesis induced by PWM were negligible (Figure 1b), except for cultures stimulated by the lowest concentration of PWM (< 0.05) under these conditions, DNA synthesis was inhibited (P < 0.05) under these conditions, DNA synthesis was still -45 times greater in these stimulated cultures than in unstimulated control cultures.

**Effects of 9,13-di-cis-RA on Proliferation and Phenotype**

Supplementation of cultures stimulated by OVA with 9,13-di-cis-RA at the physiologic concentration of 10⁻⁸ M was not associated with any change in the total number of cells or the number of cells expressing specific surface antigens (Figure 2). In contrast, 9,13-di-cis-RA at 10⁻⁸ M caused a moderate reduction (P < 0.05) in the total number of cells, CD2⁺ T cells,
and IL-2 r+ cells in cultures stimulated by PWM (Figure 2). Although the number of CD8+ T cells was unaffected in these cultures, the number of CD4+ T cells was decreased (P < 0.05).

The percentages of individual cell types in cultures stimulated by PWM and OVA were unchanged (P > 0.05) by 9,13-di-cis-RA at 10^{-8} M (data not shown).

**Effects of 9,13-di-cis-RA on Cytokine Secretion**

Unstimulated and stimulated cultures of PBML produced detectable concentrations of IL-2, although cultures stimulated by OVA and PWM produced more (P < 0.05) IL-2 than did unstimulated cultures (Figure 3a). Interferon-γ was undetectable in supernatants from unstimulated cultures and was present at higher (P < 0.05) concentrations in cultures stimulated by PWM than in cultures stimulated by OVA (Figure 3b). Supplemental 9,13-di-cis-RA at the physiologic concentration of 10^{-8} M did not affect (P > 0.05) the amount of IL-2 or IFN-γ secreted by cultures (Figure 3).

**Effects of 9,13-di-cis-RA on Ig Secretion**

The concentration of polyclonal IgM in control cultures stimulated by PWM was greater (P < 0.05) than the concentration in unstimulated cultures (Figure 4a). The 9,13-di-cis-RA did not affect (P > 0.05) IgM secretion (Figure 4a) in unstimulated cultures or in cultures stimulated by PWM.

Ovalbumin-specific IgG in control cultures stimulated by OVA was greater (P < 0.05) than that in unstimulated cultures (Figure 4b). The 9,13-di-cis-RA (10^{-9}, 10^{-8}, and 10^{-7} M) did not affect (P > 0.05)
TABLE 2. Contrasting effects of 9,13-di-cis-retinoic acid (RA) (0 to 10 \(^{-6}\) M) on DNA synthesis in 4- and 6-d cultures of bovine mononuclear leukocytes stimulated by pokeweed mitogen at 1 \(\mu\)g ml\(^{-1}\) (n = 4).

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>9,13-di-cis-RA (cpm (\times 10^{-3}))</th>
<th>Mean</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 M</td>
<td>X</td>
<td>SEM</td>
</tr>
<tr>
<td>4 d</td>
<td>102 (\pm) 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 d</td>
<td>64 (\pm) 12</td>
<td></td>
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<tr>
<td></td>
<td>10(^{-10}) M</td>
<td>X</td>
<td>SEM</td>
</tr>
<tr>
<td>4 d</td>
<td>103 (\pm) 5</td>
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<tr>
<td>6 d</td>
<td>67 (\pm) 12</td>
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<td>10(^{-9}) M</td>
<td>X</td>
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<td>4 d</td>
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</tr>
<tr>
<td>4 d</td>
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<tr>
<td>6 d</td>
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<tr>
<td></td>
<td>10(^{-7}) M</td>
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</tr>
<tr>
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<td>6 d</td>
<td>39 (\pm) 11</td>
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</tr>
<tr>
<td></td>
<td>10(^{-6}) M</td>
<td>X</td>
<td>SEM</td>
</tr>
<tr>
<td>4 d</td>
<td>90 (\pm) 8</td>
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<td></td>
</tr>
<tr>
<td>6 d</td>
<td>23 (\pm) 7</td>
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<td></td>
</tr>
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\(^{1}\)Means differ from the control value within a row (P < 0.05).

secretion (Figure 4b) in unstimulated cultures or in stimulated cultures.

**Uptake of RA by Mononuclear Leukocytes**

Intracellular accumulation of RA isomers in PBML cultures stimulated by PWM and treated with 10\(^{-9}\) M \([3H]\)-RA (9,13-di-cis-RA, all-trans-RA, or 9-cis-RA) is shown in Figure 5. Of the treatments, supplemental 9,13-cis-RA had the least effect on cellular RA concentrations; there was no detectable RA in 8-h cultures and very little in 24-h cultures (Figure 5a). All-trans-retinoic acid, rather than 9,13-di-cis-RA, was the predominant isomer in these cultures. In contrast, treatment with all-trans-RA or 9-cis-RA produced measurable concentrations of 9-cis-RA and all-trans-RA at 8 h and even greater concentrations of all three isomers after 24 h. In these cultures (Figure 5, b and c), the predominant isomer associated with cells was the treatment isomer. Cellular 9,13-cis-RA was detectable in all 24-h cultures, although at much lower concentrations than all-trans-RA and 9-cis-RA.

**DISCUSSION**

The capacity of 9,13-di-cis-RA to modulate the reactivity and phenotype of bovine PBML was evaluated in vitro using leukocytes from nongravid Holstein heifers with a mean plasma 9,13-di-cis-RA concentration of 0.11 ng ml\(^{-1}\) (3.7 \(\times\) 10\(^{-10}\) M), which was <2% of the concentration in maternal bovine plasma during the immediate postpartum period (11). Using this in vitro model, exogenous 9,13-di-cis-RA approximating the highest plasma concentrations occurring in vivo had no effect on DNA synthesis, IL-2 or IFN-\(\gamma\) secretion, or Ig secretion induced by mitogen or antigen. These results suggest that the elevated concentrations of 9,13-di-cis-RA in maternal plasma immediately after parturition would not influence these functions of the PBML population and probably do not contribute to the previously reported (12, 13, 15, 28, 29) reduction in functional capacity of the PBML population immediately after parturition.

9,13-di-cis-Retinoic acid is also the most abundant RA isomer in the plasma of the neonatal calf (10). The elevated concentration of this isomer is dependent on the ingestion of whole colostrum by the calf. In a related study (25) that examined the effects of 9,13-di-cis-RA on the activity of PBML from precolos-
Figure 4. Polyclonal IgM secretion (a) and ovalbumin (OVA)-specific IgG secretion (b) in 14-d cultures of bovine mononuclear leukocytes supplemented with 9,13-di-cis-retinoic acid (RA) at 0 M (solid bar), 10⁻⁹ M (patterned bar), 10⁻⁸ M (open bar), and 10⁻⁷ M (striped bar). Supplemental RA did not affect (P > 0.05) secretion of IgM or antigen-specific IgG. PWM = Pokeweed mitogen.

Figure 5. Cellular retinoid acid (RA) concentrations in mononuclear leukocytes stimulated by pokeweed mitogen (PWM) and treated with 10⁻⁹ M [³H]-9,13-di-cis-RA (a), [³H]-all-trans-RA (b), or [³H]-9-cis-RA (c). Data are expressed as counts per minute produced by a single culture flask incubated for 8 or 24 h. Figure legend: 9-cis-RA (solid bar), all-trans-RA (patterned bar), and 9,13-di-cis-RA (open bar).

Centrals calves, physiologic concentrations of this isomer did not alter the functional capacity (i.e., mitogen-induced IFN-γ and Ig secretion) of the PBML population. Those results and results from the present study provide a strong indication that the predominance of 9,13-di-cis-RA in the circulation of periparturient dairy cows and neonatal calves does not modulate the function of their PBML.

Percentages of cells in cultures after stimulation by mitogen or antigen that expressed specific cell surface antigens also were unaffected by supplemental 9,13-di-cis-RA. Although this RA did not alter the proliferation of cells in cultures stimulated by antigen, it did have an antiproliferative effect in cultures stimulated by mitogen. The CD2⁺ T cell population, specifically the CD4⁺ T cell in this population, was most affected by treatment with RA. This modest effect of 9,13-di-cis-RA on a specific subset of lymphocytes was apparently of insufficient magnitude to influence the functional capacity of the PBML population. Overall, the relative inactivity of 9,13-di-cis-RA in this system is in marked contrast to previous studies (6, 20, 22), indicating that other isomers of RA profoundly influence the proliferation, functional capacity, and phenotype of bovine PBML stimulated by mitogen. Reasons for the inactivity of 9,13-di-cis-RA are not clear. Retinoic acids exert their effects by binding to high affinity intracellular receptors with ultimate effects on gene transcription. Two distinct families of receptors have been designated: RA receptor (7, 24) and retinoid X receptor (RXR) (17); each has multiple isoforms. All-trans-retinoic acid and 9-cis-RA have similar affinities for RA receptor; however, the principal ligand for RXR seems to be 9-cis-RA. Studies considering the biosynthesis and
metabolism of all-trans-RA (2, 9, 18, 19) indicated that 13-cis-RA and the glucuronides of all-trans-RA and 13-cis-RA may provide reservoirs of slowly metabolized, low biologically active metabolites that contribute to the maintenance of steady-state concentrations of all-trans-RA. The 9,13-di-cis-RA may function in a similar role.

Recent studies (11, 26) suggest that 9,13-di-cis-RA is a major product of 9-cis-RA metabolism. Administration of 9,13-di-cis-RA to rats also elevated plasma 9-cis-RA (11), indicating that conversion of 9,13-di-cis-RA to 9-cis-RA may occur. Subtle effects of 9,13-di-cis-RA on bovine PBML (Figures 1 and 2; Table 2) in the present study might also have been the result of a low rate of conversion of 9,13-di-cis-RA to 9-cis-RA, a ligand for both RXR and RA receptor. Further research is necessary to clarify the degree of interconversion between RA isomers.

In the present study, treatment of PBML stimulated by PWM with [3H]-9,13-di-cis-RA was not associated with any detectable cellular [3H]-RA after 8 h and only marginally detectable concentrations of 9-cis-RA, all-trans-RA, and 9,13-di-cis-RA after 24 h, suggesting that 9,13-di-cis-RA converts at a very low rate to other forms of RA. Treatment of identically prepared cultures with 9-cis-RA or all-trans-RA, in contrast, was associated with detectable cellular [3H]-9-cis-RA and all-trans-RA at 8 h. After 24 h, the abundance of cellular [3H]-RA was greater than that at 8 h and exceeded the abundance of [3H]-RA in 24-h cultures originally treated with 9,13-di-cis-RA. These results indicate that all-trans-RA and 9-cis-RA, unlike 9,13-di-cis-RA, readily accumulate in the cell and convert more quickly to other forms of RA. Similarly, Reinhardt and Horst (26) have demonstrated that treatment of confluent cultures of T-47 human breast cancer cells with 10-9 M [3H]-9,13-di-cis-RA caused an elevation in its concentration in culture medium, but there was no cellular accumulation of 9,13-di-cis-RA or other isomers of RA. The low affinity for binding of 9,13-di-cis-RA to cellular RA binding proteins (11) and the RXR (R. L. Horst and T. A. Reinhardt, 1995, unpublished data) might account for the low rate of accumulation of 9,13-di-cis-RA in bovine PBML. These results may also explain the minimal impact of 9,13-di-cis-RA on bovine PBML in vitro.

CONCLUSIONS

Overall, the results of the present study suggest that the elevated concentration of 9,13-di-cis-RA in the plasma of dairy cows during the immediate postpartum period minimally affects the reactivity and phenotype of circulating mononuclear leukocytes. Conceivably, metabolic events leading to the increased concentrations of 9,13-di-cis-RA in the plasma of the dam may influence immune function by mechanisms not directly associated with 9,13-di-cis-RA. Future studies will investigate this possibility.

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REFERENCES

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