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Estradiol-17 β and Progesterone Increase Ovine Uterine Suppressor Cell Activity^{1,2}

Edward C. Segerson³, Hong Li, and Charles W. Talbott

Department of Animal Science, North Carolina A&T State University, Greensboro 27411

ABSTRACT: We evaluated the regulation of ovine uterine (UT) suppressor cell activity by progesterone (P₄), estradiol-17 β (E₂), and P₄ + E₂ in ovariectomized (OVX) ewes. Following 14 d of steroid injections, endometrial cells (designated as UT cells) were recovered postmortem, and unfractionated and fractionated cells were assessed for suppression of autologous phytohemagglutinin (PHA)-treated peripheral blood lymphocytes (PBL). Supernatants from cultured UT cells were also assessed for suppressor activity. In other experiments, UT cells recovered from nontreated OVX ewes were cocultured with PHA-treated PBL and varying concentrations (1×10^{-11} to 1×10^{-5} M) of each steroid preparation. Supernatants from separate cultures that contained UT cells and steroids were evaluated for suppressor activity. Uter-

ine cells from control and steroid-treated ewes suppressed proliferative responses of PHA-treated PBL; however, suppressor activity of UT cells was greater ($P < .05$) for E₂ - treated than for control and P₄-treated ewes. Uterine suppressor cells from steroid-treated ewes sedimented in Percoll within a density range of 1.002 to 1.056 g/mL. Uterine cells from all ewes released suppressor factor(s) into the culture medium; however, the activity of the supernatant from the cultured cells was not increased for the steroid-treated ewes. For cocultures that contained steroids and cultures that contained supernatant, suppressor activity of the UT cells was increased by specific concentrations of each steroid preparation. These findings demonstrate that reproductive steroids augment ovine UT suppressor cell activity.

Key Words: Sheep, Immunosuppression, Uterus, Reproduction, Steroids

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Introduction

Uterine (UT) suppressor cell activity (i.e., suppression of specific lymphocyte responses) has been identified in mice (Slapsys and Clark, 1982; Clark et al., 1985), humans (Daya et al., 1985a,b), swine (Croy et al., 1987), and sheep (Segerson and Libby, 1984; Segerson and Gunsett, 1992). Lysis of preattachment ovine conceptuses by autologous lymphokine activated killer (LAK) cells (Segerson and Gunsett, 1994) suggests that UT suppressor cells may be requisite for maintenance of pregnancy in ruminant livestock.

Uterine mononuclear cells from estrous and d-14 cyclic ewes responded less to the mitogen phytohemag-

glutinin (PHA), than UT cells from ovariectomized (OVX) ewes (Segerson and Libby, 1984). Proliferative responses of PHA-treated peripheral blood lymphocytes (PBL) were suppressed to a greater degree by cocultured UT cells from d-14 cyclic than by those from OVX ewes (Segerson and Gunsett, 1992). In addition, UT cells from the cyclic ewes released a \geq 248-kDa soluble suppressor macromolecule into the culture medium (Segerson and Gunsett, 1992). These findings suggested that suppressor responses of UT cells were regulated, at least in part, by an ovarian component, presumably steroid hormones. Previously, Segerson and Gunsett (1992) reported that 14 d of progesterone (P₄) injections in OVX ewes failed to affect the suppressor activity of UT cells. This finding was considered preliminary and necessitated a more detailed investigation of the possible roles of P₄ and estradiol-17 β (E₂) in ovine UT suppressor cell function.

The primary objectives of this investigation were to determine whether injections of P₄ and E₂ in OVX ewes and the addition of these steroids to cultures of UT cells affected the activity of UT suppressor cells. The secondary objective involved the determination of the relative densities of UT suppressor cells.

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³To whom correspondence should be addressed.

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Experimental Procedures

Animals and Surgical Procedure. Nulliparous yearling ($n = 16$; 50.3 ± 1.7 kg BW [$\bar{x} \pm \text{SEM}$]) and multiparous ($n = 4$; 8 to 9 yr; 63.4 ± 3.7 kg BW) Suffolk-crossbred ewes were used for the investigation. Ewes were maintained in total confinement and fed alfalfa hay to meet nutritional requirements and were given ad libitum access to water. All procedures involving the ewes were conducted in compliance with university and federal guidelines. Before the breeding season, each ewe was ovariectomized using a midventral laparotomy procedure with xylazine + ketamine (100 mg/mL of each) anesthesia, which was administered through an indwelling jugular catheter. Feed and water were restricted for 24 h before laparotomy. Ewes were allowed to recover for 60 d before experimentation.

Uterine Suppressor Cell Activity of Steroid-Treated Ewes. Yearling ewes were allocated, according to weight, to one of the following treatment groups, resulting in four ewes/group: Control, P_4 , E_2 , and $P_4 + E_2$. The steroids (Sigma Chemical, St. Louis, MO) were initially dissolved in absolute ethanol (10% of final volume) followed by the addition of corn oil to yield P_4 and E_2 concentrations of 20 mg/mL and 12.5 $\mu\text{g/mL}$, respectively. The control preparation consisted of the vehicle. Ewes received i.m. injections of 1.0 mL at 0800 and 1400 daily for 13 d, followed by one injection on d 14 at 0800 of either the control preparation, P_4 (40 mg daily), E_2 (25 μg daily), or $P_4 + E_2$.

A 10-mL blood sample was collected from the jugular vein of each ewe at 0730 on d 1 and at 1000 on d 5, 10, and 14. After overnight incubation at 4°C, serum was separated by centrifugation and stored at -20°C until it was assayed for concentrations of P_4 and E_2 .

On d 14, a 50-mL blood sample was collected from each ewe and injected into a heparinized serum vial containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY) for separation of PBL for the proliferation experiments. The uterus was then removed postmortem and transported to the laboratory. Within 30 min, each uterus was flushed with 20 mL of DMEM at room temperature to recover uterine luminal secretions (ULS). Secretions were membrane-filtered (.45 μm), dialyzed (1,000 molecular weight cutoff) and vacuum-concentrated to approximately 1.0 mL, estimated for protein content (Lowry et al., 1951), and stored at -20°C until needed. A sample of DMEM (20 mL) was dialyzed separately and considered a control sample.

Using aseptic conditions, endometrial tissue from both uterine horns was removed and cut into fragments (< 3 mm) within sterile Petri dishes (Corning 100 mm \times 20 mm; Fisher Scientific, Norcross, GA) that contained 10 mL of DMEM. The fragments were pushed gently through sterile stainless steel sieves

(120- followed by a 90- μm sieve) using a rubber policeman and medium. Dissociated cells, designated as UT cells, were washed twice and resuspended in RPMI-1640 (GIBCO) containing 10% fetal bovine serum (vol/vol), 2% (vol/vol) extra 200 mM glutamine, 100 IU of penicillin, and 100 μg of streptomycin/mL. An aliquot was removed and diluted to 1×10^6 cells/mL, and the remaining suspension was centrifuged and the pellet was resuspended in 2.0 mL of DMEM for Percoll fractionation. Collagenase pretreatment of endometrial cell fragments was not done before sieving because it was ineffective in enhancing cellular dissociation during the sieving process in another investigation (Segerson, unpublished data).

Dissociated cells from P_4 -, E_2 -, and $P_4 + E_2$ -treated ewes were fractionated with a discontinuous Percoll (Pharmacia LKB Biotechnology; Uppsala, Sweden) gradient, as described by Gutierrez et al. (1979) and Kurnick et al. (1979). Control ewes did not provide enough UT cells for fractionation. Because UT suppressor cells were apparent for control- and steroid-treated ewes, we reasoned that cells from the control ewes were not essential for this component of the investigation. Percoll was made iso-osmotic with 1.5 M PBS (pH 7.4), and it was diluted with H_2O to formulate solutions ranging from 5 to 70% Percoll. Each percentage solution (1.5 mL) was layered into a 17- \times 100-mm polypropylene culture tube beginning with the 70% solution. Each endometrial cell suspension was layered on top of the gradient (Figure 1). With one exception, fraction numbers were assigned to each percentage solution. Fraction 8 represented the cellular pellet. The gradient was centrifuged, without braking, at room temperature for 10 min at $1,898 \times g$. Beginning with fraction 1, each fraction was removed and placed into a separate tube. Incidentally, epithelial cells sedimented in a distinct interface between the 5 and 10% layers (fractions 1 and 2, respectively). The majority of these cells were dead, and the cellular layer was removed with a Pasteur pipet and discarded. Cells within each fraction were washed twice and observed microscopically for relative sizes. To ensure adequate numbers of fractionated cells for the lymphocyte proliferation experiments, cells within Percoll fractions 1 to 5 and 6 to 8 were combined for each ewe because previous data for d-14 cyclic and pregnant ewes (Segerson, unpublished data) revealed similarities in suppressor activities and sizes of cells among fractions within these two groups. Cells within each group of combined fractions were diluted to 1×10^6 cells/mL with RPMI-1640. Colored marker beads of known densities (measured in grams/milliliter) were used to compute a linear regression equation. The beads were suspended together and fractionated using procedures identical to those described for the UT cells. Sedimentation distances, measured in millimeters from the base of a tube, and densities of the marker beads were used for the equation $y = b_0 - b_1(x)$, where y = sedimentation

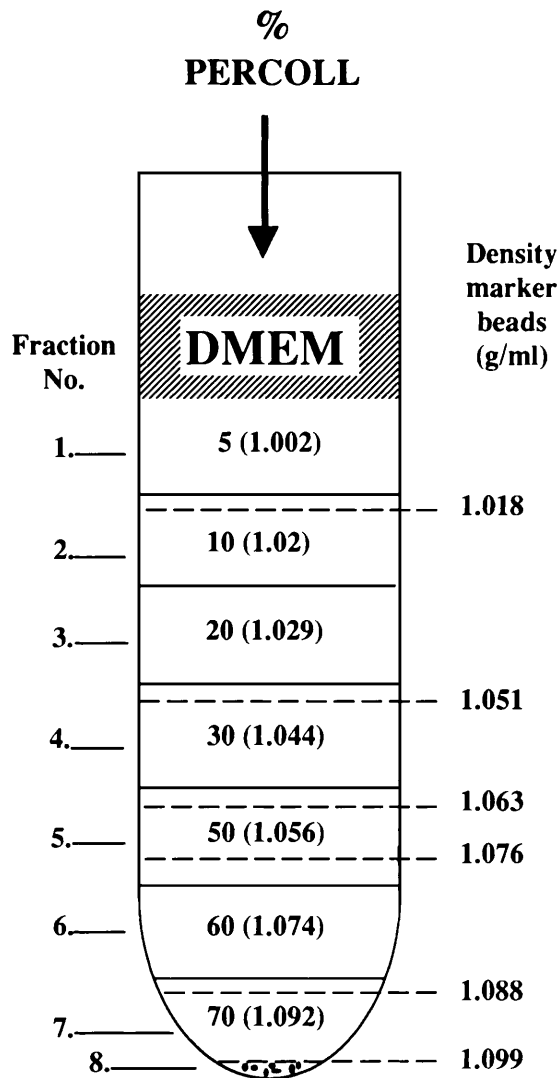


Figure 1. Endometrial cells from steroid-treated, ovariectomized ewes were fractionated with a discontinuous Percoll (5 to 70%) gradient. With one exception, each percentage solution is designated with a fraction number. Fraction 8 represents the cellular pellet. Broken lines represent the sedimentation of colored marker beads of known densities that were used to calculate a regression equation for the gradient. Numbers in parentheses represent the densities (grams/milliliter) of the specific Percoll fractions.

distance, b_0 = the intercept, b_1 = the slope, and x = density. The computed equation follows: $y = 693.6 - 630.9(x)$, $R^2 = .97$ ($P < .001$). The midpoint of each fraction was used to compute the relative density of cells recovered from the fraction (Figure 1).

Peripheral blood lymphocytes were extracted from blood samples with Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), using the procedures of Segerson and Libby (1984) and Segerson and Gunsett (1992). In addition, erythrocytes were lysed with .83% NH_4Cl . The PBL were diluted to 1×10^6 cells/mL and estimated for viability (consistently > 97%

viable) using trypan blue dye exclusion. Within the lymphocyte proliferation experiments, each treatment contained an additional culture well that was used to estimate the viability of PBL at the end of the culture period.

Procedures for the PBL and UT cell coculture experiments were similar to those of Segerson and Gunsett (1992). Briefly, numbers of autologous PBL ($1 \times 10^5/1$ mL) and unfractionated and fractionated (Percoll fractions 1 to 5 and 6 to 8) UT cells (1.0×10^5 , 5.0×10^4 , and $2.5 \times 10^4/1$ mL) from respective ewes were each added to individual culture wells, in quadruplicate, of 96-well plates with and without PHA ($.08 \mu\text{g}/1 \times 10^5$ cells; Wellcome Research Labs, Beckenham, U.K.). Coculture wells received, in quadruplicate, PHA and PBL (1×10^5) with either 1.0×10^5 , 5.0×10^4 , or 2.5×10^4 unfractionated and fractionated UT cells (ratios of 1:1, 1:5, and 1:25, respectively). At 48 h of culture, $.1 \mu\text{Ci}$ of [^3H] thymidine (TdR; methyl, 6.7 Ci/mmol; DuPont NEN Research Products, Boston, MA) was added in .05 mL, in triplicate, to the cultures. Cells were harvested (using filter mats) with trichloroacetic acid 18 to 20 h later and assessed for the incorporation of TdR. Values for percentages of suppression were calculated by first subtracting background (i.e., cultures without PHA) incorporation from values that represented PHA-treated cultures and then dividing mean incorporation for the sum of the values that represented each cellular source and number minus the value for ratios of cocultured cells by the sum of the values for each cellular source and number and multiplying by 100.

Parallel experiments were conducted to determine whether unfractionated UT cells released a soluble suppressor factor(s) into the culture medium. Uterine cells ($1 \times 10^6/\text{mL}$) were cultured, in duplicate, in 30-well plates. At 68 to 72 h of culture, each cellular suspension (1.0- mL volume) was removed and centrifuged, and the supernatant was membrane-filtered ($.2 \mu\text{m}$). Volumes (10 to 50 μL) for each sample of supernatant were added to cultures of 1×10^5 PHA-treated PBL (collected from a donor ewe), and excluding calculations, the remaining procedures were identical to those previously described. Following subtraction of background incorporation from PHA-treated cultures, percentages of suppression were calculated by dividing the value for mean incorporation of TdR for the control (i.e., PHA-treated PBL) culture minus the value for cultures containing supernatant by the value for the control culture and multiplying by 100.

Uterine luminal secretions and DMEM (control sample) were tested for suppression of lymphocyte proliferative responses. Because of unequal sample volumes following dialysis, volume equivalents were calculated to standardize the volume (based on a 1.0-mL volume) of each ULS sample and DMEM with respect to apparent concentrations of materials. Volume equivalents (equal to the number of

microliters needed for 10-, 25-, or 50- μ L treatment volumes \times the volume of each dialyzed sample/1.0 mL) and protein (25 μ g) for each sample were cultured with 1×10^5 PHA-treated PBL. The remaining procedures have been described.

Concentrations of P_4 and E_2 within serum were quantified using RIA kits (Diagnostic Products, Los Angeles, CA). Serum E_2 was extracted with ethyl ether before assay, whereas the measurement of serum P_4 did not require an extraction procedure. The P_4 and E_2 assays were validated following the recovery of added amounts of P_4 and E_2 , respectively, to pooled serum samples from OVX ewes ($n = 4$). Mean percentages of recovery of added P_4 and E_2 were 105.5 and 85.5, respectively, and the lower limits of sensitivity for each assay were .1 ng/mL and 2.5 pg/mL, respectively. Inter- and intraassay CV for P_4 were 7.3 and 9.2%, respectively, and the CV for E_2 were 10.6 and 11.2%, respectively.

Suppressor Activity of Uterine Cells Treated with Steroids. Nonsteroid-treated multiparous OVX ewes were used for these experiments because they provided a greater number of UT cells than yearling ewes. With one exception, the procedures for dissociation of UT cells were identical to those previously described. To minimize concentrations of P_4 and E_2 within RPMI-1640, fetal bovine serum was replaced with steer serum. Stock solutions (1×10^{-3} M) of P_4 , E_2 , and $P_4 + E_2$, were prepared in absolute ethanol. A 1.0-mL aliquot was diluted to 25 mL with RPMI-1640, and further dilutions were made at .1 decrements to yield experimental solutions ranging from 4×10^{-5} to 4×10^{-11} M. The addition of .05 mL of each steroid solution to cocultures that contained PHA-treated PBL (1×10^5 /.1 mL) and unfractionated UT cells (1×10^5 /.05 mL; PBL:UT cell ratio of 1:1, respectively) resulted in final steroid concentrations for the majority of the culture period of 1×10^{-11} to 1×10^{-5} M. Steroid solutions of 1×10^{-5} M contained 1% ethanol, whereas ethanol was diluted at .1 decrements for steroid solutions ranging from 1×10^{-6} to 1×10^{-11} M. For these experiments, .025 mL of TdR was added to the cultures.

In parallel experiments, unfractionated UT cells (5×10^5 /.375 mL) were added to individual wells of 30-well plates with .125 mL of either control medium or solutions of P_4 , E_2 , and $P_4 + E_2$ to produce final concentrations of steroids that ranged from zero (control) to 1×10^{-5} M. At approximately 72 h of culture, cellular suspensions were removed and centrifuged, and the supernatants were membrane-filtered. Volumes (10 and 25 μ L) of each supernatant were added to cultures of PHA-treated PBL (1×10^5), and the remaining procedures were identical to those previously described.

Preliminary experiments ($n = 3$) were conducted to determine whether 1×10^{-11} to 1×10^{-5} M of each steroid preparation or ethanol (1 and .1 % solutions in culture) affected proliferative responses of PHA-treated PBL. Values for percentages of suppression of

TdR incorporation for PHA-treated PBL cultures that contained steroids were subtracted from percentage values for UT cellular cocultures and supernatant cultures to remove any effects of the steroids on PBL.

Statistical Analysis. Percentages of suppression and viability data were subjected to arcsin transformation (SAS, 1992) before analysis. Before transformation, percentage of suppression values < 0 and > 100 were assigned values of 0 and 100, respectively. Data from all experiments were analyzed by least squares ANOVA using the mixed model procedure of SAS (1992) with animal as a random effect. Between-animal variation was accounted for by the restricted maximum likelihood (REML) procedure. Differences ($P < .05$) among mean values for fixed effects (i.e., type of fraction, steroid treatment and concentration, cellular coculture ratio, supernatant volume, and appropriate interactions) were determined by confidence limits. Percentage data were back-transformed for presentation.

Results

Uterine Suppressor Cell Activity of Steroid-Treated Ewes. Serum concentrations of P_4 and E_2 were increased ($P < .001$) by d 5 in steroid-injected ewes, and these concentrations remained constant throughout the remainder of the treatment period (Figure 2).

The incorporation of TdR into PBL was 12 times greater for cultures that contained PHA. Mean (\pm SEM) counts per minute for cultures that contained PBL and PHA-treated PBL were 321.1 ± 39.5 and $3,924 \pm 405.5$, respectively. Suppressor activity was evident for cocultures of unfractionated UT cells for ewes in all treatment groups, and the mean percentage of suppression value for the combined groups was greater ($P < .05$) at the 1:1 than at the 1:25 ratio (67.0 and 34.7, respectively, Figure 3). At the 1:1 ratio, the activity was greater ($P < .05$) for UT cells from E_2 -treated than from control and P_4 -treated ewes and tended ($P < .1$) to be greater for cells from E_2 - than from $P_4 + E_2$ -treated ewes (Figure 3). A similar treatment pattern was observed at the 1:5 ratio, although there was no trend for greater activity of UT cells from E_2 -treated than from $P_4 + E_2$ -treated ewes. Viability of PBL was unaffected by treatment at these ratios and the mean estimate combined for cocultures of UT cells for control and steroid-treated ewes was $53.4 \pm 1.8\%$ viable cells. Suppression was attributed to UT cells recovered from Percoll fractions 1 to 5 (Figure 3) that sedimented within a density range of 1.002 to 1.056 g/mL, as shown in Figure 1. The predominant cells within these fractions were considered small cells (≤ 5.2 μ m in diameter). At the 1:1 ratio, the mean percentage of suppression value for the steroid treatments combined was greater ($P < .05$) for the small cells than the corresponding combined value for cells recovered from Percoll fractions 6 to 8, with densities > 1.056 g/mL (Figure 1). Although data

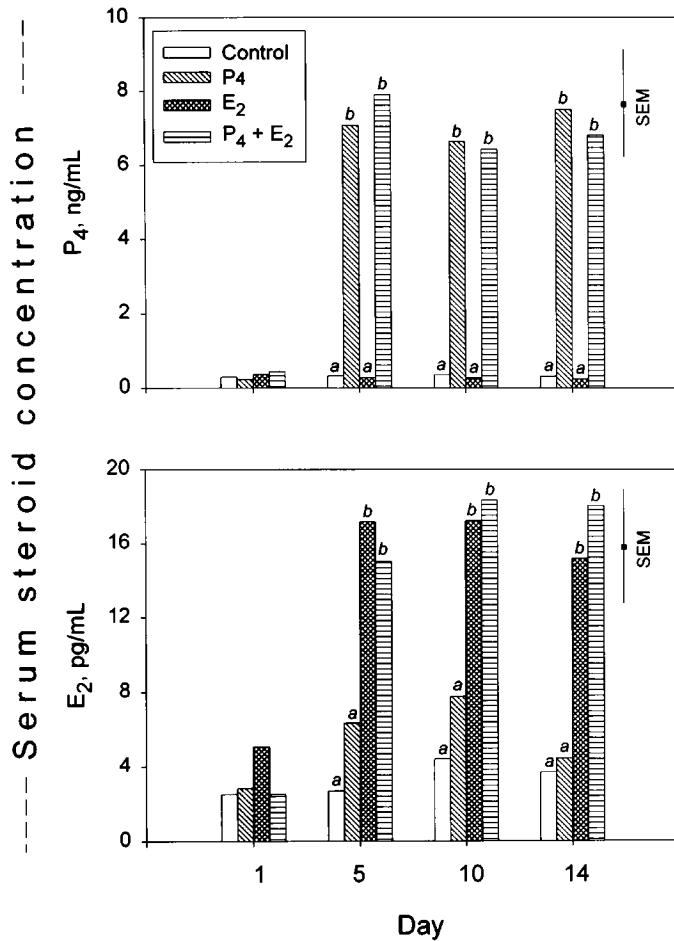


Figure 2. Serum concentrations of progesterone (P₄) and estradiol-17 β (E₂) are presented for specific days of an injection regimen for control and steroid-treated, ovariectomized ewes. Mean values among treatments within day with no superscript in common are different ($P < .05$).

were unavailable for fractionated UT cells from control ewes, it is likely that suppressor responses of fractionated cells from these ewes would have been similar to those of the P₄- and P₄ + E₂-treated ewes because of their similar responses for the unfractionated cells. Suppression of PHA-treated PBL tended ($P < .1$) to be greater for cocultured UT cells from Percoll fractions 1 to 5 for E₂-than for P₄-treated ewes.

Unfractionated UT cells from ewes in all treatments released a soluble suppressor factor(s) into the culture medium. The activity of the factor(s) within the supernatant cultures was dependent only on the volume of supernatant added to the cultures (Figure 4). For treatments combined, percentage of suppression values increased ($P < .05$) in a volume-dependent manner (27.4, 44.8, and 66.1% for volumes of 10, 25, and 50 μ L, respectively). Viability of PBL was not affected by either volume or treatment, and the estimate for volumes and treatments combined was $67.9 \pm 2.1\%$ viable cells.

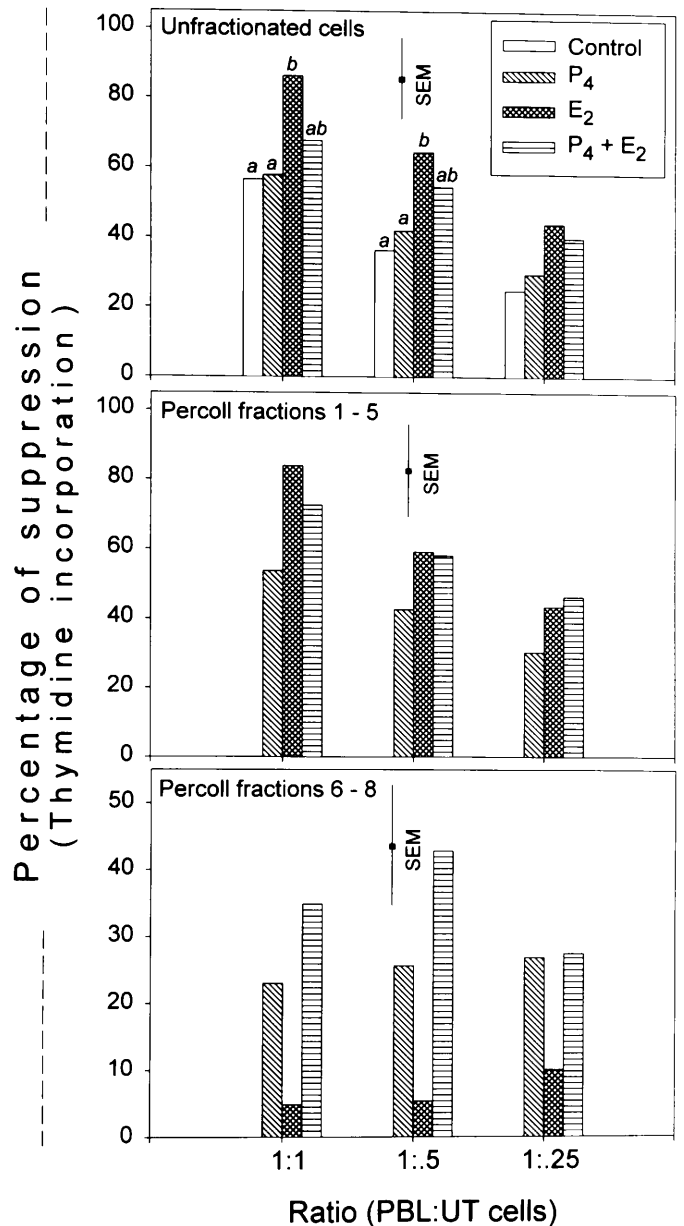


Figure 3. Thymidine incorporation data are presented for phytohemagglutinin-treated cocultures containing peripheral blood lymphocytes (PBL; 1×10^5) with either 1×10^5 , 5×10^4 , or 2.5×10^4 unfractionated or fractionated (combined Percoll fractions 1 to 5 and 6 to 8) uterine (UT) cells (PBL:UT ratios of 1:1, 1:5, and 1:25, respectively) recovered from control and progesterone (P₄)-, estradiol-17 β (E₂)-, and P₄ + E₂-treated, ovariectomized ewes. Treatment differences were apparent for unfractionated UT cells. Mean values among treatments within ratio with no superscript in common are different ($P < .05$).

Uterine luminal secretions from control ewes failed to suppress the incorporation of TdR of PHA-treated PBL, whereas suppression was evident and comparable among steroid treatments (Figure 5). For steroid treatments combined, suppression was greater ($P <$

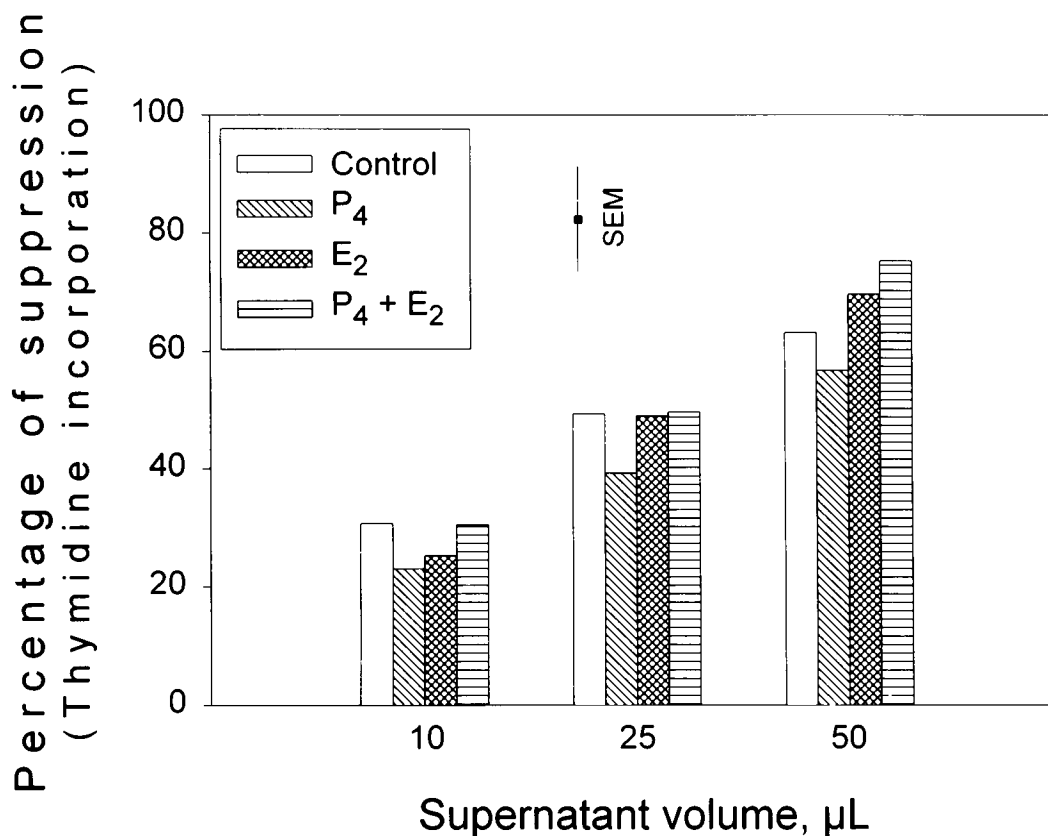


Figure 4. Thymidine incorporation data are presented for phytohemagglutinin-treated peripheral blood lymphocytes (1×10^5) cultured with supernatant from cultured uterine cells recovered from control and progesterone (P_4)-, estradiol- 17β (E_2)-, and $P_4 + E_2$ - treated, ovariectomized ewes. There were no differences for mean values among treatments within volumes; however, percentage of suppression values for treatments combined increased ($P < .05$) in a volume-dependent manner.

.05) for 50 than for 10 μL (88.5 and 48.7%, respectively) of ULS. The suppressor pattern for 25 μg of ULS protein (Figure 5) was similar to those observed for the volume data. Suppression was not mediated by apparent cytotoxicity of PBL, although the mean percentage of viable cells for combined cultures of ULS from E_2 - treated ewes (45.3) tended ($P < .1$) to be less than the percentages (range of 56.4 to 60.2) for the remaining treatment groups. The DMEM sample failed to suppress the incorporation of TdR.

Suppressor Activity of Uterine Cells Treated with Steroids. Thymidine incorporation of steroid-treated PBL was affected ($P < .03$) by experiment, steroid concentration, and treatment. Peripheral blood lymphocytes were stimulated ($P < .05$) by 1×10^{-5} M concentrations of each steroid. Increased TdR incorporation may have resulted, in part, from the 1% ethanol within this concentration of each steroid preparation because TdR incorporation was increased by 20% for these cultures. For solutions of steroids $< 1 \times 10^{-5}$ M, the volume of ethanol was considered negligible with respect to its effect on lymphocyte proliferation because the percentage of suppression value for

cultures that contained .1% ethanol was zero. The viability of PHA-treated PBL was unaffected by steroid treatment.

Uterine cells from four untreated OVX ewes suppressed ($P < .01$) the incorporation of TdR into cocultured PHA-treated PBL. Percentage of suppression values ranged from .7 to 52.7 ($31. \pm 5.9$; mean \pm SEM), and the mean was considered a control value. The subtraction of percentage values for steroid-treated PBL from values for steroid-treated cellular cocultures revealed that the incorporation of TdR was affected by steroid treatment ($P < .02$) and concentration ($P < .003$). For concentrations combined, the mean percentage of suppression value was greater ($P < .05$) for the $P_4 + E_2$ -treated than for the P_4 -treated coculture (54.1 and 31.7, respectively). In addition, values were greater ($P < .05$) than the control value for cocultures that contained 1×10^{-5} M of each steroid, whereas 1×10^{-10} M of $P_4 + E_2$ tended ($P < .1$) to increase suppressor responses of the UT cells (Figure 6).

For PHA-treated PBL cultures that contained 10 and 25 μL of supernatant (from nonsteroid-treated UT cell cultures), percentages of suppression values were

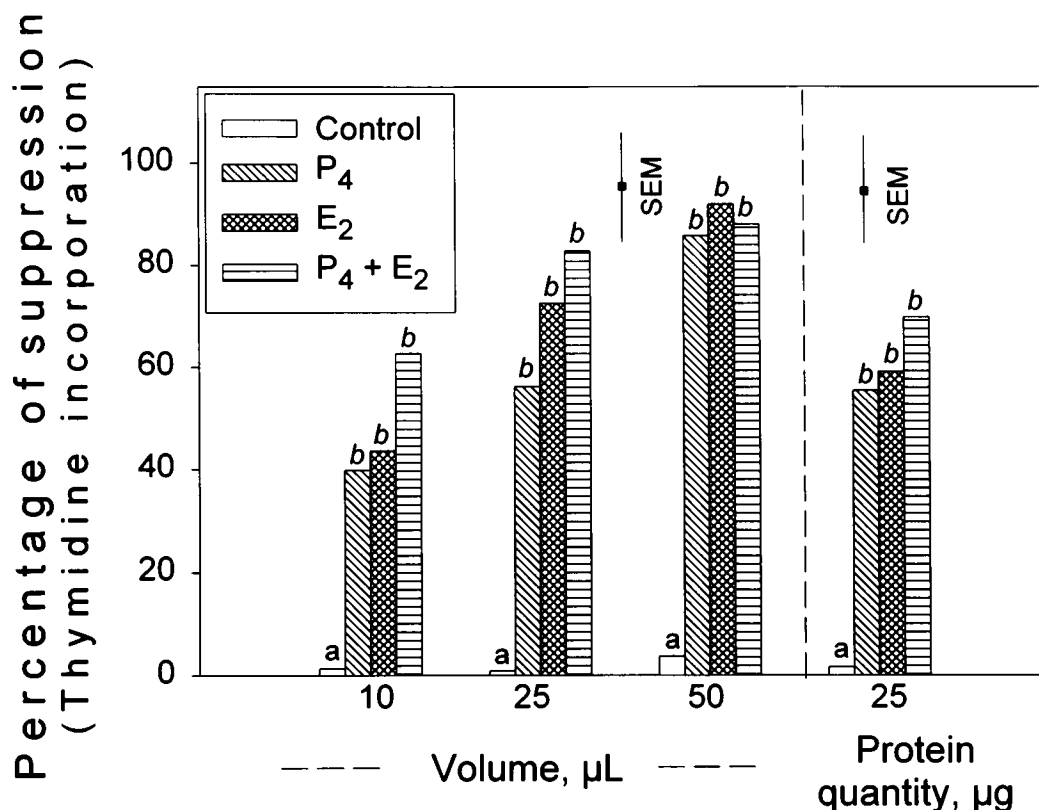


Figure 5. Thymidine incorporation data are presented for phytohemagglutinin-treated peripheral blood lymphocytes (1×10^5) cultured with volumes (10 to 50 μL) and protein (25 μg) from uterine luminal secretions recovered from control and progesterone (P_4)-, estradiol- 17β (E_2)-, and $\text{P}_4 + \text{E}_2$ -treated, ovariectomized ewes. Mean values among treatments within volumes and protein quantity with no superscript in common are different ($P < .05$).

45.8 and 53, respectively. Supernatant (10- and 25- μL volumes) from UT cells treated with 1×10^{-5} M of P_4 and 1×10^{-9} M of E_2 suppressed lymphocyte proliferative responses greater ($P < .05$) than the response observed for the control culture (Figure 6).

Discussion

Uterine suppressor cells inhibited proliferative responses of autologous PHA-treated peripheral blood lymphocytes. Suppressor cell activity was not dependent on ovarian function because cells from control ewes suppressed proliferative responses of cocultured PHA-treated PBL and released a soluble suppressor factor(s) into the culture medium. The degree of activity, however, may be dependent on ovarian function because suppressor activity for cocultured UT cells was increased in E_2 -treated ewes. Suppressor activity was not increased for supernatant from cultured UT cells from the steroid-treated ewes. Suppressor responses of UT cells from E_2 -treated ewes were not mediated by their apparent cytotoxicity upon PBL. Injections of the steroids resulted in serum concentrations of P_4 (6.3 to 8.5 ng/mL) that approximated those of Baird et al. (1976) and Hauger et al.

(1977) for luteal-phase ewes, whereas serum concentrations of E_2 (15.2 to 18.4 $\mu\text{g}/\text{mL}$) approximated those reported by Hauger et al. (1977) for a 4- to 6-d period around estrus. The periods of P_4 and E_2 domination were greater in duration following steroid treatment than observed during the estrous cycle. One of the primary objectives of this investigation was to determine whether steroid treatment for a 14-d period was capable of affecting UT suppressor cell function. The findings may necessitate the subsequent investigation of suppressor cell function using steroid treatments that better reflect the physiological serum concentrations of P_4 and E_2 that occur during the estrous cycle. The findings from this investigation extend those in which mononuclear cells harvested from luminal blood of OVX ewes, following endometrial curettage, responded greater to PHA than cells harvested from estrous and d-14 cyclic ewes (Segerson and Libby, 1984). Greater suppressor activity of UT cells from d-14 cyclic than from OVX ewes (Segerson and Gunsett, 1992) further suggested a possible role for ovarian function in intrauterine immunosuppression. A preliminary finding from that study (Segerson and Gunsett, 1992) relative to the ineffectiveness of injections of P_4 in increasing UT suppressor cell activity has been corroborated in this investigation.

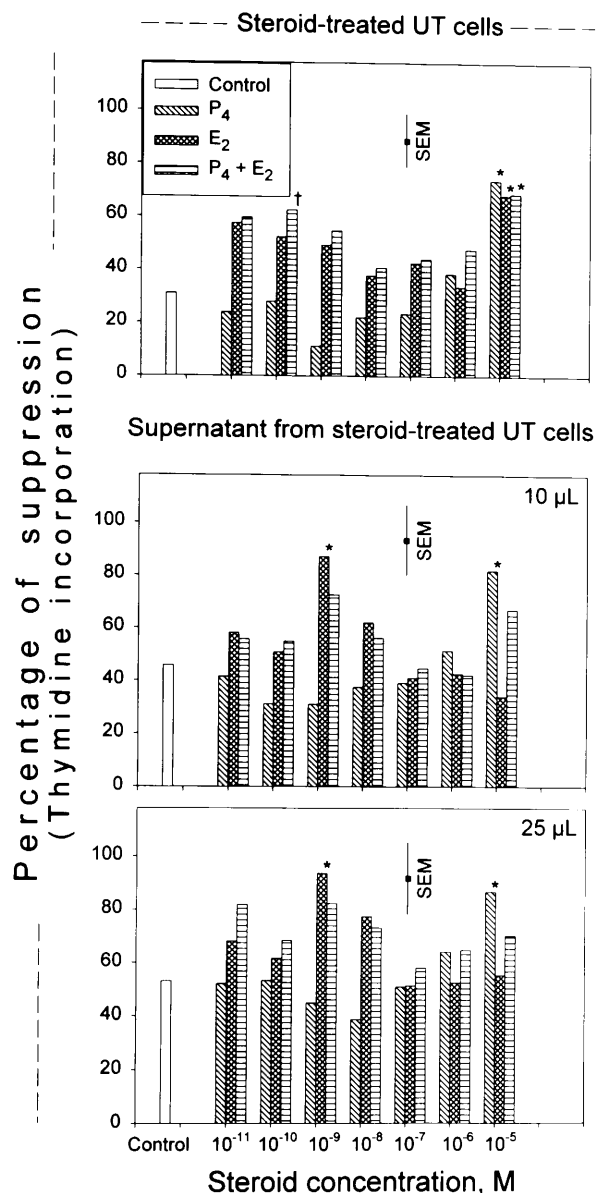


Figure 6. Thymidine incorporation data are presented for phytohemagglutinin (PHA)-treated peripheral blood lymphocytes (PBL; 1×10^5) cultured with either uterine (UT) cells (1×10^5) from nontreated, ovariectomized ewes and solutions (1×10^{-11} to 1×10^{-5} M) of progesterone (P_4), estradiol- 17β (E_2), and $P_4 + E_2$ or volumes (10 and 25 μ L) of supernatant (from the culture of UT cells and solutions of the steroids). Control cultures represented either UT cells cocultured with PHA-treated PBL without steroids or PHA-treated PBL with supernatant from nonsteroid-treated UT cell cultures. Mean values with an asterisk are greater ($P < .05$) than the value for the control culture.

The secondary objective involved the determination of the range in relative densities for UT suppressor cells. The coculture of PHA-treated PBL with two groups of fractionated UT cells revealed that suppressor activity was predominantly associated with cells

that sedimented within a density range of 1.002 to 1.056 g/mL (Percoll fractions 1 to 5, respectively). Typically, this density range is attributed to cells that are smaller than conventional lymphocytes. Before combining cells within these fractions, microscopic observation revealed a preponderance of small cells (i.e., ≤ 5.2 μ m in diameter). In a previous investigation, these cells were evaluated morphologically and placed into categories in which cellular diameters ranged from 1.3 to 5.2 μ m, with the majority having a diameter of approximately 5.0 μ m (Segerson, unpublished data). It is unlikely that the UT suppressor cells comprise the conventional T- and (or) B-lymphocyte lineages because the range in densities for the suppressor cells is less than the range reported for murine and human T- and B-lymphocytes within peripheral blood (Kurnick et al., 1979). In addition, complement-mediated lysis of T-, B-, and natural killer-like-lymphocytes within preparations of unfractionated UT cells failed to abrogate suppressor responses of the UT cells (Segerson, unpublished data). Further investigation is needed to determine whether the nonspecific release of suppressor factor(s) from UT cells is related to the ≥ 248 -kDa macromolecule identified within supernatant from cultured UT cells of d-14 cyclic ewes (Segerson and Gunsett, 1992). In this investigation, the possibility that suppressor activity resulted from changes in the composition of the medium due to the presence of the UT cells is remote because cell culture experiments included appropriate control cultures.

Suppression of TdR incorporation by ULS was increased following daily injections for 16 d of P_4 and E_2 , but not $P_4 + E_2$, in OVX beef heifers (Segerson et al., 1986) and daily injections for 10 d of P_4 in OVX ewes (Hansen and Skopets, 1992). Secretions from control ewes in this investigation did not affect TdR incorporation, whereas all steroid preparations suppressed incorporation in a similar manner. Suppressor activity of ULS was not mediated by apparent cytotoxicity of PBL. The suppressor mechanism for ovine and bovine ULS has been investigated by this laboratory. Ovine macromolecules of ≥ 248 , 70, and 14 kDa within ULS recovered on d 14 of pregnancy (Segerson, 1988) and bovine uterine luminal macromolecules of $\geq 4 \times 10^6$ Da and 21 kDa recovered on d 17 of pregnancy (Segerson and Gunsett, 1990) suppressed interleukin-2 (IL-2)-mediated proliferation of PBL. Suppression of the bovine macromolecules likely occurred through the inhibition of binding of IL-2 to the p75 β component of the IL-2 receptor (Segerson and Libby, 1990). In addition, each of the bovine macromolecules suppressed the lytic activity of LAK cells on K-562 target cells (Segerson and Gunsett, 1993). It is not known whether suppressor activities of ULS, UT cells, and supernatant within this investigation were mediated by inhibition of IL-2 activation of PBL. Differences in steroid-mediated

suppressor activity between UT suppressor cells and ULS leads one to question whether the suppressor factor(s) within ULS originated from the suppressor cells. If not, it is conceivable that different mechanisms may regulate the activity of each of the suppressors.

Although steroid-mediated suppressor responses of UT cells were observed following administration of the steroids to ewes and cell cultures, responses varied according to the route of administration. Treatment of ewes with E_2 resulted in increased suppressor activity of UT cells without an apparent increase in the release of soluble suppressor factor(s) into the culture medium. However, the addition of E_2 to PHA-treated PBL cocultured with UT cells failed to increase the suppressor activity of the UT cells (with the exception of 1×10^{-5} M of E_2 , which may be considered a pharmacological concentration), but apparent increased release of soluble suppressor factor(s) was observed for UT cells cultured with 1×10^{-9} M of estradiol-17 β . Injections of P_4 and $P_4 + E_2$ did not affect suppressor responses of cocultured UT cells, but responses were either increased or tended to increase following the addition of specific concentrations of these steroid preparations to single cultures and(or) cocultures of UT cells. Contrasting responses have been reported for UT suppressor cells and supernatants recovered from other species during various reproductive stages. Small murine (Clark et al., 1983) and human (Daya et al., 1985a) suppressor cells were shown to be pregnancy-dependent and capable of releasing soluble suppressor factors into the culture medium. Large murine and human suppressor cells were recovered during pseudopregnancy and the luteal phase, respectively, and both were incapable of releasing soluble suppressor factors (Clark et al., 1986a). Croy et al. (1987) reported that UT cells recovered on d 28 of pregnancy exhibited suppressor activity, but they failed to release soluble suppressor factor(s).

The origin of ovine UT suppressor cells is unknown at this time, but one may postulate that they originate either within the endometrium or become sequestered there following trafficking of cells within peripheral blood. Data from this investigation indicate that steroid-mediated increases in suppressor activity of UT cells, including release of suppressor factor(s), may have resulted from the direct action of steroids on suppressor cells present within endometrial tissue, increased cell trafficking from peripheral blood, or both. Increased suppression of PHA-treated PBL by cocultured steroid-treated UT cells and increased release of suppressor factor(s) from UT cells cultured with specific concentrations of P_4 and E_2 suggests an interaction between the steroids and suppressor cells. Cellular trafficking patterns may have been increased in E_2 -treated ewes; Ford et al. (1979) demonstrated that blood flow to the ovine uterus was greater at

estrus than on d 14 or 15 of the cycle. Although it is unlikely that UT suppressor cells consist of CD8 suppressor lymphocytes (for reasons stated previously for T-lymphocytes), this lymphocyte represents one type of migratory cell that has been identified within the ovine endometrium (Lee et al., 1988; Segerson et al., 1991).

Although the potential relationship between ovine UT suppressor cells and the maintenance of pregnancy has yet to be determined, this relationship seems apparent for mice. Spontaneous abortion of CBA/J X DBA/2 conceptuses in the uterus of CBA/J mice was correlated with a deficiency in suppressor cell activity (Clark et al., 1986b). Increased death rates of murine conceptuses have been observed in studies in which monoclonal antibodies were directed against pregnancy-associated natural suppressor cells (Gronvik et al., 1987) and T-lymphocyte suppressor factors (Ribbing et al., 1988).

Implications

Uterine suppressor cells may prevent lymphocytes from damaging or destroying ovine conceptus tissues during early pregnancy. The results of this investigation provide continuing evidence of a potential relationship between suppressor cell activity and ovarian steroid hormones. The administration of estradiol-17 β increased suppressor cell activity when administered to ovariectomized ewes, and progesterone, estradiol-17 β , and the combination of these hormones increased specific aspects of suppressor cell activity when cultured with uterine cells. If conceptus mortality is, in some way, related to suboptimal uterine suppressor cell function, elucidation of the regulatory mechanisms of these cells would seem necessary to optimize their function.

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