Effect of Estradiol and Progesterone on Lymphocyte and Neutrophil Functions in Steers

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Polymorphonuclear leukocyte function and lymphocyte blastogenesis in response to mitogens were evaluated in castrated male cattle after the repeated administration of estradiol or progesterone. Polymorphonuclear leukocyte function was evaluated with the following five parameters: (i) random migration under agarose, (ii) ingestion of 125I-labeled Staphylococcus aureus, (iii) nitroblue tetrazolium reduction, (iv) iodination, and (v) antibody-dependent cell-mediated cytotoxicity. The administration of high dosages of estradiol cypionate produced no measurable effect on the total or differential leukocyte count, neutrophil function, lymphocyte blastogenesis, or blood cortisol levels. The administration of high dosages of progesterone caused a significant enhancement of random migration by neutrophils and a depression of the activity of the myeloperoxidase-H2O2-halide antibacterial system (iodination) of the neutrophil. Progesterone administration did not cause a measurable effect on the lymphocyte blastogenic response to mitogens or the ability of polymorphonuclear leukocytes to ingest S. aureus, reduce nitroblue tetrazolium, or mediate antibody-dependent cell-mediated cytotoxicity. Progesterone did not cause a change in blood cortisol concentrations; therefore, the observed effects on polymorphonuclear leukocyte function were not due to alterations in blood cortisol concentrations. Impairment of the iodination reaction indicates that high dosages of progesterone interfere with an important bactericidal mechanism of the neutrophil.

The effect of estrogens and progesterone on the immune system is not well understood. High concentrations of estradiol in vitro have been reported to modify the activity of the myeloperoxidase-mediated microbicidal system of the neutrophil (7). High concentrations of progesterone in vitro have been reported to labelize neutrophil lysosomal membranes (8), and both estrogens and progesterone in vitro have been reported to inhibit the oxidative metabolism of the neutrophil (1) and to inhibit lymphocyte reactivity in the mixed lymphocyte reaction and the reaction to nonspecific mitogens (3, 13). This previous work was performed by treating isolated leukocytes in vitro with high concentrations of the steroid hormones. The effect of physiological or pharmacological levels of these hormones in vivo on neutrophil and lymphocyte function is not clear.

The purpose of the present experimentation was to determine whether alterations in neutrophil function or lymphocyte responsiveness to mitogens could be detected after the administration of high dosages of estradiol or progesterone to cattle. Castrated male cattle were used to avoid interference from physiological levels of estradiol, progesterone, and testosterone found in intact female or male animals.

MATERIALS AND METHODS

Animals and hormonal treatment. Ten head of apparently healthy 12- to 16-month-old Holstein-Friesian and Brown Swiss steers, weighing 400 to 600 kg each, were used. They were serologically negative for infectious bovine rhinotracheitis and bovine viral diarrhea virus antibody. The estradiol and progesterone experiments were conducted separately, using the same animals. The animals were rested for 4 days between experiments. For each experiment, the 10 animals were housed together; 5 of the animals were randomly assigned to a control group, and 5 were assigned to a hormone treatment group. Estradiol-treated cattle were injected intramuscularly with 30 mg of estradiol cypionate (The Upjohn Co., Kalamazoo, Mich.) at 0700 h and 20 mg of estradiol cypionate at 1600 h daily for 4 days. Progesterone-treated cattle were injected intramuscularly with 10 ml of a solution containing 750 mg of progesterone (Sigma Chemical Co., St. Louis, Mo.) in ethanol-propylene glycol (50:50) at 0700 h and 1600 h daily for 4 days. The control animals in the progesterone experiment received similar injections containing vehicle only. Blood for evaluation of lymphocyte and neutrophil function was collected at 0900 h daily for 3 days beginning 26 h after the first injection of hormone.
Leukocyte preparation. Mononuclear cells were isolated by a modification of the procedure of Böyum (2). A total of 20 ml of blood was collected aseptically into 2 ml of acid–citrate dextrose solution. This anticoagulated blood was diluted with 20 ml of sterile 0.015 M phosphate-buffered saline solution, pH 7.2, and layered over a column of 1.077 (specific gravity) Ficol-Hypaque (Histopaque-1077; Sigma) in a screw cap (25 by 150 mm) siliconized test tube and centrifuged at 500 × g for 45 min. The lymphocytes were removed from the Ficol-Hypaque plasma interface, washed in Hanks balanced salt solution, and counted.

Polymorphonuclear leukocytes (PMNs) were isolated from the peripheral blood as previously described (9). Briefly, the anticoagulated blood was centrifuged, the plasma and buffy coat layer were discarded, and the erythrocytes were removed by hypotonic lysis with distilled water to yield a cell preparation of generally greater than 90% pure PMNs. The cells were adjusted to a concentration of 5.0 × 10⁷ PMNs per ml in phosphate-buffered saline solution (pH 7.2) for use.

Lymphocyte blastogenesis. Lymphocytes were cultured in microtiter plates (3596; Costar, Cambridge, Mass.) with 2.0 × 10⁵ mononuclear cells in 0.15 ml of culture medium per well. The culture medium consisted of Medium 199 with Earle salts (GIBCO Laboratories, Grand Island, N.Y.) containing 15% heat-inactivated fetal calf serum and 1% antibiotic-antimycotic solution (GIBCO Laboratories). Control and mitogen-stimulated cultures were assayed in triplicate wells. Mitogen-stimulated cultures received 25 μl of a dilution of mitogen (phytohemagglutinin-P; Difco Laboratories, Detroit, Mich.), concanavalin A (Miles Laboratories, Inc., Elkhart, Ind.), or pokeweed mitogen (GIBCO Laboratories) which had been predetermined to give optimal stimulation under identical culture conditions. The microtiter plates were incubated at 37°C in a humidified 5% CO₂ atmosphere. After 48 h, 0.25 μCi of [³H]thymidine (New England Nuclear Corp., Boston, Mass.) was added to each well. At 16 h later, the cultures were harvested onto glass fiber filters with an automated sample harvester (Flow Laboratories, Inc., Rockville, Md.). The harvested wells were placed in scintillation vials containing 10 ml of toluene-based cocktail (Metric-Pak 2a70; Research Products International Corp., Elk Grove Village, Ill.), and the counts per minute of radioactivity were determined in a liquid scintillation counter (Model 2425; Packard Instrument Co., Inc., Downers Grove, Ill.).

PMN function tests. The procedures for evaluating random migration under agarose (9), ingestion of ¹²⁵I-labeled Staphylococcus aureus (9), nitroblue tetrazolium (NBT) reduction (9), iodination (9), and antibody-dependent cell-mediated cytoxicity (ADCC) (10) by PMNs have been described in detail elsewhere. All PMN function tests were conducted in duplicate. Briefly, random migration under agarose was evaluated by using plastic petri plates which contained a layer of 0.8% agarose with 10% fetal calf serum and 1% antibiotic-antimycotic in minimum essential medium (GIBCO Laboratories). Wells (diameter, 2.0 mm) were punched in the agar and filled with a sample of the PMN suspension. The migration plates were placed in an incubator under a humidified 5% CO₂ atmosphere at 37°C. After 18 h, the plates were removed, and the area of PMN migration was determined and expressed in square millimeters. Heat-killed [¹²⁵I]iododeoxyuridine-labeled S. aureus was used to evaluate ingestion (9). Opsonized [¹²⁵I]S. aureus and PMNs were incubated together at 37°C with a bacteria-to-PMN ratio of 60:1. After 10 min, lysostaphin (Sigma) was added, and the incubation was continued for an additional 30 min. The PMNs were then washed twice in phosphate-buffered saline solution (pH 7.2) by centrifugation, and the amount of PMN-associated radioactivity was determined. The results are expressed as the percent of the [¹²⁵I]S. aureus which was ingested. The quantitative NBT reduction assay was performed by adding 5.0 × 10⁶ granulocytes to a suspension of opsonized zymosan in 1.0 ml of Earle balanced salt solution containing 0.4 mg of NBT (Sigma). After a 5.0-min incubation in a 37°C water bath, the reaction was stopped by the addition of 5.0 ml of cold N-ethylmaleimide (Sigma). The purple formazan formed by the reduction of NBT was extracted with pyridine, and the optical density at 480 nm was determined. The results are expressed as optical density units per 5.0 × 10⁶ PMNs per 5 min in 5.0 ml of pyridine. The standard reduction mixture for the determination of stimulated iodination contained 2.5 × 10⁶ PMNs, 0.05 μCi of ¹²⁵I (New England Nuclear), 40 nmol of NaI, and 0.5 mg of opsonized zymosan in 0.5 ml of Earle balanced salt solution. At 20 min after the PMNs were added, the reaction was stopped by adding 2.0 ml of cold 10% trichloroacetic acid. The resulting precipitate was washed once in 10% trichloroacetic acid, and the amount of radioactivity in the precipitate was determined in a gamma counter. The results are expressed as nanomoles of NaI per 10⁷ PMNs per hour. The ADCC assay was performed by utilizing [⁵¹Cr]-labeled chicken erythrocytes as target cells. The reaction mixture contained 2.5 × 10⁵ [⁵¹Cr]-labeled chicken erythrocytes and 2.5 × 10⁶ PMNs (effector-to-target cell ratio, 10:1) in 0.5 ml of Medium 199 containing 10% bovine anti-chicken erythrocyte serum. Triton X controls, antibody controls, and PMN controls were included. After a 3-h incubation at 37°C in a humidified 5% CO₂ atmosphere, the reaction tubes were centrifuged, and a sample of supernatant solution was removed for gamma counting. The results were expressed as percent of specific [⁵¹Cr] release.

Determination of serum cortisol concentration. Blood for the cortisol assay was allowed to clot for 4 to 6 h at 25°C. The serum was then removed and stored at −20°C until the cortisol assay was performed. Serum cortisol concentration was determined by a double antibody radioimmunoassay, using a commercial kit (Diagnostic Products Corp., Los Angeles, Calif.). The samples were assayed in duplicate, and all of the samples were processed concurrently. The minimal unit of detectability of the assay system was 5.0 ng/ml. For statistical evaluation, all values below this limit were assigned a value of 5.0 ng/ml.

Hematological studies. The total leukocyte count in heparinized blood obtained by jugular venipuncture was determined by electronic counting (Coulter Electronics Co., Inc., Hialeah, Fla.). Blood films for differential leukocyte counts were prepared and stained with Wright's stain, and 100 cells were counted. Because lymphocytes and monocytes cannot be accurately differentiated in bovine blood by this method (6), they were counted together and termed mononuclear cells.

Statistical analysis. The estradiol and progesterone
experiments were each conducted by using a group of five control and five hormone-treated animals. All 10 animals were bled each day for the 3-day course of the experiment. The leukocytes from a single day’s bleeding were all processed in parallel. A mean and standard error of the mean was determined for each parameter for each group from these three bleedings. An analysis of variance procedure was performed to determine the level of significance of any differences between the two groups within an experiment. Because there was significant day-to-day variability in test results, the data was blocked by day for the analysis of variance. Conservative degrees of freedom (1, 9) were used in the F test.

RESULTS

Effect of estradiol administration. The repeated administration of high dosages of estradiol cypionate to steers produced behavioral signs which are typical for estrus in female cattle. Estradiol administration had no significant effect on the total or differential leukocyte count (Table 1), lymphocyte blastogenesis (Table 2), or any of the parameters of neutrophil function which were evaluated (Table 3). The serum cortisol concentration for the control group in the estradiol experiment was lower than the cortisol concentration for any of the other groups (Table 1). The reason for this difference is not known and was not statistically significant.

Effect of progesterone administration. The repeated administration of progesterone had no effect on total or differential leukocyte numbers (Table 1), lymphocyte blastogenesis (Table 2), or serum cortisol levels (Table 1). Progesterone administration did have an effect on certain neutrophil function parameters. Random migration under agarose by neutrophils was significantly enhanced in the progesterone-treated cattle ($P < 0.05$). Neutrophils from the control animals had a mean area of migration of 68.2 mm$^2$, whereas neutrophils from the progesterone-treated cattle had a mean area of migration of 80.0 mm$^2$ (Table 3). Neutrophils from the progesterone-treated animals had significantly decreased iodination (37.5 nmol of NaI per 10$^7$ PMNs per h) as compared with the control animals (45.1 nmol of NaI per 10$^7$ PMNs per h; $P < 0.05$ [Table 3]). There was no significant difference in the ability of neutrophils from control and progesterone-treated animals to ingest S. aureus, reduce NBT, or mediate ADCC (Table 3).

DISCUSSION

The repeated administration of high dosages of estradiol to steers failed to produce any measurable effect on lymphocyte blastogenesis or neutrophil function. The methods which were employed were sufficiently sensitive to detect alterations in neutrophil and lymphocyte function after the administration of adrenocorticotropic hormone (11a) or dexamethasone (11) to cattle. The actual blood level of estradiol in the estradiol-treated animals is not known, but it was apparently quite high. The recommended therapeutic dosage of estradiol cypionate in cattle is 3 to 10 mg per adult animal, with a repeat injection in 1 week if necessary (17). The steers in this study received a total of 50 mg of estradiol cypionate per day for 4 days. The signs of behavioral estrus which were observed in the steers indicate that the hormone was being absorbed into the bloodstream. There are reports that concentrations of estradiol which are greater than 10,000 times the physiological concentration will inhibit the oxidative metabolism (1) and the myeloperoxidase-H$_2$O$_2$-halide antibacterial system (7) of the human neutrophil in vitro and that estradiol concentrations of greater than 500 times the physiological concentration will inhibit the lymphocyte blastogenic response to mitogens in vitro (13). Schiff et al. concluded that estradiol has the potential to affect neutrophil

| TABLE 1. Effect of estradiol or progesterone administration on the total and differential leukocyte count and serum cortisol levels in steers$^a$

<table>
<thead>
<tr>
<th>Expt and treatment$^b$</th>
<th>No. of following type of blood cell per mm$^3$:</th>
<th>Serum cortisol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leukocytes</td>
<td>Mononuclear</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>9,200 ± 600</td>
<td>5,800 ± 400</td>
</tr>
<tr>
<td>Estradiol</td>
<td>8,000 ± 600</td>
<td>4,900 ± 300</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>9,600 ± 700</td>
<td>5,500 ± 400</td>
</tr>
<tr>
<td>Progesterone</td>
<td>9,200 ± 300</td>
<td>5,300 ± 200</td>
</tr>
</tbody>
</table>

$^a$ Numbers represent the mean ± standard error of the mean. There were no statistically significant differences between the treated and control groups within an experiment. The $n$ values represent repeated evaluation of five animals per group.

$^b$ In experiment 1, $n = 10$ for both groups; in experiment 2, $n = 15$ for both groups.
function and lymphocyte blastogenesis in vivo. However, since hormone levels this high are probably never attained in vivo, the relevance of this data to the in vivo effect of estradiol is uncertain. The NBT reduction assay which we performed evaluates an aspect of the oxidative metabolism of the neutrophil, and the iodination reaction evaluates the activity of the myeloperoxidase-H₂O₂-halide antibacterial system (9). Our results indicate that even suprapharmacological levels of estradiol in vivo failed to affect neutrophil function or lymphocyte blastogenesis.

Progesterone was used at a very high dosage in this experimentation to maximize any potential effects. The recommended therapeutic dosage of progesterone in cattle is 500 to 1,000 mg per adult animal repeated in 10 days if necessary (17). The progesterone-treated animals in this experiment received 1,500 mg per day for 4 days. Unlike estradiol, progesterone did have a significant effect on certain aspects of neutrophil function (Table 3). There was a significant enhancement of random migration and inhibition of iodination but no significant effect on the ability of neutrophils to ingest S. aureus, reduce NBT, or mediate ADCC. The administration of adrenocorticotrophic hormone to steers produced a nearly identical effect on neutrophil function (11a). This finding suggests that the two steroid hormones, progesterone and cortisol, may influence neutrophil function through a similar mechanism. Synthetic progestins have been reported to have weak glucocorticoid-like activity in the rat (5). Our results indicate that progesterone also has cortisol-like activity in cattle.

The administration of dexamethasone (11), adrenocorticotrophic hormone (11a), or progesterone to cattle will result in an increase in random migration by neutrophils. Stevenson and co-workers (15, 16) have reported that the increase in random migration by neutrophils which is associated with corticosteroid administration in humans is indirect and is due to corticosteroids causing mononuclear phagocytes to release a polymorphonuclear-stimulat-

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**TABLE 2. Effect of estradiol or progesterone administration on lymphocyte blastogenesis**

| Expt and treatment | Δcpm (SI)² for following type of mitogen: |  |
|--------------------|------------------------------------------|  |
|                    | Phytohemagglutinin | Concanavalin A | Pokeweed |
| 1 | None (control) | 32,800 ± 8,000 (116.3 ± 34.6) | 41,000 ± 8,000 (137.2 ± 28.1) | 23,200 ± 3,500 (79.2 ± 12.5) |
| Estradiol | 27,500 ± 7,400 (84.6 ± 13.0) | 44,000 ± 7,700 (138.9 ± 15.0) | 25,500 ± 2,500 (88.6 ± 9.8) |
| 2 | None (control) | 32,500 ± 6,600 (88.4 ± 20.1) | 37,600 ± 6,200 (111.8 ± 21.6) | 13,100 ± 3,000 (31.4 ± 6.0) |
| Progesterone | 36,100 ± 5,900 (95.5 ± 15.3) | 40,300 ± 5,600 (117.0 ± 20.1) | 12,200 ± 2,100 (33.0 ± 5.6) |

² Numbers represent the mean ± standard error of the mean. There were no statistically significant differences between the treated and control groups within an experiment. The n values represent repeated evaluation of five animals per group.

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**TABLE 3. Effect of estradiol or progesterone administration on neutrophil function in steers**

<table>
<thead>
<tr>
<th>Expt and treatment</th>
<th>Random migration (mm²)</th>
<th>S. aureus ingestion (%)</th>
<th>NBT reduction (OD)²</th>
<th>Iodination (nmol of Nai per 10⁵ PMNs per h)</th>
<th>ADCC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>55.5 ± 2.4</td>
<td>40.9 ± 3.2</td>
<td>0.32 ± 0.04</td>
<td>31.8 ± 2.1</td>
</tr>
<tr>
<td>Estradiol</td>
<td>53.7 ± 3.1</td>
<td>44.8 ± 3.1</td>
<td>0.32 ± 0.04</td>
<td>34.0 ± 2.6</td>
<td>94.6 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>None (control)</td>
<td>68.2 ± 3.3</td>
<td>58.5 ± 4.9</td>
<td>0.34 ± 0.03</td>
<td>45.1 ± 2.6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>80.0 ± 2.3d</td>
<td>55.0 ± 4.5</td>
<td>0.32 ± 0.02</td>
<td>37.5 ± 1.4d</td>
<td>85.6 ± 3.7</td>
</tr>
</tbody>
</table>

² Numbers represent the mean ± standard error of the mean. The n values represent repeated evaluation of five animals per group.

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a In experiment 1, n = 10 for both groups; in experiment 2, n = 15 for both groups.

b Significant difference from control group value (P < 0.05).
ing factor. This factor may act by inhibiting microtubule formation within the neutrophil. Agents which inhibit microtubule function will cause an enhancement of random migration (16) and an inhibition of degranulation (4) in neutrophils. The iodination reaction is dependent upon degranulation to release myeloperoxidase from the lysosomes. An inhibition of degranulation would result in a lower iodination value. Therefore, the observed increase in random migration and decrease in iodination may both be due to an inhibition of microtubule formation within the neutrophil. Progesterone and cortisol may have similar mechanisms for inhibiting microtubule formation. The effects on neutrophil function observed after the administration of progesterone were not due to elevated plasma cortisol levels in the progesterone-treated cattle. There was no significant difference in serum cortisol levels between the two groups of cattle (Table 1). In addition, progesterone had no significant effect on the number of neutrophils or eosinophils in the peripheral blood (Table 1). Increased blood cortisol levels will characteristically cause a neutrophilia and eosinopenia in cattle (12).

The administration of progesterone to steers had no significant effect on the lymphocyte blastogenic response to mitogens. In this respect, the effect of progesterone administration differed from the effect of adrenocorticotropic hormone administration to cattle. Adrenocorticotropic hormone administration caused an inhibition of lymphocyte blastogenesis in response to phytohemagglutinin and concanavalin A (11a). High concentrations of progesterone in vitro have been reported to inhibit human lymphocyte blastogenesis in the mixed lymphocyte reaction and in response to mitogens; physiological levels of progesterone, however, did not have any effect (3, 13). It has been postulated that the ability of progesterone in high concentrations to inhibit lymphocyte reactivity is very important for the maintenance of pregnancy (3). In most species, the trophoblast cells of the placenta produce progesterone, resulting in a high local concentration of progesterone at the maternal-fetal interface. It is postulated that the progesterone concentration in this area is high enough to inhibit lymphocyte reactivity to foreign antigens, but the substantially lower progesterone concentration in the peripheral blood will not suppress lymphocyte reactivity. We do not know the serum concentration of progesterone achieved in our study or how this relates to the local concentration of progesterone at the maternal-fetal interface, but our results indicate that suprapharmacological doses of progesterone do not inhibit lymphocyte reactivity to mitogens in steers.

It cannot be assumed that estradiol and progesterone will have the same effect or lack of effect on neutrophil and lymphocyte function in cows as that observed in steers. In this experimentation, we used steers to determine whether any effect could be observed when high dosages of hormone were used in animals that did not have significant background levels of estradiol, progesterone, or testosterone. Several points need to be considered when attempting to extrapolate these data to the effects of physiological concentrations of estradiol and progesterone in the cow. One important consideration is that the concentration and binding affinity of estradiol and progesterone receptors in the tissues of steers may be quite different from that in cows. The fact that estradiol caused behavioral signs of estrus in these steers and progesterone had certain effects on neutrophil function indicates that there were at least some hormone receptors present in the tissues of these castrated male animals. We have made the assumption that sufficient hormone was present to saturate the intracellular receptors, because we administered a very high dosage of hormone in a formulation and by a route of administration recommended for therapeutic use in cattle. Another point to consider is that estradiol and progesterone are present concurrently in cow blood in varying concentrations (14), whereas in the steers in this study, presumably only one of these hormones was present in significant concentration. The ratio of the two hormones may be an important determinant of the effect on the immune system.

In conclusion, high dosages of estradiol and progesterone, when administered in vivo, did not produce the effects on immune function which were predicted based on experimentation in which extremely high concentrations of hormone in vitro were used (1, 3, 7, 8, 13). That is, high dosages of estradiol and progesterone had no detectable effect on lymphocyte function in steers, and estradiol had no detectable effect on neutrophil function. The only alterations of immune function which were observed were the enhancement of neutrophil random migration and impairment of neutrophil iodination associated with progesterone administration. Even though these effects of progesterone on neutrophil function were not dramatic, they should not be minimized because a conservative statistical approach found them to be significant ($P < 0.05$), both the enhancement of random migration and the inhibition of iodination could be explained on the basis of an inhibition of microtubule function, and adrenocorticotropic hormone administration to cattle produced the same effects on neutrophil function as did progesterone, thus suggesting that both cortisol and progesterone may alter neutrophil function through a similar mechanism.
ACKNOWLEDGMENTS

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LITERATURE CITED