

# Progesterone Inhibits Rejection of Xenogeneic Transplants in the Sheep Uterus

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## Key Words

Progesterone · Xenotransplant · Hybridoma cells · Uterine tissue graft

## Abstract

**Objectives:** One of the proposed roles of progesterone is to prevent maternal immunological destruction of the allogeneic conceptus. Here, it was demonstrated that progesterone allows survival of a xenotransplant placed in the uterine lumen. **Methods:** Ovariectomized ewes, surgically prepared to have ligatures around each uterine horn, were given daily subcutaneous injections of 50 mg progesterone or vehicle (sesame oil). After 30 days of treatment, mouse hybridoma cells were transplanted to one ligated uterine horn and phosphate-buffered saline was injected into the other horn. The uterus was flushed after an additional 14 days of treatment and hybridoma cells were identified by immunofluorescence. **Results:** Overall, hybridoma cells were recovered from 4 of 5 progesterone-treated ewes and 1 of 5 vehicle-treated ewes. Immunohistochemical analysis of intercaruncular endometrium using antibodies towards CD8,  $\gamma\delta$ , and CD45R lymphocyte markers revealed that local presence of hybridoma cells caused a significant increase in CD8+ cells in all tissue compartments. While

not significant, the numbers of CD8+ cells in the luminal and glandular epithelium were lower for progesterone-treated ewes. Progesterone tended to increase  $\gamma\delta$  T cell numbers in the glandular epithelium. **Conclusions:** Results demonstrate that xenograft rejection in the uterus is associated with an increase in CD8+ cells in the endometrium and that progesterone can inhibit uterine tissue graft rejection responses sufficiently to allow survival or delay rejection of xenograft tissue.

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## Introduction

The essential hormone for the maintenance of pregnancy is progesterone [1]. Among its functions, progesterone may be a key hormone for preventing the destruction of the conceptus by the mother's immune system. Although the uterus possesses components of the immune system sufficient to destroy allografts placed within the uterine lumen [2, 3], the pregnant uterus becomes modified to accept antigenically distinct tissue during pregnancy. Thus, the conceptus, which is a foreign allograft, routinely survives in the uterus. In some cases, pregnancies as antigenic as those representing xenografts can be successfully maintained to term [4, 5]. The major evidence that

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progesterone plays an important role in uterine acceptance of foreign tissue during pregnancy is the wide range of immunosuppressive actions attributed to this hormone. Progesterone is inhibitory to lymphocyte proliferation at high concentrations [6, 7] and can induce secretion of immunosuppressive molecules from the endometrium such as ovine uterine serpin (OvUS) [8]. Moreover, progesterone can delay rejection of grafts placed within the uterine lumen [2, 3, 9], increase susceptibility to uterine bacterial infection [10, 11] and, at least in the ewe, reduce numbers of endometrial lymphocytes [12].

There were two objectives of the present experiment. The first was to develop a model for studying the regulation of tissue rejection responses in the uterus by progesterone. The cell used for transplantation was the mouse hybridoma cell because it can be easily identified by immunofluorescent techniques using antibodies to mouse MHC haplotypes. The second objective was to evaluate changes in endometrial lymphocyte numbers following transplantation to ascertain lymphocyte cell types that become increased upon exposure to foreign tissue and to test whether progesterone alters lymphocyte responses in the endometrium. Attention focused on lymphocytes with three major phenotypic markers present on endometrial lymphocytes in sheep [13]. These three markers, one or more of which can be present on an individual lymphocyte, are: CD45R, which identifies naïve T cells; CD8, which typically represent cytotoxic T cells, and the  $\gamma\delta$  T-cell receptor, which marks a major fraction of endometrial T cells during late pregnancy in sheep [13]. Lymphocytes of the  $\gamma\delta$  T-cell lineage have been implicated as playing an immunosuppressive role during pregnancy in mice [14, 15].

## Materials and Methods

### *Hybridoma Cells*

Several hybridoma cell lines were maintained at the University of Florida Hybridoma Facility. These included: HL-926, a cell line prepared by Charles Guy (Department of Environmental Horticulture, University of Florida) that produces an IgG1 against spinach BiP, a member of the heat shock protein 70 family [16]; a cell line (HL-869) that produces an IgG2a recognizing H2-K<sup>d</sup>, and cell lines producing antibodies to sheep CD8 (7C2), CD45R (20–96), and  $\gamma\delta$  T-cell receptor (86 D) that were originally obtained from the European Collection of Animal Cell Culture (Salisbury, UK). K562 cells (a human erythroleukemia line) were obtained from ATCC (Rockville, Md.) for use as a negative control for immunofluorescent staining of hybridoma cells.

### *Antibodies*

Antibody to CD8 was purified from cell culture supernatant using a Hi-Trap Protein G Sepharose column (Pharmacia & Upjohn, Kalamazoo, Mich.). The anti-H-2K<sup>d</sup> antibody was used as a cell culture supernatant and antibodies towards CD45R and  $\gamma\delta$  T-cell receptor were used as ascites fluid made in mice.

### *Progesterone*

This hormone was dissolved in a small amount of ethanol, mixed with 100 ml of sesame oil, and then heated overnight at 60 °C to evaporate the ethanol. The volume was adjusted to 500 ml to provide a progesterone concentration of 25 mg/ml. The sesame oil vehicle was prepared similarly except without the addition of progesterone.

### *Other Materials*

The immunohistochemistry kit (HistoScan Universal Monoclonal Detector Kit) was obtained from Biomedica (Foster City, Calif.). Sesame oil was obtained from Hain Food Group, Inc. (Uniondale, N.Y.) and Tissue-Tek OCT Compound was from Miles Diagnostic (Elkhart, Ind.). Progesterone, mouse IgG1, control mouse ascites fluid (clone NS-1), sheep anti-mouse IgG conjugated to FITC (Fab<sub>2</sub> fragment), trypan blue, and normal goat serum were from Sigma (St. Louis, Mo.). Other reagents were from Sigma or Fisher Scientific (Pittsburgh, Pa.).

### *Animals*

Rambouillet-type ewes, maintained on a diet of Bermuda hay ad libitum, were used for the experiment. Twelve ewes were bilaterally ovariectomized via midventral laparotomy and uterine ligations were placed around each horn. After >30 days, ewes received daily subcutaneous injections of either 50 mg progesterone in 2 ml sesame oil or 2 ml sesame oil vehicle for 30 days. The dose of progesterone used is sufficient to maintain pregnancy in ovariectomized ewes [17]. Reproductive tracts were then exposed via laparotomy. One uterine horn was injected with  $1.0 \times 10^7$  hybridoma (HL-926) cells suspended in 2 ml 10 mM NaPO<sub>4</sub> (pH 7.4) containing 0.9% (w/v) NaCl (PBS), and the other horn received 2 ml PBS. The HL-926 cell line produces an antibody to spinach BiP and was chosen because the antibody was found not to react in an ELISA with lysates of heat-shocked sheep peripheral blood lymphocytes (results not shown).

Hormonal treatments continued for 14 days after injection of cells. Ewes were then slaughtered via captive-bolt stunning and exsanguination. Reproductive tracts were removed and uterine fluid was aspirated from the ligated portion of the uterine horn. If uterine fluid was not observed, uterine flushes were performed with 5 ml PBS. Uterine fluid and flushes from each horn were centrifuged separately at 250 g for 5 min. Supernatant fractions were stored at –20 °C for later analysis. The cell pellet was resuspended in 100  $\mu$ l PBS containing 1% (v/v) goat serum (PBSG) and evaluated for the presence of hybridoma cells.

### *Determination of Hybridoma Cells*

Hybridoma cells were identified via indirect immunofluorescence using a monoclonal antibody (HL-869) to the MHC class-I haplotype present on HL-926 cells (H2-K<sup>d</sup>). An aliquot of 80  $\mu$ l of resuspended cells was centrifuged at 250 g for 5 min, the supernatant decanted and cells resuspended with 100  $\mu$ l of anti-H2-K<sup>d</sup> monoclonal antibody (HL-869, 10  $\mu$ g/ml in PBSG). Cells were incubated for 60 min on ice, washed, and then incubated for another 60 min on ice with 100  $\mu$ l of FITC-conjugated sheep anti-mouse IgG (Fab<sub>2</sub> frag-

Table 1. Effect of progesterone on numbers of recovered hybridoma cells in the uterus of ovariectomized ewes

Treatment	Ewe number	Hybridoma-injected horn	PBS-injected horn
Sesame oil	40	0	0
	162	0	0
	170	0	0
	28	0	0
	3	33,000	0
Mean		6,600	0
Progesterone	42	6,250,000	2,120,000
	52	13,500,000	0
	169	15,580,000	0
	41	0	0
	51	NA	97,500
Mean		8,746,000	443,500

Cell number was affected by progesterone treatment ( $p < 0.01$ ), uterine horn ( $p < 0.04$ ) and the progesterone  $\times$  horn interaction ( $p < 0.04$ ). Means are least squares means. NA = Not available for analysis.

ment; diluted 1:32). Cells were then washed, resuspended in 20  $\mu$ l staining buffer and examined using an epifluorescent microscope. Positive (cultured HL-926 cells) and negative (K562 cells) controls were included in each analysis.

The proportion of cells exhibiting fluorescence was counted. In addition, the total cell concentration in the uterine fluid or flushings was determined by fixing cells in 4% (w/v) paraformaldehyde, staining with trypan blue and counting cells with a hemacytometer. The total number of fluorescent cells was calculated by multiplying the total number of cells by the percentage of cells exhibiting fluorescence.

#### Tissue Collection and Embedding

Two tissue samples were excised from randomly chosen regions of the intercaruncular endometrium of each uterine horn. Tissue samples (~ 3 mm<sup>3</sup>) were temporarily placed in PBS and then transferred into aluminium boats (1 cm<sup>3</sup>) containing OCT freezing medium. Blocks were frozen in liquid-nitrogen cooled isopentane and later immersed in liquid nitrogen. Frozen tissue blocks were stored at -70 °C until sectioning was performed.

#### Localization of Endometrial Lymphocytes

Immunohistochemistry was performed on tissues from two separate blocks from each uterine horn of 10 ewes. Frozen blocks of tissue were sectioned on a cryostat at 5  $\mu$ m thickness. Sections were placed on polylysine-coated slides and fixed with 95% ethanol and air-dried onto the slide for 1 h. Tissue sections were rehydrated with PBSG and kept at 4 °C until processed. All histochemical steps were performed at room temperature and slides were rinsed with PBSG and blotted dry between each step. Slides were treated with 0.6% (w/v) H<sub>2</sub>O<sub>2</sub> in PBSG or 3 min to eliminate endogenous peroxides. Tissues were then treated with conditioner from the Biomeda kit for 5 min. Sections were incubated for 6 h with antibody to CD8 (6  $\mu$ g/ml), CD45R (1:800 dilution of ascites fluid) or  $\gamma\delta$ -TCR (1:300 dilution of ascites fluid) diluted in PBS. Mouse IgG1 (5  $\mu$ g/ml) and appropriately diluted control mouse ascites fluid were used as negative controls. Other steps, including incubation with anti-mouse IgG linked to bio-

tin (30 min), streptavidin-peroxidase (30 min) and amino-9-ethylcarbazole (10 min) were conducted using reagents supplied in the kit. Slides were rinsed with deionized water for several minutes and carefully blotted before applying mounting medium and coverslips.

#### Determination of Lymphocyte Numbers

Slides were observed at 400 $\times$  magnification in a bright field microscope containing a graticule in one eyepiece. The number of positive cells was counted in three tissue types: luminal epithelium; glandular epithelium, and stroma. For each tissue type, three randomly selected sites were chosen for morphometric analysis and the number of positive cells in 30 squares of the graticule (each square = 625  $\mu$ m<sup>2</sup> surface area) were counted. For epithelium, squares were chosen for counting so as to ensure the entire area of the squares was within the epithelium. For stroma, squares were chosen for counting so as to evaluate a cross-sectional area extending from the lumen to the deep stroma.

#### Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Model procedures of the Statistical Analysis System Institute [18]. The main effects were treatment, animal within treatment, and uterine horn. The animal was considered random and other main effects were considered as fixed, and all interactions were included in the model. Data on hybridoma cell number were log transformed before analysis. Data on the proportion of samples with hybridoma cells were analyzed by  $\chi^2$  analysis.

## Results

#### Survival of Hybridoma Cells

Data from uterine fluid were only available for 10 of the 12 ewes because two reproductive tracts were damaged in the abattoir following slaughter. For 1 of the

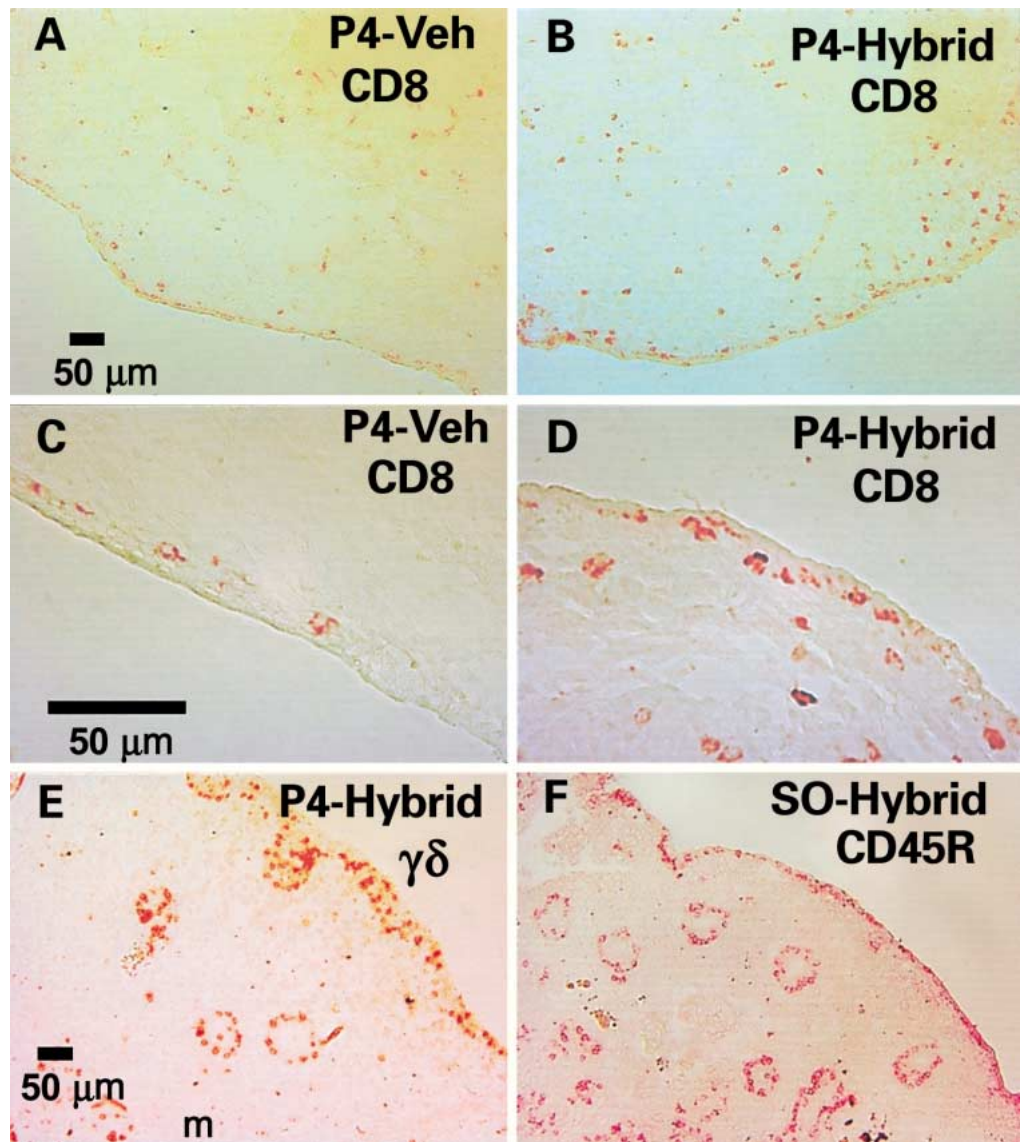


Fig. 1. Immunohistochemical localization of T cells in the sheep endometrium. Shown are lymphocytes reacting with antibody to CD8 (A–D),  $\gamma\delta$  (E) and CD45R (F). The scale for each row is shown in the left panel. Sections of endometrium were from uterine horns injected with vehicle (Veh) or hybridoma cells (Hybrid) and from ewes treated with sesame oil vehicle (SO) or progesterone (P4).

remaining 5 progesterone-treated ewes (ewe 169), 11 ml of fluid that resembled ascites fluid accumulated in the hybridoma-injected horn. In the other 4 progesterone-treated ewes and in the 5 vehicle-treated ewes, visible fluid was not present and each uterine horn was flushed to recover uterine contents.

The presence of hybridoma cells in the flushings was detected by immunofluorescence; overall numbers of recovered hybridoma cells are summarized in table 1. The

least-squares mean for number of hybridoma cells recovered from ewes injected with sesame oil was 0.0 for the PBS-injected horn (hybridoma cells were recovered from 0/5 ewes) and 6,600 for the hybridoma-injected horn (hybridoma cells were recovered from 1/5 ewes). In comparison, the least-squares mean for numbers of hybridoma cells recovered from ewes injected with progesterone was 443,500 for the PBS horn (hybridoma cells were recovered from 2/5 ewes; the ligatures were patent in the ewes

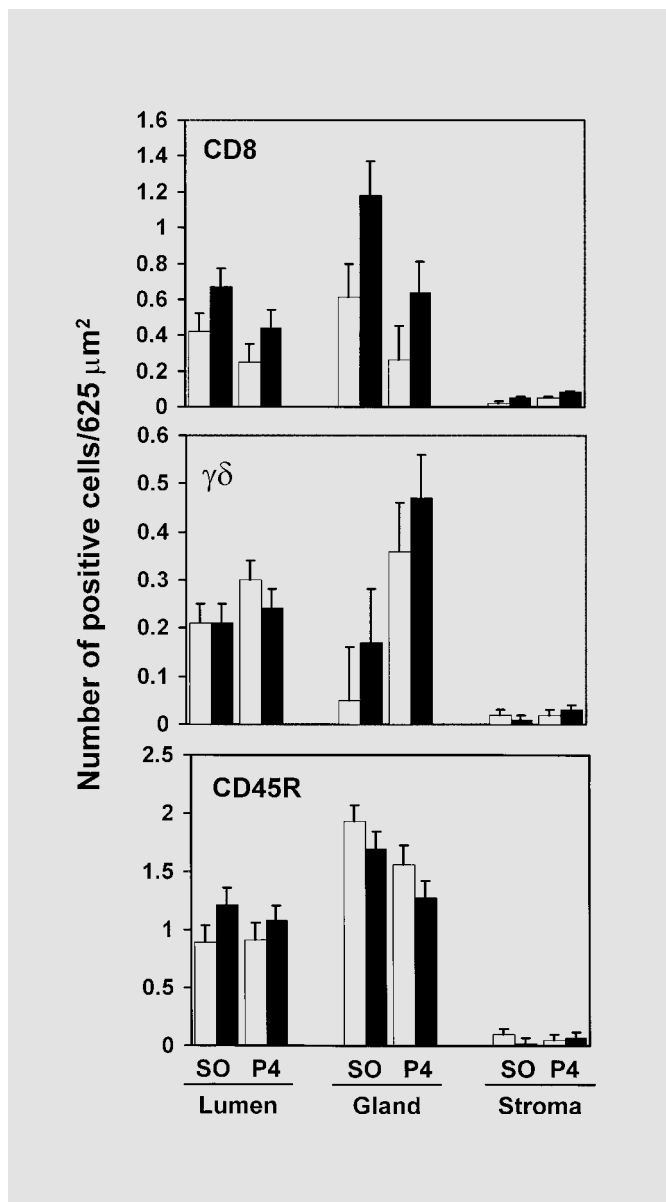


Fig. 2. Effect of progesterone treatment and infusion of hybridoma cells on the number of lymphocytes in the uterine endometrium of ovariectomized ewes. Ewes were treated with either sesame oil vehicle (SO) or progesterone (P4). Hybridoma cells were injected into one ligated uterine horn (■) while the other ligated uterine horn received PBS (□). After 14 days, tissue was removed and subjected to immunohistochemistry. As compared to vehicle, CD8+ cells were higher in the horn treated with hybridoma cells (lumen,  $p = 0.05$ ; glands,  $p < 0.05$ ; stroma,  $p = 0.05$ ). While not significant, cells in epithelium tended to be lower in number for progesterone-treated ewes. Treatment with hybridoma cells did not affect the number of  $\gamma\delta$  T cells but progesterone increased ( $p = 0.07$ )  $\gamma\delta$  T-cell numbers within the uterine glands. There were no significant effects on the number of CD45R+ cells in any region of the endometrium.

with hybridoma cells in the PBS-injected horn) and 8,746,000 cells for the hybridoma-injected horn (hybridoma cells were recovered from 3/4 ewes; cells were lost during processing for the 5th ewe and were not examined). Cell number was affected by progesterone treatment ( $p < 0.01$ ), uterine horn ( $p < 0.04$ ) and the progesterone  $\times$  horn interaction ( $p < 0.04$ ). Using  $\chi^2$  analysis, progesterone treatment affected the proportion of hybridoma-injected horns with recovered hybridoma cells ( $p < 0.10$ ) and the proportion of ewes with hybridoma cells (1/5 vehicle-treated ewes and 4/5 progesterone-treated ewes;  $p < 0.07$ ).

#### Endometrial Lymphocytes

For all three types of lymphocyte examined, positively stained cells were primarily located in the lumen and glandular epithelium or in the immediately adjacent stroma; fewer lymphocytes were dispersed within the remainder of the stroma. Representative photomicrographs illustrating the localization of lymphocytes is shown in figure 1 and results on the quantitative analysis of lymphocyte numbers are summarized in figure 2. The number of CD8+ lymphocytes was higher in the endometrium from the uterine horn injected with hybridoma cells as compared to endometrium from the uterine horn injected with vehicle (compare fig. 1A with 1B and fig. 1C with 1D). As shown in figure 2, this difference was present for luminal epithelium ( $p = 0.05$ ), glandular epithelium ( $p < 0.05$ ), and stroma ( $p = 0.05$ ). Although not significant, there was a tendency for injection of hybridoma cells to increase the number of  $\gamma\delta$  T cells in the glandular epithelium (fig. 2). There was no effect of injection of hybridoma cells on the number of endometrial  $\gamma\delta$  T cells in the luminal epithelium or the number of CD45R+ cells in any tissue (fig. 2).

While not significant, the mean number of CD8+ lymphocytes in luminal and glandular epithelium was numerically lower for progesterone-treated ewes as compared to ewes treated with vehicle (fig. 1). For example, the number of cells in the luminal epithelium of the hybridoma-injected horn was  $0.44 \pm 0.10$  cells/625  $\mu\text{m}^2$  for progesterone-treated ewes versus  $0.67 \pm 0.10$  cells/625  $\mu\text{m}^2$  for ewes treated with vehicle. Progesterone treatment tended to increase ( $p = 0.07$ ) the number of  $\gamma\delta$  T cells in the glandular epithelium (fig. 1). There was no effect of progesterone on the number of  $\gamma\delta$  T cells in the luminal epithelium or stroma or on the number of CD45R+ cells in any region.

## Discussion

In this experiment, progesterone increased the survival time of xenogeneic tissue placed within the uterine lumen. The presence of hybridoma cells in the uterus of progesterone-treated ewes is indicative of either survival of the cells or, as for skin grafts [3], a delay in their rejection time. Thus, these results strengthen the idea formulated based on results in sheep [3] and other species [2, 9] that progesterone can inhibit tissue rejection responses in utero. This action of progesterone is likely to be important for the survival of the immunologically foreign conceptus. The graft used in the present study, a mouse hybridoma cell line, was a xenograft and was thus more immunogenic than would be the case during allogeneic intra-species pregnancies. While the frequent failure of xenogeneic pregnancies may be caused by immunological rejection of the conceptus by the mother, such a mechanism for pregnancy loss, if occurring, does so despite the inhibitory actions of progesterone on uterine immune function. Indeed, the birth of chimeric individuals [4] as well as hybrid animals such as mules and hinnies [5] argues for an effective mechanism for inhibiting xenogeneic immune responses in utero. Progesterone is likely to represent an important component of this mechanism.

While graft rejection in utero has been reported [2, 3], the lymphocyte populations that mediate rejection have not been described. In the present experiment, CD8+ lymphocytes were the only population of endometrial lymphocytes to experience a statistically significant increase in the number in responses to local injection of hybridoma cells. Thus, it is likely that, as for other grafts [19, 20], CD8+ cytotoxic cells are involved in rejection responses. In contrast to the increase in the number of CD8+ cells in response to hybridoma cells, there was no hybridoma-induced increase in the number of  $\gamma\delta$  T cells in the luminal epithelium or in CD45R+ cells in either luminal or glandular epithelium.

It is possible that local lymphocyte responses to the hybridoma cells were obscured somewhat because there was some movement of hybridoma cells between uterine horns. It is not known whether this movement occurred in utero, because of inadequate ligation, or was an artefact induced by the flushing procedure after collection of the reproductive tract. In addition, lymphocyte numbers were examined at one time after grafting only and lymphocyte responses to the presence of hybridoma cells or to progesterone treatment may have been different at other times. However, the failure to see an increase in  $\gamma\delta$  T cells at the site of graft placement (i.e., in the luminal epithe-

lium) is consistent with other models of graft rejection in which there is non-involvement of  $\gamma\delta$  T cells in tissue rejection [21, 22]. In contrast,  $\gamma\delta$  T cells with natural killer cell-like activity have been isolated from other allografts [23]. In certain grafts,  $\gamma\delta$  T cells may play an immunoregulatory role to promote graft survival [24]. Perhaps, the nonsignificant increase in numbers of glandular  $\gamma\delta$  T cells in response to hybridoma cells represents such an immunosuppressive population of cells.

While not significant, progesterone tended to lower numbers of CD8+ cells in all regions of the endometrium. Thus, progesterone may have blocked the proliferation or migration of CD8+ cells into the uterus and this action of progesterone may have contributed to its graft-promoting action. There was no inhibitory effect of progesterone on numbers of CD45R+ cells or  $\gamma\delta$  T cells in the endometrium. Indeed, progesterone tended ( $p = 0.07$ ) to increase the number of  $\gamma\delta$  T cells in the glandular epithelium. The failure of progesterone to reduce the  $\gamma\delta$  T-cell number is not surprising since this population of T cell is unresponsive to the inhibitory actions of OvUS [25], the endometrial secretory protein which is believed to mediate many effects of progesterone on uterine immune function. In addition, rather than decrease during pregnancy, there is a large increase in the number of  $\gamma\delta$  T cells in the luminal endometrial epithelium during mid to late pregnancy [26]. In contrast, failure to observe a decrease in the number of CD45R+ cells in the luminal and glandular epithelium is inconsistent with results from an earlier experiment in which progesterone treatment caused a reduction in these cells in the endometrium [12]. The CD45R marker, which is characteristic of naïve T cells, is expressed on several populations of endometrial lymphocytes [13] and failure to observe effects of progesterone on the number of cells positive for CD45R may reflect the differential regulation of various lymphocyte populations.

There are at least three possible mechanisms by which progesterone could inhibit lymphocytes involved in xenograft rejection. First, progesterone could act directly on endometrial lymphocytes to inhibit their proliferation or activation. This is unlikely, however, unless endometrial lymphocytes are more sensitive to progesterone than are peripheral blood lymphocytes. The concentrations of progesterone in the blood of ewes receiving injections of 100 mg progesterone (twice the dose used in this study) peaked at 31 ng/ml ( $10^{-7}$  M) at 6 h after injection [27]. This concentration of progesterone causes only slight or no inhibition of proliferation of mitogen-stimulated, peripheral blood lymphocytes [6, 7, 28, 29]. For ovariectomized ewes received 50 mg progesterone/day, concentra-

tions of progesterone in uterine fluid were 0.2 ng/ml [3]. Progesterone could also induce secretion of endometrial molecules that in turn inhibited endometrial lymphocytes involved in graft rejection. Progesterone can induce immunosuppressive factors in uterine secretions [27, 30]. The most abundant of these lymphocyte-inhibitory factors is OvUS (formerly known as uterine milk protein), which can inhibit a variety of lymphocyte responses in vivo and in vitro [for review see, 8]. In the current experiment, progesterone was administered at an adequate dose and for a sufficient time before introduction of the graft (30 days) to induce OvUS secretion [3, 31, 32]. Finally, progesterone may act to inhibit graft rejection, at least in part, by inducing the presence of suppressive  $\gamma\delta$  T cells in the endometrium. This hypothesis is suggested by the fact that progesterone tended to increase the number of  $\gamma\delta$  T cells in the glandular epithelium and suppressive  $\gamma\delta$  T cells that secrete transforming-growth factor are present in the mouse uterus during pregnancy [14, 15]. In addition, there is evidence for the existence of  $\gamma\delta$  T cells that promote graft survival [24].

In conclusion, the results indicate that xenograft rejection in the uterus is associated with an increase in the

number of CD8+ T cells and that this rejection response can be inhibited by progesterone. The possibility that inhibitory responses of progesterone are mediated, in part, by activation of a population of suppressive  $\gamma\delta$  T cells should be considered based on the observation that progesterone tended to increase  $\gamma\delta$  T-cell numbers in glandular epithelium.

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