

PROSTAGLANDINS MODULATE THE UTERINE RESPONSE
TO INFECTIOUS BACTERIA IN POSTPARTUM AND
ESTROUS CYCLIC EWES

by

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ABSTRACT: The uterine immune system is down regulated when exposed to P₄. Five experiments were conducted to determine the *in vivo* and *in vitro* role(s) of P₄ and prostaglandins in immune function(s) associated with uterine infections. In Exp. 1, postpartum ewes (d 0 = parturition) were supplemented with either OIL or P₄ (beginning on d 10) or were SHAM or OVEX. Vena caval blood and lymphocytes were collected on d 14, and 16 to 19. All ewes received intrauterine inoculations of bacteria on d 15 and uteri were collected on d 20. Ewes receiving P₄ developed infections. Lymphocytes were incubated with mitogens, PGE₂, indomethacin (INDO; a PG synthesis inhibitor) or both in a 3 × 2 × 2 arrangement. Concanavalin A-stimulated blastogenesis in P₄-OVEX ewes and PGE₂ and PGE₂ + INDO treated lymphocytes was inhibited ($P < .05$). Cyclic ewes in their follicular or luteal phase received either intrauterine inoculations of saline or bacteria, vena caval blood was collected for 3 d, and uteri were collected. Lymphocytes were incubated with mitogens, PGE₂ (Exp. 2), PGF_{2α} (Exp. 3) and(or) INDO in a 3 × 2 × 2 arrangement. Only luteal phase ewes that received bacteria developed infections. In Exp. 2, Con A- and LPS-stimulated blastogenesis were greater for luteal than for follicular phase ewes. T lymphocyte proliferation was inhibited in ewes inoculated with bacteria. T lymphocyte proliferation tended to be higher ($P = .09$) when incubated with INDO. In Exp. 3, T lymphocyte proliferation in response to PGF_{2α} was greater for follicular than for luteal phase ewes. Neutrophils were lower in ewes inoculated with

bacteria. In Exp. 4 and 5, uteri of luteal-phase (d 6) ewes were inoculated with bacteria. Ewes received either 15 mg of Lutalyse or saline on d 9, and uteri were collected on d 11. Lutalyse reduced P_4 , tended to decrease neutrophils, allowed ewes to clear infections, and had no effect on blastogenesis. Methods for modulating uterine prostaglandins seem to reduce susceptibility to uterine infections.

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Abbreviations

The following abbreviations were used throughout this dissertation:

BW	Body weight
cAMP	Cyclic AMP
cfu	Colony forming units
cGMP	Cyclic GMP
Con A	Concanavalin A
cpm	Counts per minute
CL	Corpus luteum or corpora lutea
CV	Coefficient of variation
d	Day(s)
dpm	disintegrations per minute
E ₂	Estrogen or estradiol-17 β
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
hr	Hour(s)
IL	Interleukin
i.m.	Intramuscular
IP ₃	Inositol triphosphate
LTB ₄	Leukotriene B ₄
LH	Luteinizing hormone
LPS	Liposaccharides
min	minute(s)
NBT	Nitroblue tetrazolium
OT	Oxytocin
P ₄	Progesterone
PBS	Phosphate buffered saline
PG	Prostaglandin
PGE ₂	Prostaglandin E ₂
PGEM	13,14-dihydro-15-keto-PGE ₂
PGF _{2α}	Prostaglandin F _{2α}
PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PGFM	13,14-dihydro-15-keto-PGF _{2α}
PKA	Protein Kinase A
PKC	Protein Kinase C
RIA	Radioimmunoassay
S	second(s)
SEM	Standard error of the mean
wk	week(s)
WBC	White blood cell
yr	year(s)

Chapter I

STATEMENT OF THE PROBLEM

Uterine infections pose an economic threat to reproductive and overall efficiency of livestock. Mechanisms that modulate uterine susceptibility to infection in response to infectious bacteria are unknown. Early studies suggested that the stage of the estrous cycle can influence susceptibility of the uterus to infection. Furthermore, it seems that rabbits, sheep, and cattle will not develop a uterine infection unless they have a functional CL or are administered P₄ (Rowson *et al.*, 1953; Black *et al.*, 1954; Broome *et al.*, 1959; Hawk *et al.*, 1960a; Hawk *et al.*, 1961; Brinsfield *et al.*, 1964; Matsuda *et al.*, 1985; Del Vecchio *et al.*, 1992). Some investigators suggested that this observation was due to actions of E₂, which could stimulate the clearance of the infections. However, estrogen had little or no effect on the development or clearance of uterine infections unless administered at supraphysiological doses (Brinsfield *et al.*, 1964; Holdstock *et al.*, 1982; Staples *et al.*, 1983; Matsuda *et al.*, 1985). Early *in vivo* and *in vitro* studies offered no clear explanation of the uterine mechanism(s) in the regulation of an infection. Moreover, the relationship of other hormones (specifically arachidonic acid metabolites) and the activity of the uterine immune system was not determined.

Inflammation (specifically in the endometrium) is one of the early signs during the course of a uterine infection. Several arachidonic acid metabolites, the prostaglandins and leukotrienes, are direct mediators of inflammation (Fantone *et al.*, 1985). Based upon these observations, the effect of arachidonic acid metabolites on leukocyte activity (specifically lymphocytes and neutrophils) has attracted considerable attention. Investigations on the direct action of arachidonic acid metabolites on immune cells

demonstrated that eicosanoids can change lymphocyte and neutrophil activities (Vaillier *et al.*, 1992; Roper and Phipps, 1992; Roper *et al.*, 1994; Watson *et al.*, 1987a; Hoedemaker *et al.*, 1992) but, these effects seem to vary across species and investigations. Prostaglandin $F_{2\alpha}$, the primary luteolysin in ruminants, decreases P_4 and presumably can up-regulate the uterine immune system and help clear uterine infections. Because prostaglandins have a short half-life, research has been performed mostly with *in vitro* techniques concerning the effect of arachidonic acid metabolites on immune cells.

This project was designed to study the effects of ovarian hormones by using either postpartum or estrous cyclic ewes, with emphasis on the effects of arachidonic acid metabolites, PGE_2 (Exp. 1, 2, and 4) and $PGF_{2\alpha}$ (Exp. 3 and 5), on immune cell activities. Experiments were conducted to elucidate the effects of *in vivo* hormonal changes and on the *in vitro* activity of neutrophils and lymphocytes when the uterus is exposed to infectious bacteria. The role of exogenous $PGF_{2\alpha}$ in the treatment of uterine infections was also examined (Exp. 4 and 5).

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

Uterine infections pose a severe threat to livestock reproductive efficiency and overall profitability. Uterine infections can cause extended calving intervals, uterine damage, and possibly lead to permanent sterility, causing a cow to be culled (Griffen *et al.*, 1974). Regardless of management intensity, approximately 10% of the postpartum dairy cows will develop a uterine infection; however, the occurrence may surpass 40% (Arthur *et al.*, 1989; Lewis, 1997). Perhaps 85 to 90% of cow uteri are contaminated with bacteria for the first 2 wk after parturition (Elliot *et al.*, 1968; Griffin *et al.*, 1974). The most common bacteria associated with uterine infections are *Arcanobacterium pyogenes* and *Escherichia coli* (Olson *et al.*, 1986). Bartlett *et al.* (1986) reported that a producer loses an average of \$106 for each cow that develops a uterine infection.

Progesterone is the dominant hormone of pregnancy in cattle. Concentrations of progesterone steadily increase until about d 250 of gestation and then begin to decrease until the day of parturition (reviewed by Goff and Horst, 1997). On the day of parturition, progesterone decreases to undetectable concentrations, and estrogen increases to 4 to 6 ng/mL (reviewed by Goff and Horst, 1997).

During the periparturient period, the uterine immune system is typically up-regulated until it is exposed to progesterone. After this exposure, the uterine immune system is down-regulated, and the incidence of uterine infections often increases. Several factors can predispose a cow and increase her chances of developing a uterine infection. These would include dystocia (difficult birth), poor nutritional status of the

dam, inadequate hygiene at the time of calving, and retained fetal membranes. Fetal membranes are considered to be retained if they have not been expelled within 24 h after parturition.

Definition of a Uterine Infection

Researchers have published thousands of manuscripts that have some reference to uterine infections, but there seems to be no universal definition of the infection that is being investigated. Methods used to diagnose and classify these infections vary among investigators, making comparisons among studies and interpretation of the data difficult. Lewis (1997) defined a uterine infection as an indication that the uterus is contaminated with bacteria. Endometritis is an inflammation of the endometrium. Metritis is inflammation of the uterus.

Phase of the estrous cycle seems to have a major impact on the ability of bacteria to contaminate and establish an infection. During the follicular phase, when P_4 concentrations are low and E_2 concentrations are high, the uterus is seldom infected. However, during the luteal phase, when P_4 concentrations are high and E_2 concentrations are low, the uterus becomes easily infected when challenged with bacteria.

Ramadan *et al.* (1997) suggested that progesterone inhibits lymphocyte proliferation and that during the luteal phase, progesterone concentrations are great enough to cause immunosuppression in the uterus. Estrogen seems to play a less important role. One could propose that simply decreasing progesterone would reverse

immunosuppression in the uterus. However, this does not seem to be the case during the periparturient period.

Studies during the last 45 yr suggest that uterine infections (natural or induced) do not usually develop in animals that lack an active CL. Uterine infection incidences increase when CL develop or when progesterone is administered to rabbits (Black *et al.*, 1953a, 1954), sheep (Brinsfield and Hawk 1968a,b), and cows (Black *et al.*, 1953b, 1954; Del Vecchio *et al.*, 1992). However, most cows do not have an active CL during the periparturient period and do not develop uterine infections. Lewis (1997) proposed that the uterine immune system is up-regulated during the periparturient period and remains up-regulated until there is exposure to progesterone. After the exposure to progesterone, the uterine immune system is down-regulated, and the incidence of uterine infections increases.

Neutrophil chemotaxis, superoxide anion production (a measure of destruction of foreign material), and iodination (a measure of ingestion of foreign material) were decreased during the first week after parturition, compared with cells from control steers (Kehrli *et al.*, 1989a,b). The phagocytic ability of neutrophils is decreased before and after calving in cows that developed metritis and in cows with retained placentas (Cai *et al.*, 1994; Hussain, 1989). Neutrophil activity (superoxide anion production and stimulus-induced shape change) was significantly less in cows with retained placentas during the first week postpartum. Lymphocyte blastogenesis in response to mitogens was reduced during the periparturient period, compared with cells from steers used as controls (Kehrli *et al.*, 1989a,b). Thus, the *in vitro* impairment of immune cell activities corresponds to an increased susceptibility to infections.

Endocrine Events of the Ovine Estrous Cycle

The ovine estrous cycle consists of two distinct phases: follicular and luteal. The follicular phase (proestrus and estrus) is characterized by decreasing P_4 concentrations, estrus, and development of a functional CL. Estrus is the result of increased E_2 , which is produced by the ovulatory follicle and induces estrus and the LH surge. The luteal phase (metestrus and diestrus) is characterized by CL maturation and P_4 production, and lasts from the time of ovulation until the CL undergoes luteolysis. During metestrus, P_4 concentrations plateau, and there is a slight increase in uterine $PGF_{2\alpha}$ (Kindahl *et al.*, 1976, Wilson *et al.*, 1972). Diestrus is associated with increased P_4 along with a maturing CL. Decreasing concentrations of P_4 and increasing concentrations of E_2 and $PGF_{2\alpha}$ are associated with proestrus (for review see Hansel and Convey, 1983).

During ovulation, follicles rupture due to a surge of LH, and theca and granulosa cells luteinize and become small and large luteal cells, respectively (Niswender and Nett, 1988). Weight and size of the CL increase during metestrus and the first portion of diestrus (Henricks *et al.*, 1972). A mature CL will produce P_4 concentrations of ≥ 6.0 ng/mL of serum. Luteal secretion of P_4 is essential until d 50 of gestation in sheep (Lauderdale, 1986; Niswender and Nett, 1988). In each case, E_2 must prime the reproductive tract, by inducing formation of P_4 receptors (Muldoon, 1980) and support the environment required for normal embryonic growth and development (Niswender and Nett, 1988). Three days before estrus, P_4 decreases rapidly, E_2 increases (reviewed by Hansel and Convey, 1983), and a new estrous cycle begins.

Estradiol exhibits a biphasic pattern during the estrous cycle. The first rise in serum E_2 from the dominant follicle of the first follicular wave (Ireland and Roche, 1983) reaches concentrations of 10 pg/mL in cows. After atresia of the dominant follicle, E_2 decreases to basal levels (2 to 5 pg/mL). The second rise in E_2 occurs about 2 to 3 d before estrus (Hansel and Convey, 1983). This rise in E_2 serves to induce the formation of OT receptors in the endometrium such that a “positive feedback loop” can occur between $PGF_{2\alpha}$ and OT. For ovulation to occur, a decrease in P_4 (i.e., less than 1.0 ng/mL of serum) and an increase in E_2 must occur, which induces a LH surge.

Biosynthesis and Secretion of Prostaglandins

Milvae (1986) reviewed several pathways in which prostanoids are synthesized from arachidonic acid. These pathways include the cyclooxygenase pathway, which produces prostaglandins and thromboxanes, and the lipoxygenase pathway, which produces fatty hydroxylacids and leukotrienes. Several pathways can lead to the production of $PGF_{2\alpha}$, but the most energy efficient pathway is through the cyclooxygenase pathway.

The primary precursors of all prostaglandins are fatty acids obtained from the diet or membrane lipids, mainly arachidonic acid, which is converted from linoleic acid by desaturase enzymes (Moore, 1985). Arachidonic acid is found mainly in phospholipids and triglycerides in the cell membrane. The rate limiting step of prostaglandin biosynthesis is the liberation of arachidonic acid by the action of phospholipase A_2 . The free arachidonic acid can be metabolized by two enzymes, cyclooxygenase and lipoxygenase. Arachidonic acid is converted to PGG_2 by cyclooxygenase in the

presence of O_2 , and PGG_2 is converted to PGH_2 by peroxidase activity. Prostaglandin H_2 is converted to $PGF_{2\alpha}$ and PGE_2 by PGF synthase and PGE synthase, respectively. Also, $PGF_{2\alpha}$ can be converted to PGE_2 and vice versa by 9-hydroxy-PG-dehydrogenase and 9-keto-PG-reductase, respectively (Oliw *et al.*, 1983). Figure 1 shows an overview of the prostaglandin synthesis pathway.

Prostaglandins are often increased during uterine manipulation and irritation, typically encountered with artificial insemination and embryo transfer techniques (Schallenberger *et al.*, 1989; Seguin *et al.*, 1974), following heat stress (Malayer *et al.*, 1990), and in short luteal phase cows (Ramirez-Godinez *et al.*, 1981; Pratt *et al.*, 1982; Cooper *et al.*, 1991). Cows with Gram negative mastitis have elevated $PGF_{2\alpha}$ concentrations in milk and shortened interestrus intervals (reviewed by Cullor, 1990).

Luteal Regression

Prostaglandin $F_{2\alpha}$ has been implicated as the primary luteolysin in many ruminant animals (Goding, 1974). Prostaglandin $F_{2\alpha}$ must be released in a series of five to eight pulses to initiate luteolysis in cattle (Kindahl *et al.*, 1976). These pulses appear just before the initiation of luteolysis (i.e., significant decreases in serum P_4). Transfer of uterine $PGF_{2\alpha}$ to the ovary has been proposed to occur by a countercurrent mechanism between the uteroovarian vein and ovarian artery (McCracken *et al.*, 1972). McCracken *et al.* (1972) provided the first evidence of a countercurrent mechanism by observing that [3H] $PGF_{2\alpha}$ infused into the ovarian pedicle was transferred from the uterine vein to the ovarian artery.

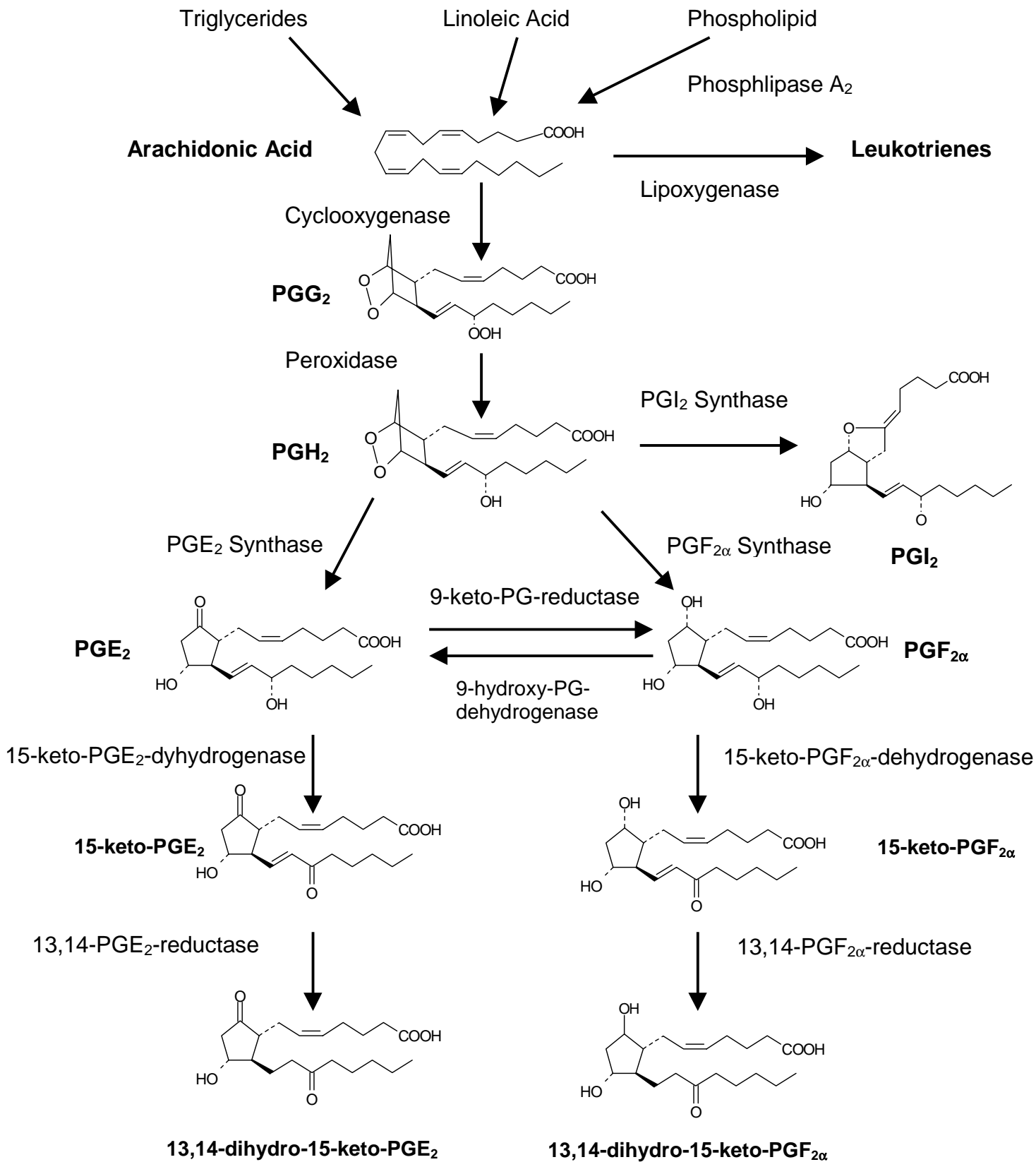


Figure 1. Biosynthesis and prostaglandin production pathway.

For spontaneous luteolysis to occur, the uterus requires a period of P₄ exposure (Lafrance and Goff, 1990; Homanics and Silvia, 1988). This period is needed to allow for accumulation of lipid droplets in uterine epithelial cells and up-regulation of the cyclooxygenase enzyme, stimulation of IP₃ turnover, and pulsatile release of PGF_{2α} during spontaneous luteolysis. Furthermore, P₄ inhibits the ability of E₂ to stimulate OT receptors (as reviewed by Silvia *et al.*, 1991). McCracken *et al.* (1984) suggested that luteolysis involves the formation of endometrial OT receptors induced by a pulse of E₂ such that a positive feedback loop can occur. Following P₄ exposure, the receptors for P₄ down regulate themselves, causing the formation of OT receptors and allowing OT to enhance a pulsatile release of PGF_{2α}.

The positive feedback between OT and PGF_{2α} is associated with a decrease in P₄, growth of the ovulatory follicle, and increased E₂. Increased E₂ may help drive luteolysis to completion by inducing the formation of additional OT receptors, stimulation of arachidonic acid turnover in phospholipid and triglyceride pools, stimulation of cyclooxygenase enzyme production, and further release of PGF_{2α} (Raw *et al.*, 1988; as reviewed by Silvia *et al.*, 1991).

Cellular Mechanisms of PGF_{2α}

The gene coding for the for PGF_{2α} receptor has been cloned, and its presence has been characterized throughout the estrous cycle in sheep and cattle (Rao *et al.*, 1979; Graves *et al.*, 1995; Wiltbank *et al.*, 1995). The PGF_{2α} receptor is a G-protein coupled receptor containing seven hydrophobic transmembrane spanning regions that represent α-helices (Ostrowski *et al.*, 1992). The PGF_{2α} receptor has been localized primarily to the

large luteal cells of the CL in sheep (Balapure *et al.*, 1989). Receptors for $\text{PGF}_{2\alpha}$ are present as early as d 3 after estrus in cattle (Rao *et al.*, 1979; Wiltbank *et al.*, 1995) and increase in concentration and binding affinity throughout the estrous cycle. However, the CL is not responsive to administration of $\text{PGF}_{2\alpha}$ until d 5 postestrus in cattle (Rowson *et al.*, 1972). Furthermore, this effect does not seem to be due to a lack of high-affinity receptors for $\text{PGF}_{2\alpha}$ on the CL (Wiltbank *et al.*, 1995).

When $\text{PGF}_{2\alpha}$ binds to its receptor, phospholipase C is activated, and the receptor- $\text{PGF}_{2\alpha}$ complex is translocated to the plasma membrane (Wiltbank *et al.*, 1990). Prostaglandin $\text{F}_{2\alpha}$ increases phosphatidylinositol hydrolysis, specifically hydrolysis of phosphoinositol bisphosphate to IP_3 and diacylglycerol (DAG), within the luteal cell (Jacobs *et al.*, 1991). Diacylglycerol can act as a second messenger through activation of PKC (Nishizuka, 1984). Protein kinase C may phosphorylate, and thereby activate, enzymes involved in liberating arachidonic acid from intracellular storage pools. Cytosolic free calcium is increased from the intracellular pool in the large luteal cells in response to $\text{PGF}_{2\alpha}$ (Wegner *et al.*, 1990), and luteal OT is released. Sustained intracellular concentrations of free calcium are cytotoxic in numerous cell systems (Rasmussen and Barrett, 1984), suggesting that the luteolytic effects of $\text{PGF}_{2\alpha}$ are mediated in this manner.

Prostaglandin $\text{F}_{2\alpha}$ also exhibits antisteroidogenic activity in luteal tissue. Prostaglandin $\text{F}_{2\alpha}$ blocks LH-induced stimulation of adenylate cyclase, thus reducing cAMP concentrations in luteal tissues (Lahav *et al.*, 1976). Henderson and McNatty (1977) suggested that this reduction in cAMP concentrations causes the cholesterol esterase enzyme to be converted from its active phosphorylated form to an inactive

dephosphorylated form. The net effect of this step is a reduction of free cholesterol for P₄ biosynthesis by luteal cell mitochondria. Furthermore, mRNA for the LH receptor on rat CL is decreased with the process of luteolysis (Bjurulf and Selstam, 1996).

Luteinizing hormone is the major luteotropic hormone in cows and ewes (Hansel *et al.*, 1973; Niswender *et al.*, 1981). Luteinizing hormone is known to stimulate P₄ production by luteal tissues *in vitro* (Kaltenbach *et al.*, 1967), and frequent injection of LH at midcycle prolonged the functional lifespan of the CL and the estrous cycle.

Immunological Functions of Neutrophils and Lymphocytes

The immune system has five cell types, specifically leukocytes, that are ultimately responsible for the destruction and elimination of foreign antigens that invade the body. These leukocytes are the neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Classification of the leukocytes is based on the presence (granulocytes) or absence (agranulocytes) of granules. The granulocytic cells can be further subdivided into neutrophils, basophils, and eosinophils, depending on their affinity for basic or acidic dyes (Klebanoff and Clark, 1978). The agranulocytes can be further subdivided into lymphocytes and monocytes. Only the neutrophils and lymphocytes will be discussed, because they are the most numerous in general circulation and have the most control over of foreign invaders. Neutrophils functions as an active phagocyte and increase exponentially during acute infections. Lymphocytes are concerned with immunologic responses in the body; the B lymphocytes are responsible for the production of immunoglobulins, and T lymphocytes play a regulatory role and destroys grafts, tumors, and virus-infected cells (Elgert, 1996).

Neutrophils. Neutrophils are short-lived cells (1 to 3 d) and have the ability to migrate and engulf many foreign invaders. In order to engulf the invader, neutrophils must travel to site of the invader. Neutrophils are attracted to that site by the release of chemoattractants either produced by the bacteria or the inflammation caused by the bacteria. In the literature, there are several references to the type of neutrophil movements including random locomotion, chemotaxis, and chemokinesis. Neutrophils can exhibit random locomotion, which is defined as a type of locomotion that is random in direction and the axis of the moving cell is not oriented in relation to the stimulus. Chemotaxis is a reaction by which the direction of locomotion of cells is determined by substances in their environment. Chemokinesis is a reaction by which the speed or frequency of locomotion of cells and(or) the frequency and magnitude of turning (change of direction) of cells moving at random is determined by substances in their environment (reviewed by Smith and Lumsdum,1983).

Both oxygen dependent and oxygen independent pathways destroy the foreign invaders. The oxygen dependent pathway involves production of various oxidizing agents (i.e., superoxide free radical, $\cdot\text{O}_2^-$) by the reduction of molecular oxygen, in a process called the respiratory burst. The respiratory burst includes an increased usage of glucose via the hexose monophosphate shunt and the generation of reactive free radicals and metabolic products (Fantone and Ward, 1982). The basis for the respiratory burst is the activation of a membrane-bound flavoprotein oxidase that catalyzes the following reaction: $2\text{O}_2 + \text{NADPH} \rightarrow 2\cdot\text{O}_2^- + \text{NADP}$ (Gabig and Babior, 1981). The dismutation of $\cdot\text{O}_2^-$ to H_2O_2 occurs spontaneously or is catalyzed by superoxide dismutase. Several aspects of the respiratory burst can be measured in the

laboratory. Methods include oxygen consumption, fixation of inorganic halides to proteins (specifically iodine), chemiluminescence, and increased reduction of a dye called NBT (Roth and Kaeberle, 1981).

Other methods by which neutrophils utilize to kill invaders via the oxygen dependent pathway include generation of a hydroxyl radical ($\text{OH}\cdot$), singlet oxygen ($^1\text{O}_2$), and hydrochlorous acid (HOCl ; reviewed by Bertram, 1985). Hydroxyl radicals can be generated by the interaction between $\cdot\text{O}_2^-$ and H_2O_2 in the presence of a trace metal (Fee and Valentine, 1977) and the reaction of $\cdot\text{O}_2^-$ with hydroxyperoxides of lipid peroxidation (Babior, 1978a,b). Formation of HOCl is accomplished by combining myeloperoxidase with H_2O_2 to form an enzyme-substrate complex that oxidizes halides (probably chloride) to form toxic products. These N-chloroamines react with chemotactic peptides and protease inhibitors and allow for local and systemic regulation of inflammatory events (Weiss *et al.*, 1983).

Lymphocytes. The uterine immune system consists of two main components: cell-mediated and humoral. The cell-mediated component consists of the thymus-dependent cells (T cells) and the humoral component consists of the bone marrow-dependent cells (B cells). After an exposure to an antigen, macrophages and B cells phagocytize the antigen, process the antigen, and present the processed antigen to T cells. There are two subsets of T cells: T helper and T cytotoxic cells. The T helper cells cause B cell proliferation and secretion of copious amounts of specific immunoglobulins that are specific for the antigen. The T cytotoxic cells are responsible for destruction and elimination for the specific antigen that the immune system comes in contact with.

Effects of Steroids on Uterine Infection Development and Lymphocyte Functions in Response to a Bacterial Challenge

In this section, I will discuss research of the past 50 yr that is associated with the influence of the estrous cycle on the development of uterine infections when exposed to infectious bacteria. The early research from the 1950s and 1960s was often crude and subjective, and it offers no clear answers as to the mechanism(s) that regulate how the uterus manages an infection.

Effects of progesterone. Rowson *et al.* (1953) used semen samples that were contaminated with *A. pyogenes* and inseminated cattle that were in the follicular and luteal phase of the estrous cycle. Infections (determined by the presence of pus and reculture of bacteria) developed only in cows that were inseminated in the luteal phase. In their second experiment, three ovariectomized cows were given a sensitizing dose of 25 mg of stilbestrol (a synthetic estrogen), followed by four daily injections of 50 mg of progesterone to simulate the luteal phase, inseminated, and were killed 2 d later for collection of their reproductive tracts. Two ovariectomized cows were given 50 mg of stilbestrol (to simulate the follicular phase), inseminated when they were detected in estrus, and were killed 2 d later. Four cows were treated as controls and were inseminated without any hormone treatment and killed 2 d later. Infections only developed in cows that were treated with both stilbestrol and progesterone. In an attempt to prevent uterine infections in a third experiment, six cows were inseminated with semen that was or was not contaminated with bacteria and diluted with egg yolk phosphate buffer and penicillin, streptomycin, and sulphanilamide and stored for 24 h. Cows inseminated with contaminated semen had slight pus present but bacteria could

not be recultured indicating that the uterus cleared the bacterial challenge. In a fourth experiment, their luteal phase cows received a culture of bacteria in their uterus. After 2 d, cows received 25, 50, 75, or 100 mg of stilbestrol and were slaughtered 2 d later. Only cows that received 100 mg of stilbestrol had cleared the infections. The researchers hypothesized that estrogen may have a governing role in clearing a uterine infection in the presence of a CL. In a fifth experiment, cows received bacteria as in the fourth experiment, had their CL removed, were allowed to come into estrus, and were killed 2 d later. Cows that were detected in estrus had no signs of a uterine infection at slaughter. Thus, the uteri seemed to be resistant to infections in the absence of P₄ and susceptible in the presence of P₄. The addition of antibiotics to the contaminated semen prevented the development of an infection; however, the authors suggested that this result may be a false negative because pus was still present. Lastly, infections can be cleared if the CL (source of P₄) is removed and the cattle come back into estrus.

Black *et al.* (1954) investigated the bactericidal activity of the uterus in rabbits. Rabbits that were either in estrus, ovariectomized, or pseudopregnant had their uteri inoculated with *E. coli*, and their cervixes were ligated to prevent drainage from the uterus. All rabbits were killed 24 h after inoculation, and reproductive tracts were collected to determine whether an infection had developed. Pseudopregnant rabbits had more recovered bacteria, compared with rabbits in estrus or with ovariectomized rabbits. These researchers suggested that the uteri of the pseudopregnant rabbits had lost their ability to kill bacteria because of the high P₄ concentrations. They also suggested that it was not necessarily a function of estrogen, because the

ovariectomized rabbits and the rabbits in estrus showed a similar pattern of bactericidal activity.

Broome *et al.* (1959) evaluated the importance of the granulocyte system in the uterine defense against bacterial exposure. In three experiments, rabbits in estrus or pseudopregnant rabbits had their cervixes ligated and uteri inoculated with *E. coli*. Rabbits were killed, and their uteri were collected at various time intervals to measure the rate of elimination of bacteria from the uterus and the rate of leukocyte accumulation following uterine inoculation. There were significant differences in the bacterial contents of uteri from rabbits in estrus and pseudopregnant rabbits in as little as 2 h following inoculation, and mobilization of leukocytes occurred more rapidly in the uteri of rabbits in estrus. There was also a negative relationship between numbers of bacteria and leukocytes in the rabbits in estrus. Using the same experimental methods as above, Hawk *et al.* (1960a,b) reported similar results. Overall, pseudopregnant rabbits had greater uterine bacterial counts 4 h after inoculation.

Data to this point had puzzled many investigators and led to the question of whether this inhibition of bactericidal activity by leukocytes was systemic or a local effect. Hawk *et al.* (1960a) inoculated uteri and pleural cavities of estrus and pseudopregnant rabbits and with *E. coli*. Rabbits that had their uteri inoculated were killed at 4 and 16 h after inoculation; pleural inoculated rabbits were killed at 4 and 6 h after they were inoculated. Pleural cavities and uteri were flushed with saline and the number of live bacteria and the bacterial activity of the exudates were determined. The exudates were heated at 56°C for 30 min to rule out the possibility that thermolabile blood serum bactericidins killed the *E. coli* in the bactericidal activity of the exudates.

Therefore, only the more thermostabile substances were related to leukocyte influx. More live bacteria were found in the uteri of pseudopregnant rabbits at both 4 and 16 h after inoculation. Exudates were highly bactericidal at both 4 and 16 h from the rabbits in estrus. Exudates from pseudopregnant rabbits were only slightly bactericidal at 4 h, but, after the development of an inflammatory response, their exudates had powerful bactericidal properties. However, the authors suggested that this observation could be because the numbers of luminal leukocytes increased between 4 and 16 h. Unlike the uterine flushings, lung exudates often appeared bloody, but were more of a serum color after centrifugation. In pleural cavity exudates, no differences were observed in the number of live bacteria and bactericidal activity between estrus and pseudopregnant rabbits at 4 and 6 h after inoculation. Thus, the endocrine status did not influence the bactericidal activity of the pleural cavity exudates. In contrast, ovarian status had a marked effect on the number of recovered live bacteria and bactericidal activity of the exudates. The authors suggested that there was a local effect of ovarian hormones on the inhibition of leukocytes.

Hawk *et al.* (1961) inoculated the uteri of 40 sheep with *E. coli* during estrus (d 0) or the luteal phase (d 7, 8, or 9 of the cycle). Sheep were killed at 4, 8, 12, 16, 24, and 48 h after inoculation, and reproductive tracts were collected and flushed with saline to determine the number of recovered *E. coli* and leukocytes. Also, the uteri were processed for histological determinations for leukocyte infiltration into the endometrium. At 4 h, there were more polymorphonuclear leukocytes within the endometrium and in the uterine lumen in the estrus ewes. At 8 h post-inoculation, ewes in estrus had a greater intensity of leukocyte staining in the endometrium and had fewer recovered live

bacteria. By 12 and 24 h, the staining intensity for leukocytes and number of recovered live bacteria were comparable between the estrus and luteal ewes. There were somewhat greater numbers of live *E. coli* in the luteal phase sheep at 12 to 24 h after inoculation. The authors suggested that this was probably due to the multiplication of the bacteria, but it could also be due to less effective bactericidal activity by leukocytes in the luteal phase sheep. Also, the difference in the early leukocytic response was much less in the luteal phase sheep, but investigators could not determine whether the leukocytes passed through the endometrium of the two physiologically different types of ewes at different rates.

To answer the question, "Is the rate of leukocytic passage through the endometrium influenced by hormones?", Brinsfield *et al.* (1963) inoculated *E. coli* into the uterus of ewes in estrus, luteal, and ovariectomized ewes. Ovarian hormones did not change the rate of passage of leukocytes into the uterine lumen. The authors also suggested that the hormones possibly delayed the uterine immune response to the bacterial challenge and that ovariectomy, by removing ovarian hormones, improved the uterine immune response.

Brinsfield *et al.* (1964) conducted an experiment to determine whether factors other than endogenous estrogen (possibly exogenous steroids) could slow leukocytic migration in estrus ewes. Ovariectomized ewes received E₂, P₄, or both. All ewes received uterine inoculation of *E. coli* at the end of the hormonal treatments. Ewes in estrus and untreated ovariectomized ewes served as control. Before the ewes were killed, they received an i.v. injection of trypan blue dye to stain tissue leukocytes. More leukocytes were present in the uterine lumen, and leukocytic activity was enhanced in

ewes that received estrogen. Ovariectomized ewes that received estrogen and P₄ were similar to the ewes in estrus in their leukocytic responses; ovariectomized ewes treated with P₄ had the least leukocytic response.

At this point, several researchers had asked the question: Do uterine infections affect the development of the CL? In a study by Coudert and Short (1966), ewes had their uteri inoculated with *E. coli* on d 10 of the estrous cycle. The lifespan of the CL in some of the ewes was significantly longer. In contrast, Brinsfield and Hawk (1968a) ligated the uterine horn and inoculated either the uterus or the broad ligament with *E. coli*. Thirty-six hours after estrus, ewes had their uterine horns ligated and inoculated with either saline or *E. coli*. The ewes were killed on d 7 of the estrous cycle, and reproductive tracts were collected to determine CL weight and the presence of bacteria. No differences were noted in ewes that received inoculations in the broad ligament compared with ewes that received a uterine inoculation. Six of the seven ewes that received a uterine bacterial inoculation had already reovulated at slaughter and had smaller CL (92 vs 500 mg for bacteria and saline, respectively). The authors suggested that infection on d 1 (36 h after estrus) may have activated luteolytic mechanisms or inhibited luteotropic mechanisms (or both) and led to inhibited CL growth. It is possible that the uterine infection induced on d 10 of the estrous cycle may have delayed the luteolytic mechanisms or it may have activated the luteotropic mechanisms.

Brinsfield and Hawk (1968b) conducted an experiment to further investigate whether the inhibition of CL growth was acting unilaterally or a local acting luteolytic mechanism. On d 1, the uterine horn either adjacent or opposite to the ovary that contained the CL was ligated and received either saline or *E. coli*. All ewes were killed 6

d later, reproductive tracts were collected and flushed, and CL were weighed.

Inoculation with bacteria inhibited CL development in both ovaries, indicating that this was not a local effect. However, the authors also found debris at slaughter in the control uterine horn that was not inoculated. This would suggest that bacteria did indeed migrate from the ligated horn. They suggested that it was possible that the ligatures may have loosened due to the uterus becoming more flaccid as the ewe entered the luteal phase.

Matsuda *et al.* (1985) demonstrated that the survival period of *E. coli* was longer in luteal phase rabbits than in follicular phase rabbits. When luteolysis was initiated with PGF_{2α} or hCG, P₄ concentrations were reduced, and bacteria was rapidly cleared from the uterus. When the uteri of ovariectomized rabbits were inoculated with *E. coli*, no sign of uterine infections was present after 6 d. However, *E. coli* survived longer in the uteri of ovariectomized rabbits treated with progesterone. When formalin killed *E. coli* were inoculated into uteri, the number of heterophils phagocytizing the bacteria was less in the P₄-treated rabbits 4 h after inoculation. The authors suggested that E₂ alone had no effect on the bacterial survival in the uterus, but it may have heightened the P₄ effect of inhibiting the bactericidal activity of the uterus. The authors also observed high concentrations of E₂ and P₄ during the luteal phase and suggested that both hormones may have inhibited the bactericidal activity of the uterus.

Monterroso and Hansen (1993) used lectin-stimulated sheep and cow lymphocytes to test whether inhibitors of steroid receptors block the suppressive effects of P₄. Neither RU38486 nor RU43044 (P₄ receptor antagonists) prevented the suppressive effects of P₄ on lymphocyte proliferation; however, these antagonists were

inhibitory themselves. Moreover, the effects of P₄ and antagonists were additive. The percentage of inhibition caused by P₄ was similar with and without the antagonists.

Effect of estrogens. Many attempts have been made to explain the differences in uterine susceptibility to bacterial infections during the estrous cycle. It has been hypothesized that estrogens have a major influence over the uterine immune system. During high estrogen concentrations, some mechanisms that have been suggested are an increased blood flow to the uterus (Rowson *et al.*, 1953), intensified polymorphonuclear leukocyte activity (Hawk *et al.*, 1964), and a high flow of mucus (possibly carrying the bacteria away from the uterus) from the reproductive tract (Rowson *et al.*, 1953). In previous studies from Brinsfield *et al.* (1964) and Matsuda *et al.* (1985), rabbits and ewes were administered supraphysiological doses of estrogen, and leading to an increase in leukocyte activity.

Holdstock *et al.* (1982) investigated the possible actions of testosterone, estradiol, and progesterone on lymphocyte transformation. Lymphocytes were collected from healthy human subjects and their blastogenic ability in a mitogen-induced suppressor T cell system was examined. Lymphocytes were incubated with testosterone, E₂, and P₄ at a concentration of 12, 40, and 20 ng/mL, respectively. The addition of P₄ and not testosterone or estradiol produced significantly greater T cell suppressor activity. The preincubation of lymphocytes with testosterone, but not P₄ or E₂, in the absence of mitogens resulted only in a modest suppression of T cell suppressor activity.

Staples *et al.* (1983) evaluated the possible actions of progestagens, androgens, estrogens, and corticoids on mitogen-induced lymphocyte transformation in sheep.

Progesterone and its metabolites and androgens were equally inhibitory, whereas corticosteroids were more effective than the other steroids. Estrogens, except for diethylstilbestrol, had little effect on the inhibition of lymphocyte transformation. The authors suggested that the C-19 and C-21 steroid families that had the 4-en-3-one configuration in ring A inhibited lymphocyte transformation and that inhibitory activity was enhanced by C-17 α substitution as in 17 α -hydroxyprogesterone.

In conclusion, uterine immune responses to infectious bacteria were similar in ewes and rabbits. Overwhelmingly, ewes and rabbits that were inoculated when they were in estrus had greater leukocytic activity than luteal ewes and rabbits, measured as killing efficiency and leukocyte infiltration into the uterine lumen (Black *et al.*, 1954). Also, preculture of bacteria with antibiotics reduced the ability of *E. coli* to establish a uterine infection in cattle (Rowson *et al.*, 1953). Uteri from ovariectomized ewes and rabbits had the greatest bactericidal activity, followed by uteri from animals in estrus. The bactericidal activity of the uterus could be lessened when the animals were in the luteal phase or were administered P₄ (Hawk *et al.*, 1961). The steroid that had the most modulatory activity over the uterus was P₄, whereas E₂ (unless administered at supraphysiological doses) had no or little effect on the ability to clear a bacterial challenge (Rowson *et al.* 1953; Brinsfield *et al.* 1964). Lymphocyte blastogenesis was inhibited by P₄, whereas E₂ had little effect (unless lymphocytes were cultured at supraphysiological doses; Matsuda *et al.* 1985). Removal of P₄ treatment (Brinsfield *et al.* 1968b) or luteolysis caused by PGF_{2 α} (or PGF_{2 α} analogues; Matsuda *et al.*, 1985) in ewes and rabbits inoculated with *E. coli* caused a rapid decrease in total bacterial counts and allowed females to clear their infections.

Effects of Steroids on Neutrophil Activity

In this section, I will present research that was reported over the last several years that is associated with the influence of ovarian steroids on neutrophil activity and how this may govern development of a uterine infection from exposure to infectious bacteria. Unlike research with lymphocytes, new techniques have somewhat improved our understanding of the mechanism(s) that regulate how the uterus manages an infection. However, our understanding of those mechanism(s) is incomplete.

Guidry *et al.* (1975) asked the question: "Do increased concentrations of E_2 in the blood at or just before estrus affect the concentration of circulating neutrophils or the phagocytic ability of those neutrophils". Cows were either in estrus or injected with either ethyl alcohol (control) or E_2 (0.05 mg/kg of BW twice a day for 2 d) beginning on d 10 of the estrous cycle. Blood samples were collected and used for a neutrophil phagocytosis assay for 10 d after the initiation of treatments. The injections of ethyl alcohol or E_2 did not affect neutrophil phagocytosis compared with cows in estrus.

Roth *et al.* (1983) designed an experiment to determine whether physiological concentrations of P_4 and E_2 during a "normal" estrous cycle could alter neutrophil activity in cattle. Chemotactic ability, reduction of NBT (a measure of superoxide anion production), and iodination were evaluated. Neutrophils were collected from cows three times a week during an estrous cycle. Increased serum concentrations of P_4 were associated with a depression in NBT reduction and iodination, but random migration of neutrophils was not affected. Increased E_2 concentrations were associated with high cortisol concentrations and enhanced random migration of neutrophils. The authors

suggested that E₂ enhances random movement, whereas P₄ down-regulated the ability of neutrophils to produce superoxide anion and to destroy a target.

Buyon *et al.* (1984) demonstrated that E₂ at a concentration of 3 µg/mL decreased the chemotactic response to a peptide (f-Met-Leu-Phe) and superoxide anion production. Neutrophil activity was inhibited 32 and 38% at dosages of E₂ at 1.5 and 3 µg/mL, respectively. However, there was no inhibition of neutrophil activity at lower dosages of E₂ (0.0003, .03, or .3 µg/mL).

Watson *et al.* (1987b) investigated the effect of physiological concentrations of ovarian steroids on phagocytosis and migration of blood and uterine-derived neutrophils. Ovariectomized horses were either treated with oil, P₄ (100 mg/d), or E₂ (1 mg) for 7 d. This treatment produced changes in the morphology of the endometrium to represent diestrus (P₄), estrus (E₂), and anestrus (oil). On d 6 of treatment, their uteri were infused with *Streptococcus zooepidemicus*. Neutrophils were collected 18 h after uterine inoculation, and their migratory ability was determined. The random migration of blood neutrophils was reduced by P₄ treatment. *In vitro* addition of P₄ to blood neutrophils from a cyclic mare also inhibited migration. Uterine neutrophils from E₂-treated and control mares had greater migratory ability than that by blood neutrophils. In P₄-treated mares, phagocytosis by uterine neutrophils was less than for the other treatments. The authors suggested that P₄ and E₂ have significant modulatory activities in both migration of blood neutrophils and phagocytosis of uterine neutrophils.

Angel *et al.* (1992) assessed the effect of E₂ on hematological values and the chemiluminescence response (a measure of the respiratory burst reaction) of neutrophils. Variables were measured in steers (n = 14) on d -14, -7, and -1 before E₂

implantation, and blood samples were collected on d 1, 2, 3, 4, 8, 15, 22, 29, 36, 43, and 50. Concentrations of E₂ were higher in steers implanted with E₂ implants.

Chemiluminescence and hematological indices were not affected by E₂ treatment. This study suggests that E₂ has no effect on the neutrophils' ability to produce superoxide anions as measured by chemiluminescence.

Subandrio and Noakes (1997) compared the chemotactic ability of bovine neutrophils to migrate to the uterus in response to two chemoattractants, 1% oyster glycogen and cell-free filtrate of an *A. pyogenes*. The two chemoattractants were infused into uteri at estrus and diestrus (d 10). The average viability of active cells recovered from the uterus was 85%, and 95% were neutrophils. The bacteria-free filtrate produced a greater migratory response of neutrophils into the uterus than that of oyster glycogen. Cows that had their uteri infused with the chemoattractants on d 10 had a greater migratory response than cows that were infused on d 0. Treatment of ovariectomized cows with P₄ caused an increased migratory response when the chemoattractants were infused into the uteri. The authors suggested that the increased migratory ability of neutrophils on cows during diestrus or administered P₄ was possibly due to a compensatory response caused by reduced phagocytosis and bactericidal activity or to the suppression of other uterine defense mechanisms.

In conclusion, there seems to be no consistent finding for the impact of ovarian steroids on neutrophil activities. Some studies report that E₂ has an effect on neutrophil activities, whereas others report that E₂ has little or no effect. However, neutrophils from animals that are in a P₄-dominated environment have greater abilities to travel into the uterus. However, the bactericidal ability of those neutrophils is decreased; these two

findings would suggest that the overall action of the bactericidal ability of neutrophils is nil.

Effects of Arachidonic Acid Metabolites on Lymphocyte and Neutrophil Activities

One of the early signs of a uterine infection is a generalized inflammation of either the endometrium or the uterus. Several arachidonic acid metabolites, the prostaglandins and leukotrienes, act as direct mediators of inflammation (Fantone *et al.*, 1985). Based upon these observations, the effect of arachidonic acid metabolites on leukocytes (specifically neutrophils) has attracted considerable attention. Much of this attention has focused on the use of $\text{PGF}_{2\alpha}$ (or its analogues), because it can decrease P_4 concentrations, presumably up-regulate the uterine immune system, and cause clearance of uterine infections. However, few have investigated the direct action of arachidonic acid metabolites on the immune cells (specifically on neutrophil and lymphocyte activities). Because prostaglandins have a short half-life in tissue or in circulation, most research on arachidonic acid metabolites has been performed *in vitro*.

Lymphocytes. Lymphocyte proliferation assays are the technique that has been used the most frequently to evaluate the effects of arachidonic acid metabolites on lymphocytes.

Vaillier *et al.* (1992) conducted an experiment to determine the influence of PGE_2 or LTB_4 on natural killer cell activity by using either fresh or cultured mouse spleenocytes. Also, these investigators wanted to determine the effects of killing efficiency when enhancing or suppressive antagonists act together on cytotoxic cells. In a dose-dependent manner, natural killer cell activity was suppressed by PGE_2 (10^{-6} to

10^{-8} M) and was enhanced by the addition of LTB₄ (10^{-8} to 10^{-10} M). Presumably, PGE₂ increases intracellular cAMP whereas LTB₄ increases cGMP. When cells were cultured with forskolin (a cAMP inducer) and a cGMP analogue (8 bromo-cGMP), similar results were obtained as in the above experiment. The authors suggested that PGE₂ plays a role in the decrease of natural killer cells, whereas LTB₄ plays a role in the increased activity of natural killer cells.

Roper and Phipps (1992) reported that incubation of resting B cells with PGE₂ inhibited the cellular enlargement normally induced by IL-4 or bacterial LPS or both. Prostaglandin E₂ also suppressed activation-induced class II major histocompatibility complex (MHC) up-regulation by 35%. Also, B-cell activation and class II MHC up-regulation decreased when B cells were incubated with agents that increase intercellular cAMP (cholera toxin and dibutyryl cAMP). These results would also suggest that PGE₂ uses cAMP as a second messenger system. One surprising finding was that cholera toxin and dibutyryl cAMP inhibited B cell activation by as much as 90 to 100%, which suggests that all B cells are cAMP sensitive but that only some are sensitive to PGE₂.

Roper *et al.* (1994) further characterized the mechanism of PGE₂ action in B cells. As in the previous study, these researchers showed that PGE₂ inhibits B-cell activation events that include cell enlargement, class II MHC up-regulation, and the expression of the low-affinity IgE receptor in a dose-dependent manner. Prostaglandin E₂ also inhibited enlargement and up-regulation of activation even if B-cells were preincubated with cells and then washed out prior to B-cell stimulation. Changes in B-cell phenotype were inhibited when B cells were incubated with cyclohexamide (a

protein synthesis inhibitor) and PGE₂. These findings suggest that some factor (probably a specific protein) that PGE₂ induces causes long-lasting inhibition in the B cells. Using two-dimensional gel electrophoresis, PGE₂-induced proteins were detected in whole cell extracts from B cells incubated with PGE₂. After 3 h of treatment, two putative PGE₂-inducible regulatory proteins were synthesized: one protein with a molecular weight of 80 kDa and a pI (isoelectric point) of 5.9; one protein with a molecular weight of 20 kDa and a pI of 6.8. After 12 h of PGE₂ incubation, one more protein was synthesized with a molecular weight of 18 kDa and a pI of 6.2. These authors suggested that B-cell inhibition was not only primarily modulated by cAMP induction by PGE₂ but was also modulated by secondary PGE₂-inducible regulatory proteins. However, there was no attempt to determine whether the PGE₂-inducible regulatory proteins were responsible for the inhibition of B cells caused by PGE₂ incubation.

In conclusion, lymphocyte cytotoxicity was inhibited as well as cell enlargement and up-regulation of class II MHC molecules. However, the addition of LTB₄ to the culture media reverse these activities. The inhibition of lymphocytes is due to increased intercellular cAMP. Moreover, there are two populations of lymphocytes: those that are sensitive to PGE₂ and those sensitive to cAMP. Culturing lymphocytes with PGE₂ induced three proteins, and it is possible that these are responsible for the long-term inhibition of lymphocytes.

Neutrophils. To evaluate changes in neutrophil activity in response to arachidonic acid metabolites, I will only discuss changes that occur in neutrophil ability to undergo random locomotion, travel toward chemoattractants (chemotaxis), produce superoxide

anion (a measure of the respiratory burst), and oxidize halides to proteins (a measure of the final destruction of bacteria).

Watson *et al.* (1987a) used an “under agarose” chemotaxis assay to determine the effects of arachidonic acid metabolites (specifically LTB₄, PGE₂, and PGF_{2α}) on neutrophil migration in horses. Leukotriene B₄ was chemotactic between all final concentrations of 0.1 and 1,000 ng/mL, and PGE₂ was at 1 and 10 ng/mL. Prostaglandin F_{2α} was not chemotactic at any concentration. Random migration was inhibited when neutrophils were suspended in PGE₂ (0.1 and 1 ng/mL). No differences were observed in migration when neutrophils were suspended in uterine flushings from control mares or mares with persistent endometritis. Moreover, there was no relationship between migration and concentrations of PGF_{2α} and PGE₂ in the uterine flushings in control mares. However, there was a direct correlation between neutrophil migration and concentrations of PGF_{2α} and PGE₂ in uterine flushings from mares with persistent endometritis. The authors suggested that arachidonic acid metabolites affected equine neutrophil migration and may play a role in the recruitment of neutrophils to sites of inflammation.

Hoedemaker *et al.* (1992) collected neutrophils from ovariectomized cows and incubated them with 0, 10⁻⁸, 10⁻⁷, and 10⁻⁶ M arachidonic acid metabolites of the cyclooxygenase and lipoxygenase pathway for 30 min (PGI₂, PGE₂, PGF_{2α}, LTB₄, 5-, 12-, and 15-hydroeicosatetraenoic acid) or with steroids (P₄, E₂, estrone, or cortisol) for 2 h. The neutrophils were then subjected to neutrophil activity assays. Prostaglandin F_{2α} was chemoattractant and stimulated ingestion of [¹²⁵I]IdUR-*Staphylococcus aureus*. Prostaglandin E₂ stimulated superoxide production, whereas PGI₂ suppressed

iodination of proteins. Leukotriene B₄ was chemoattractant and stimulated random migration. Cortisol increased antibody-dependent cell-mediated cytotoxicity, whereas E₂, P₄, and estrone increased superoxide anion production. The authors suggested that steroids and eicosanoids can directly influence neutrophil activity (either stimulatory, inhibitory, or both), and they may act as direct or indirect modulators of neutrophils.

Watson (1988) collected and incubated neutrophils from ovariectomized mares with PGE₂, PGF_{2α}, or LTB₄ to determine whether these had an effect on neutrophil phagocytosis and bactericidal activities. The final concentrations of PGE₂ and PGF_{2α} used in the phagocytosis assay were 1.7 and 167 ng/mL and were 2.5 and 250 ng/mL for the bactericidal assay. Leukotriene B₄ was added at a final concentration of 1.3 and 2.0 ng/mL for phagocytosis and bactericidal assays, respectively. Addition of PGE₂ had no effect on phagocytic or bactericidal activities, whereas addition of LTB₄ enhanced the phagocytic and bactericidal activities of neutrophils. Addition of PGF_{2α} increased the bactericidal activity but had no effect on their phagocytic ability. The author suggested that the effect of prostaglandins on neutrophils is greater on the phagocytic and bactericidal activities than on migratory activities, whereas LTB₄ may be involved more as a chemotactic agent.

In summary, members of the eicosanoid family have differing effects on neutrophil activities. Cyclooxygenase products (specifically PGE₂ and PGF_{2α}) seemed to have opposite effects on neutrophils; generally, PGE₂ inhibited and PGF_{2α} enhanced neutrophil activities. However, LTB₄ (a lipoxygenase product) had powerful chemotactic properties as well as enhancing phagocytic and bactericidal activities of neutrophils.

Endocrine Profiles in Cattle With and Without Uterine Infections

The release of $\text{PGF}_{2\alpha}$ from the uterus may reflect the amount of damage and (or) repair of the endometrium. Cattle with uterine infections have a significantly different hormonal profile than cows without a uterine infection. In particular, a relatively stable metabolite of $\text{PGF}_{2\alpha}$ (PGFM; 13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$) is increased in cows with uterine infections (Watson, 1984; Thompson *et al.*, 1987; Del Vecchio *et al.*, 1992, 1994; Bekana *et al.*, 1996).

Thompson *et al.* (1987) reported that PGFM concentrations are increased (as much as five times compared with controls) during the first 5 d postpartum in cows that developed a uterine infection. Del Vecchio *et al.* (1994) reported that plasma concentrations of PGFM were positively correlated with the development of a uterine infection in postpartum cows. On average, cows were detected with uterine infections between 21 and 28 d postpartum. The authors also suggested that PGFM measurements could be used to help detect subclinical uterine infections.

Retained placental membranes are a major factor in the predisposition of cows to uterine infections. Bekana *et al.* (1994) reported that *A. pyogenes*, *Fusobacterium spp.*, and *Bacteriodes spp.* could be consistently cultured from uteri of cows that retained their placental membranes. Heuwieser *et al.* (1992) investigated the prostaglandin metabolite, PGFM and PGEM (PGE_2 metabolite), relationships between cows with “normal” and spontaneous calving versus cows with dystocia and the development of retained fetal membranes. The PGFM concentrations decreased within 3 h after parturition only in cows that underwent spontaneous calving. Three hours after calving, cows that had dystocia and shed their placental membranes had higher PGFM

concentration than cows that experienced dystocia and retained their placental membranes. However, this observation was expected because cows that have dystocia during calving typically have more uterine trauma than cows with spontaneous calving. Interestingly, cows that retained their placental membranes had a slower decrease in PGEM concentrations. The authors suggested that the negative effects of increased PGE₂ (by measurement of PGEM) was associated with the proper expulsion of the placenta. These observations suggested that there is either a deficiency in PGF_{2α} synthase or a failure of the placenta to shift from PGE₂ to PGF_{2α} production (Horta, 1984; Gross *et al.*, 1985) when the placenta is retained.

Current Treatment Regimens for Uterine Infections

The rationale for treatment of uterine infections in livestock is to provide an economic return for the owner. Because bacteria cause uterine infections, most people have a “knee-jerk” reaction and automatically think that antibiotics are the only practical treatment. However, the use of PGF_{2α} analogues are gaining favor as we learn more about how P₄ is responsible for the down-regulation of the uterine immune system. Most cows with uterine infections will resolve them spontaneously, but some will have prolonged calving intervals, produce less milk, and have fewer calves over their lifetime (reviewed by Lewis, 1997). Evaluation of therapy should include a comparison of days open and services per conception in treated and untreated cows that meet a strict classification of uterine infections. Problems associated with evaluation of the economic return of treatment include small numbers of animals used in those studies, lack (or absence) of untreated controls, and the variation between and within herds (reviewed

by Bretzlaff, 1987). In this section, I will discuss some of the experiments that were conducted to treat uterine infections.

Antibiotics. Fuquay *et al.* (1975) placed 1 g of neomycin sulfate into the uteri of dairy cattle 24 h after parturition. Uteri and ovaries of all cows were palpated 17 to 24 d after parturition and at 14-d intervals until uterine involution was complete. All cows were inseminated at the first estrus after 60 d postpartum and at each estrus thereafter until conception. Antibiotic-treated cows required more services per conception (1.7 vs 1.4 for controls) and had more open days (100.5 vs 88.5) than controls. Thus, the authors did not suggest routine intrauterine treatment of cows with neomycin sulfate.

Steffan *et al.* (1984) used four dairy herds with a high prevalence of metritis. Cows diagnosed with a uterine infection 30 d after parturition were assigned to receive intrauterine treatment (200 mg of chloramphenicol, 300 mg of framycetin [a kanamycin-like compound]), 10 mg of hydrocortisone, and 254,000 IU of vitamin A) once a week for 3 wk, Lutalyse (a PGF_{2α} analogue; 2 x 25 mg doses at 14 d apart), or saline (twice a day as in Lutalyse treated cows). *Arcanobacterium pyogenes* was isolated from 51% of the cows. Two thirds of the cows resumed ovarian activity by 30 d after parturition. The cycling cows that received antibiotics, PGF_{2α} analogue, or a placebo conceived at 124, 116, and 131 d after parturition; whereas the noncycling cows that received antibiotics, PGF_{2α} analogue, or a placebo conceived at 166, 137, and 188 d after parturition. The average recovery time was greater for the antibiotic-treated cows than for cows treated with PGF_{2α} (54 vs 46 d). The authors suggested that the ovarian status of the cows greatly improved the interval from calving to conception.

Thurmond *et al.* (1993) investigated the possibility that either penicillin G (1 million IU in 40 mL of sterile water) or oxytetracycline (500 mg in 20 mL of sterile water) infused into the uteri of dairy cows with a postpartum uterine infection could reduce the calving to conception interval. Cows were randomly allotted to receive either nothing or one of the two antibiotics. No differences were observed between the control cows or cows infused with the antibiotics. From these results, it does not seem efficacious to treat cows with a uterine infection with intrauterine antibiotics.

Prostaglandin F_{2α} Analogues. Although used originally for estrus synchronization, PGF_{2α} analogues are increasingly used to treat postpartum uterine infections, especially in cows without CL. However, the mechanism is not completely understood. Prostaglandin F_{2α} probably induces luteolysis in cows with CL, and this reduces P₄ and allows E₂ to increase (Fogwell *et al.*, 1978). These changes in P₄ and E₂ concentrations should up-regulate the immune system and allow the animal to clear the uterine infection. Wade and Lewis (1996) reported that a PGF_{2α} analogue induced the uterus to secrete more PGF_{2α}. It is possible that the exogenous PGF_{2α} increases endometrial availability of free arachidonic acid, which can be converted to PGF_{2α} through the cyclooxygenase pathway. Free arachidonic acid can also be converted through the lipoxygenase pathway to LTB₄. Both PGF_{2α} and LTB₄ can act as chemotactic agents, and LTB₄ is a potent activator of neutrophil activity (Nilsson *et al.*, 1991; Hoedemaker *et al.*, 1992). Exogenous PGF_{2α} may also increase uterine release of PGE₂, which is consistently associated with decreased neutrophil and lymphocyte proliferation (Nilsson *et al.*, 1991). However, uterine PGE₂ typically increases blood flow, and it may enhance diapedesis (Resnik and Brink, 1978).

In a prospective study, Wenzel *et al.* (1993) sent questionnaires to 332 beef producers and 279 dairy producers in Alabama about their use of PGF_{2α} analogues. Questionnaire response rate among beef producers, dairy producers, and veterinarians was 64.5, 61.6, and 75.5%, respectively. Only 7.9% of beef producers used PGF_{2α} analogues, and this was used predominantly for estrus synchronization. In contrast, 66.5% of dairy producers used PGF_{2α} analogues for unobserved estrus, uterine infections, retained fetal membranes, cystic ovaries, estrus synchronization, and induction of parturition.

Etherington *et al.* (1995) designed an experiment to compare the effects on reproductive performance of three commercially available PGF_{2α} analogues (dinoprost [Lutalyse], cloprostenol [Estrumate], and fenprostalene [Synchrocept-B]) administered to dairy cows. Cows (n = 301) were randomly assigned to receive either a placebo or one of the three PGF_{2α} analogues between 24 and 31 d postpartum. Cows were artificially inseminated at the first estrus after d 50 postpartum. There were no differences among treatments with respect to the incidence of retained fetal membranes, endometritis, pyometra, anestrus, number of services per conception, calving-to-first estrus interval, culling percentage, parity, and all factors combined. The calving-to-conception interval was less in cows that received any of the three PGF_{2α} analogues than in controls. Therefore, the use of PGF_{2α} analogues administered between 24 and 31 d postpartum was beneficial for reproductive performance.

Pankowski *et al.* (1995) compared three programs for reproductive management of the postpartum period for reproductive performance and net economic benefit. In one program, cows received a PGF_{2α} analogue at 24 to 36 d postpartum for reproductive

therapy. In the second program, cows received an additional injection of a $\text{PGF}_{2\alpha}$ analogue at 36 to 49 d postpartum. These programs were compared with a postpartum program of rectal palpation based on veterinary intervention. Survival analysis indicated that cows that received $\text{PGF}_{2\alpha}$ had a 11% higher rate of first AI and 10% higher rate of pregnancy than cows receiving rectal palpation. Costs for the $\text{PGF}_{2\alpha}$ (a single injection at 25 to 32 d postpartum) were \$4.46 less per cow than the costs for the rectal palpation program. Furthermore, costs for the $\text{PGF}_{2\alpha}$ treatment at a scheduled interval (a single injection at 25 to 32 d postpartum and again 14 d after the initial injection) were \$3.61 less per cow than the rectal palpation program, and it saved six median days open per cow compared with those on the rectal palpation program. Thus, the authors suggested that a $\text{PGF}_{2\alpha}$ program, in which $\text{PGF}_{2\alpha}$ analogues are used at scheduled intervals, is cost effective and may improve herd reproductive performance compared with more traditional (rectal palpation) programs.

To justify treating uterine infections, there must be an economic return for the owner. One common reaction is to use antibiotics to treat uterine infections; however, with increased usage of antibiotics, chances of bacterial resistance increases. Issues concerning antibiotic residues as well as costs of the antibiotics and discarded milk must be considered. Using antibiotics to treat uterine infections offers no additional profit potential compared with letting cows spontaneously clear the infection. It has been hypothesized that the ovarian status of the cows can also have an influence on treatment protocol efficiency. Cows that began cycling earlier and cleared their infections had a decreased calving-to-conception interval. By using $\text{PGF}_{2\alpha}$ analogues, one can avoid the possibility of bacterial resistance and having antibiotic residues in

milk. Analogues of $\text{PGF}_{2\alpha}$ are cheaper than a program with intervention from a veterinarian. There also seems to be a consistent positive effect of using $\text{PGF}_{2\alpha}$ analogues on calving-to-conception intervals at specific days after parturition.

Summary

Uterine infections cost livestock producers millions of dollars each year in lost milk production, prolonged parturition intervals, and reduced calf crops. Studies indicated that livestock do not usually develop a uterine infection unless they are under the influence of P_4 . Progesterone down-regulates the uterine immune system, specifically lymphocyte and neutrophil activities. Under the influence of P_4 , lymphocytes have a reduced blastogenic response to mitogens. Also, neutrophil chemotaxis, superoxide anion production, and final destruction of a pathogen are reduced. When P_4 concentrations are low and(or) livestock are in estrus, uterine infections seldom develop. Several investigators have suggested that this response is due to the actions of E_2 . However, estrogens have little or no effect (unless administered at supraphysiological doses) on the immune system.

The uterus is generally inflamed during the development and presence of a uterine infection. Due to the discovery that many prostaglandins and other arachidonic acid metabolites are directly involved with inflammation, considerable attention has been focused on the actions of these compounds on the immune system. Prostaglandin E_2 is generally considered to down-regulate, whereas $\text{PGF}_{2\alpha}$ is considered to up-regulate, immune cell activities. Prostaglandin $\text{F}_{2\alpha}$ is considered to be the major luteolysin in livestock. It is possible that $\text{PGF}_{2\alpha}$ analogue injections could reduce P_4

concentrations, cause up-regulation of the uterine immune system, and promote clearance of a uterine infection. In the United States, use of $\text{PGF}_{2\alpha}$ analogues is the most commonly used treatment for uterine infections. A typical reaction to treat bacterial infections, including uterine infections, is to treat them with antibiotics. However, current data suggest that treatment of a uterine infection with antibiotics may extend calving interval.

In conclusion, P_4 seems to be the ovarian hormone with the greatest effect on uterine immune functions. Estrogen has little or no effect. Administration of $\text{PGF}_{2\alpha}$ analogues causes a decrease in P_4 (removal of its immunosuppressive effects), allows for the uterine immune system to up-regulate, and promotes a clearance of uterine infections.

CHAPTER III

PROSTAGLANDINS MODULATE THE UTERINE RESPONSE TO INFECTIOUS BACTERIA IN POSTPARTUM EWES

Introduction

Nonspecific uterine infections reduce the reproductive efficiency of livestock (Arthur *et al.*, 1989; Lewis, 1997). Regardless of management intensity, 10% of postpartum dairy cows will develop uterine infections, and this may exceed 40% (Arthur *et al.*, 1989; Lewis, 1997). Impaired or down-regulated neutrophil and lymphocyte proliferation seems to increase the susceptibility of cows to uterine infections, and bacteria that are found commonly in the environment can then become established in the uterus (Lewis, 1997).

The uterine immune system during the periparturient period is typically up-regulated until it is exposed to progesterone. After exposure to P_4 , the uterine immune system is down-regulated, and the incidence of uterine infections often increases. Several factors including dystocia (difficult birth), poor nutritional status of the dam, inadequate hygiene at the time of calving, and retained fetal membranes can predispose a cow and increase her chances of developing a uterine infection. Progesterone down-regulates the uterine immune system, specifically lymphocyte and neutrophil activities. Under the influence of P_4 , lymphocytes have a reduced blastogenic response to mitogens. Also, neutrophil chemotaxis, superoxide anion production, and final destruction of a pathogen are reduced. When P_4 concentrations are low and (or) livestock are in estrus, uterine infections seldom develop.

One of the early signs of a uterine infection is a generalized inflammation of either the endometrium or the uterus. Several arachidonic acid metabolites, the prostaglandins and leukotrienes, act as direct mediators of inflammation (Fantone *et al.*, 1985). Based upon these observations, the effect of arachidonic acid metabolites on leukocytes has attracted considerable attention. Much of this attention has focused on the effects of PGE₂ and the use of PGF_{2α} (or its analogues). Generally, PGE₂ is considered to down-regulate lymphocyte and neutrophil activities, whereas PGF_{2α} is considered to up-regulate the immune system. It is possible that PGF_{2α} up-regulates the immune system because it can decrease P₄ concentrations and cause clearance of uterine infections. However, few have investigated the direct action of arachidonic acid metabolites on the immune cells (specifically on neutrophil and lymphocyte activities) (Hoedemaker *et al.*, 1992; Kehrl *et al.*, 1989a,b). Because prostaglandins have a short half-life in tissue or in circulation, most research on arachidonic acid metabolites has been performed *in vitro*.

Materials and Methods

The objectives for this experiment were to:

- 1) Determine the role of the ovaries in the development of a uterine infection in response to infectious bacteria.
- 2) Determine the role of P₄ administration in the development of a uterine infection in response to infectious bacteria.
- 3) Monitor the *in vitro* changes in vena caval lymphocyte activity after exposure of the uterus to infectious bacteria.

- 4) Determine *in vitro* changes in lymphocyte proliferation in response to incubation with PGE₂ and(or) indomethacin (a prostaglandin synthesis inhibitor).

General. Experiment 1 was conducted during 1999. Primiparous and multiparous Dorset, Suffolk, and mixed breed ewes (2 to 5 yr old) from the Virginia Tech Sheep Center were used for this experiment. All ewes were healthy and had no history of uterine infections. In the spring, vasectomized rams were used twice daily to check ewes for signs of estrus. Ewes with at least two consecutive estrous cycles of 15 to 16 d in duration had their estrous cycles synchronized and were field mated to fertile rams. Only ewes that had an unassisted and a “normal” parturition were used for the treatment phase of this experiment. After parturition (d 0), lambs were weaned from all ewes on d 1.

In vivo Experimental Protocol. Ewes were assigned to randomized treatments in a 2 x 2 factorial arrangement. Progesterone administration (P₄ vs OIL) and ovariectomy (SHAM or OVEX) were the main effects. See Appendix A for all specific surgical techniques. Feed and water were restricted on the evening of d 8. On d 9, vena caval catheters were placed via the saphenous vein in all ewes. The catheters were positioned at 55 cm from the leading tip of the catheter to collect blood that had recently emptied from the uterus into the vena cava. By using vena caval catheters, lymphocytes that had filtered through the uterus and had been affected by the uterine environment could be collected. In our experience, as well as that of other investigators (Benoit and Dailey, 1991), 55 cm has been the average placement for ewes. On d 10 postpartum, ewes received their respective ovarian treatment (OVEX or SHAM).

Ewes began receiving either canola oil (OIL) or P₄ in oil (P₄; 5 mg/2.5 mL of canola oil i.m., 2x/d) on d 10 postpartum. The final treatment combinations were OIL-SHAM (n = 5), OIL-OVEX (n = 4), P₄-SHAM (n = 4), and P₄-OVEX (n = 5). All uteri were inoculated with 56 x 10⁷ cfu of *A. pyogenes* and 26 x 10⁷ cfu of *E. coli* using a laparoscopic procedure on d 15. The bacteria were suspended in 5 mL of saline (.9% NaCl solution) and injected into the uterus. Vena caval blood (12 mL) was collected into sterilized heparinized tubes on d 9, 14, 16, 17, 18, and 19 for determination of P₄, PGF_{2α}, and PGE₂ concentrations and lymphocyte collection. Plasma was stored at -20°C until PGF_{2α} and PGE₂ RIA could be performed. Another blood sample (12 mL) was collected at the same time for serum collection and placed into sterilized tubes. Serum was separated and stored at -20°C until the P₄ RIA could be performed.

All ewes were killed on d 20 for collection of reproductive tracts. Uteri were collected and flushed with 20 mL of PBS, and the amount of sediment was used to determine whether the uterus was resistant or susceptible to infection. See Figure 2 for a timeline of events for Experiment 1.

In vitro Experimental Protocol. Vena caval lymphocytes were collected from each ewe on d 9, 14, 16, 17, 18, and 19 to determine their blastogenic response (previously described) to mitogens (Con A and LPS). Lymphocytes were arranged in a 3 x 2 x 2 factorial arrangement with mitogens (none, Con A, or LPS), PGE₂, and indomethacin (INDO) as the main effects. The final incubation volume included 100 μL (1 x 10⁵ cells) of lymphocyte suspension, 100 μL of respective mitogen suspension (unstimulated, 1 μg/well of Con A, or .5 μg/well of LPS), and respective *in vitro* treatment (none, PGE₂, INDO, or PGE₂ + INDO; these treatments will be referred to as the well treatment)

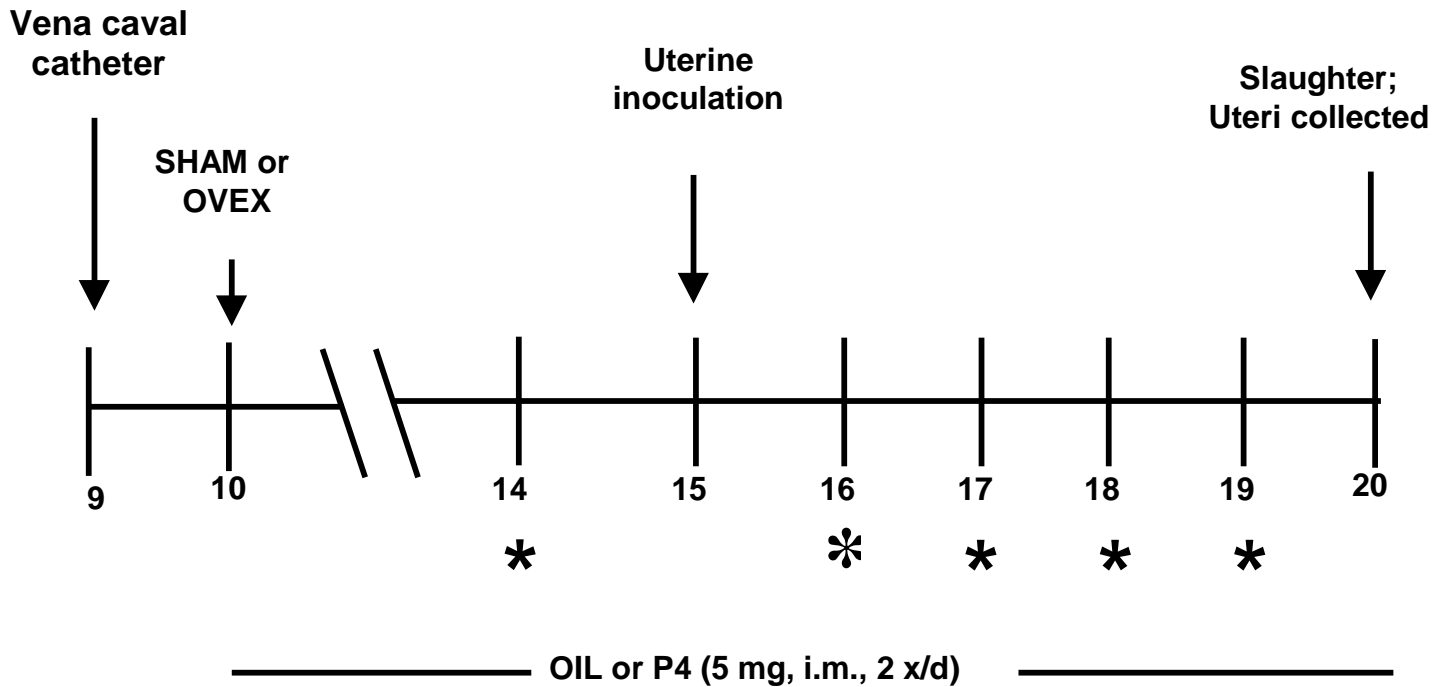


Figure 2. Timeline of events for Experiment 1. All ewes were fitted with a vena caval catheter on d 9 postpartum (d 0 = day of parturition). On d 10, ewes were either ovariectomized or sham ovariectomized. Progesterone supplementation was initiated on d 10 and continued until all ewes were slaughtered on d 20 for collection of reproductive tracts. All ewes had their uteri inoculated with bacteria (56×10^7 cfu of *A. pyogenes* and 26×10^7 cfu of *E. coli*) on d 15. Vena caval blood was collected on days marked with stars to quantify serum concentrations of progesterone, plasma concentrations of prostaglandins ($\text{PGF}_{2\alpha}$ and PGE_2), and for collection of lymphocytes for submission to a lymphocyte proliferation assay.

suspended in 10 μL of 200 proof ethanol. The final concentration of PGE_2 and indomethacin was 10^{-7} M per well.

Bacterial Culture and Inoculations. Two bacterial species were used to induce infections in ewes: *Arcanobacterium pyogenes* and *E. coli*. The strains of these bacteria were collected from a dairy cow at Virginia Tech diagnosed with endometritis, and both strains are β -hemolytic and pathogenic to cows, ewes, and pigs (Del Vecchio *et al.*, 1992; Ramadan *et al.*, 1997; Wulster-Radcliffe, 2000). Both were previously purified and kept in skim-milk-broth medium at -20°C . An aliquot of frozen bacteria was thawed, 10 to 20 μL were placed on Brain Heart Infusion agar (Difco, Detroit, MI), and these were incubated in a humidified chamber at 37°C for 24 h. A single colony from each culture was taken aseptically, transferred to another plate of agar, and incubated for another 24 h. The cultures were washed separately with 10 mL of Brain Heart Infusion broth (Difco, Detroit, MI), transferred into side-arm flasks (Bellco, Vineland, NJ), and held in a shaking 37°C water bath for at least 6 h. Every hour, the flasks were rotated, allowing broth to enter the side arm, and optical densities were measured until it reached the desired turbidity need for inoculation. The appropriate amount of broth culture was transferred into sterile glass tubes, centrifuged at $10 \times g$ for 5 min, and the pellet resuspended in 5 mL of sterile saline.

Lymphocyte Blastogenesis. Lymphocyte separation and blastogenic measurements were performed as described Burrells and Wells (1977). Briefly, vena caval lymphocytes were separated from heparinized whole blood. After centrifugation at $2,800 \times g$ for 15 min at 4°C , the buffy layer was transferred, mixed with 5 mL of filter-sterilized Hank's balanced salt solution (HBSS; Gibco BRL, Grand Island, NY), gently

layered on top of 4 mL of Histopaque (Sigma Chemical), and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte layer separated on top of the Histopaque. The lymphocytes were transferred with sterile Pasteur pipettes into other sterile tubes, washed twice with HBSS, and suspended in complete RPMI-1640 medium (Gibco) supplemented with 2 mM of glutamine, 10% of fetal calf serum (Gibco), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin (Sigma Chemical). The total number of live lymphocytes was determined with the trypan blue exclusion method and suspended to a final concentration of 1×10^6 cells/mL of medium. Two types of mitogens were used in this experiment: Con A and LPS. Concanavalin A ($1 \mu\text{g}/\text{well}$; Sigma Chemical, C-5275) is a nonspecific T-cell stimulator, and LPS ($.5 \mu\text{g}/\text{well}$; Sigma Chemical, L-3755) is a B-cell activator. Lymphocytes (1×10^5) were added to all wells in a row in sterilized 96-well culture plates (Beckton Dickenson Labware, Lincoln Park, NJ). Wells 1 to 3 were used to determine basal activity of lymphocytes, Wells 4 to 6 were used to determine T cell activity (cells plus Con A), Wells 7 to 9 were used to determine B cell activity (cells plus LPS), and Wells 10 to 12 were used to determine cell viability at the time of lymphocyte harvest. Each plate was incubated for 48 h in a humidified chamber at 37°C in 5% CO_2 . Sixteen hours before harvesting of cells, $1 \mu\text{Ci}$ of [^3H]thymidine (specific activity of 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in complete RPMI-1640 medium was added to Wells 1 to 9. At the end of the incubation period, lymphocytes were transferred to fiberglass filter paper (Whatman Ltd., Maidstone, England) with the use of a Mini Mash system (Whittaker, Walkersville, MD). Filter paper was cut according to each corresponding well and placed into scintillation vials (Mini poly-Q vials; Beckman, Fullerton, CA). Lymphocytes were dissolved by incubation with 400 μL of .3 M HCl for

30 min at room temperature; 40 μ L of NaOH was added to neutralize the solution. One milliliter of scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was added to each vial, thoroughly shaken, placed into a liquid scintillation spectrophotometer, and radioactive decay was quantified. The total amount of incorporated [3 H]thymidine was expressed in picomoles. This was calculated with the following equation:

[3 H]thymidine incorporation in pmol = cpm \times (100/E) \times [1/(S \times 2.22)], where cpm = counts per minute for each vial, E = efficiency of counting, and S = specific activity of [3 H]thymidine. The efficiency of counting was calculated from the cpm and a tritium quench curve. The quench curve consisted of a set of vials containing a known amount of [3 H]H $_2$ O, equal volume of scintillation cocktail, and increasing amounts of carbon tetrachloride (CCl $_4$; a quenching agent). The counting efficiency was calculated by the division of the measured cpm by the known dpm.

Progesterone Assay. A [125 I]progesterone RIA kit (Diagnostic Products Company, Los Angeles, CA) was used to quantify P $_4$ in all samples after treatment. Samples were assayed in duplicate. The intra-assay CV was 37%, respectively.

Prostaglandin Assays. Plasma concentrations of PGF $_{2\alpha}$ were determined with a RIA using the procedure of Pexton *et al.* (1975), and PGE $_2$ concentrations were determined with a RIA using the procedure of Lewis *et al.* (1978). The intra- and interassay CV for PGF $_{2\alpha}$ were 11.1 and 14.4%, respectively. The intra- and interassay CV for PGE $_2$ were 13.6% and 30.1%, respectively.

Statistical Analyses. The MIXED procedures of Statistical Analysis System (SAS, 1996) were used to analyze the data. Hormonal data (P $_4$, PGE $_2$, and PGF $_{2\alpha}$) and differential white blood cell counts were analyzed as a split-plot design. Ovary treatment

(SHAM vs OVEX) and hormone treatment (OIL vs P₄) were the main effects in the models. The initial model used to analyze the data included the following independent variables: ovarian treatment, hormone treatment, ovarian x hormonal treatment, ewe nested within ovarian x hormonal treatment, time, time x ovarian treatment, time x hormone treatment, time x ovarian treatment x hormone treatment, and residual. The error term for the main plot was ewe nested within ovarian x hormonal treatment, and the residual was the error term for the subplot. The initial model used to analyze blastogenic responses to mitogens and *in vitro* treatments was for a split-split-split-plot design. The model included the same independent variables as the hormonal data in addition to two split plots that included terms for the mitogens (unstimulated, Con A, or LPS), the individual well treatments (control, PGE₂, INDO, and PGE₂ + INDO), all appropriate interactions, and their error terms. The LSMEANS, PDIFF, and STDERR options in SAS were used to compare the response of each variable when the MIXED procedure indicated a significant effect of ovarian treatment (SHAM vs OVEX) or P₄ treatment (OIL vs P₄).

Results of Experiment 1

Development of Uterine Infections.

All ewes that were treated with P₄ developed uterine infections, whereas none of the ewes treated with OIL developed infections.

Lymphocyte Blastogenic Activity.

Neither P₄ nor OVEX treatment affected the unstimulated, Con A-stimulated, or LPS-stimulated incorporation of [³H]thymidine. However, OVEX ewes treated with OIL had greater average [³H]thymidine incorporation than did OVEX ewes treated with P₄ (5.8 vs 4.1 pmol, respectively, $P = .02$; Figure 3). Terms for mitogen, well treatment, and the mitogen x well treatment interaction were significant. The Con A-stimulated blastogenesis was greater ($P < .001$) than unstimulated and LPS-stimulated blastogenesis (2.3, 10.9, and 2.7 pmol for unstimulated, Con A, and LPS, respectively). Irrespective of mitogen, average lymphocyte blastogenesis was inhibited ($P < .05$) when incubated with PGE₂ or PGE₂ + INDO (6.0, 6.7, 4.0, and 4.3 for control, INDO, PGE₂, and PGE₂ + INDO, respectively; SEM = .5). There was also a mitogen x well treatment interaction. Concanavalin A-stimulated lymphocyte blastogenesis was less ($P < .01$) in PGE₂ and PGE₂+INDO (7.5 and 8.3 pmol) than in control or INDO (12.9 and 14.7 pmol; SEM = .5; Figure 4).

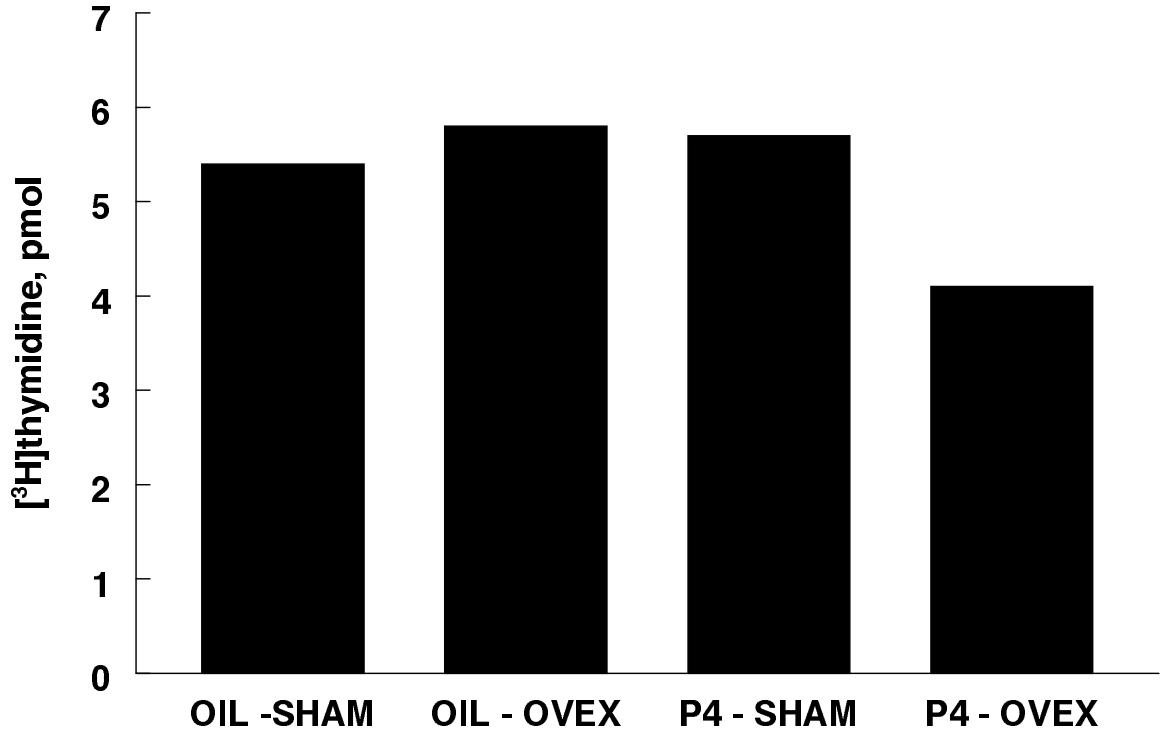


Figure 3. The effect of *in vivo* treatments on lymphocyte proliferation. Vena caval blood was collected from postpartum ewes that were given intrauterine inoculations of bacteria. Lymphocytes were recovered and incubated *in vitro*. The interaction between progesterone supplementation and ovarian treatment was significant ($P = .02$; SEM = .5). Ewes that received progesterone supplementation and were ovariectomized had the least amount of incorporated [³H]thymidine].

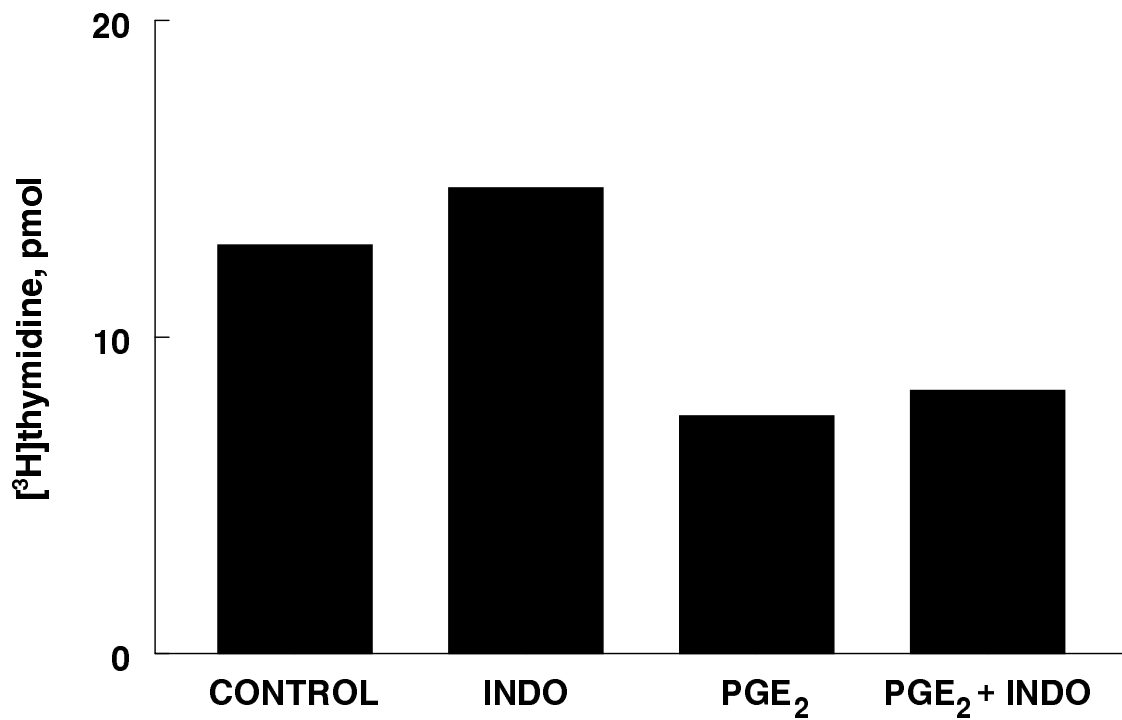


Figure 4. The effect of *in vitro* treatment on the Concanavalin A-stimulated lymphocyte proliferation. Vena caval blood was collected from postpartum ewes that were given intrauterine inoculations of bacteria. Lymphocytes were recovered and incubated *in vitro*. The interaction between mitogen and well treatment was significant ($P < .01$; SEM = .5). Lymphocytes stimulated with Con A and incubated with PGE₂ or PGE₂ + INDO had the least amount of incorporated [³H]thymidine].

Prostaglandin and Steroid Data.

There were no differences in vena caval concentrations of either $\text{PGF}_{2\alpha}$ or PGE_2 in this experiment (Figure 5). The average concentration of PGE_2 in SHAM ewes was 241 pg/mL, whereas the average was 232 pg/mL for OVEX ewes. The average concentration of PGE_2 in OIL ewes was 296 pg/mL, whereas the average was 195 pg/mL for ewes treated with P_4 . The average concentration of $\text{PGF}_{2\alpha}$ in SHAM ewes was 176 pg/mL, and the average was 245 pg/mL for OVEX ewes. The average concentration of $\text{PGF}_{2\alpha}$ in OIL treated ewes was 239 pg/mL, whereas the average was 140 pg/mL for ewes treated with P_4 .

Treatment with P_4 affected ($P < .0001$) the vena caval concentrations of P_4 (Figure 6). The average concentrations of P_4 was greater in P_4 treated ewes (.5 vs 3.6 ng/mL for OIL and P_4 treated ewes, respectively). Progesterone concentrations fluctuated across day of the experiment, but there was no significant effect of day.

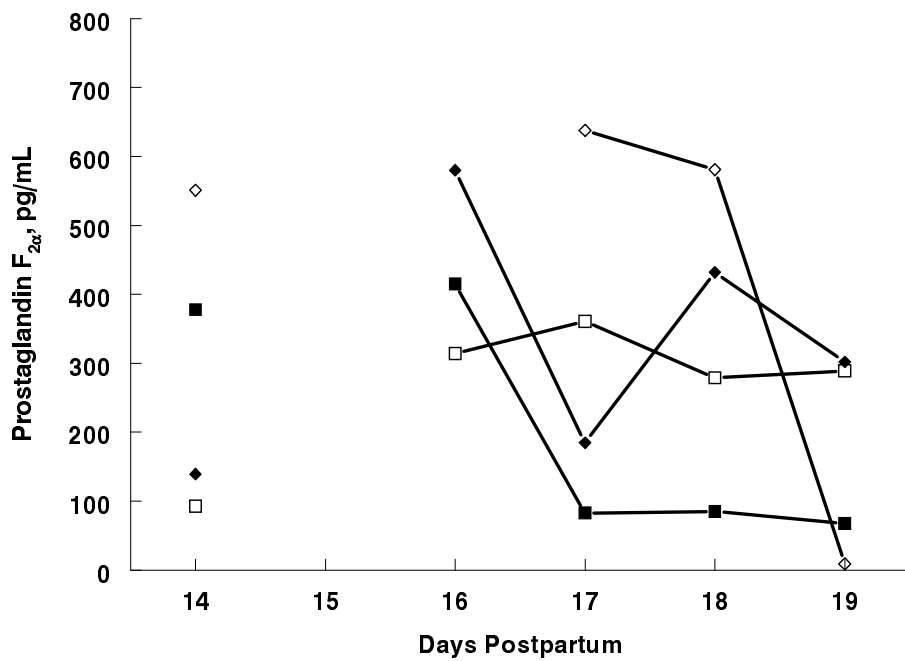
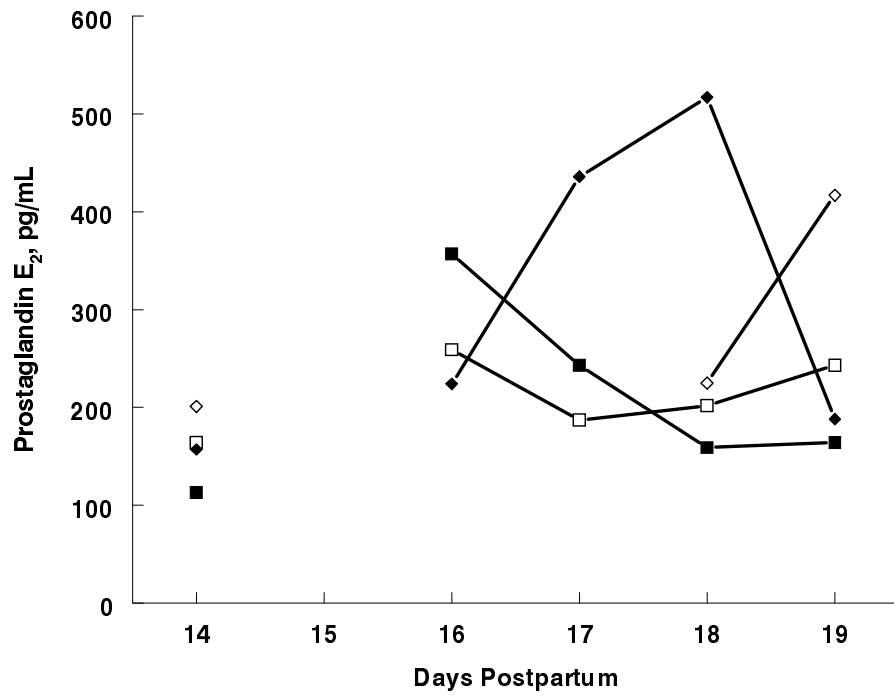


Figure 5. The average concentrations of prostaglandins in vena caval blood from ewes given intrauterine inoculations of bacteria on d 14 postpartum in Experiment 1.

PGE₂ SEM = 21 pg/mL; PGF_{2α} SEM = 37 pg/mL

◆ OIL-SHAM; ◇ OIL-OVEX; ■ P4-SHAM; □ P4-OVEX

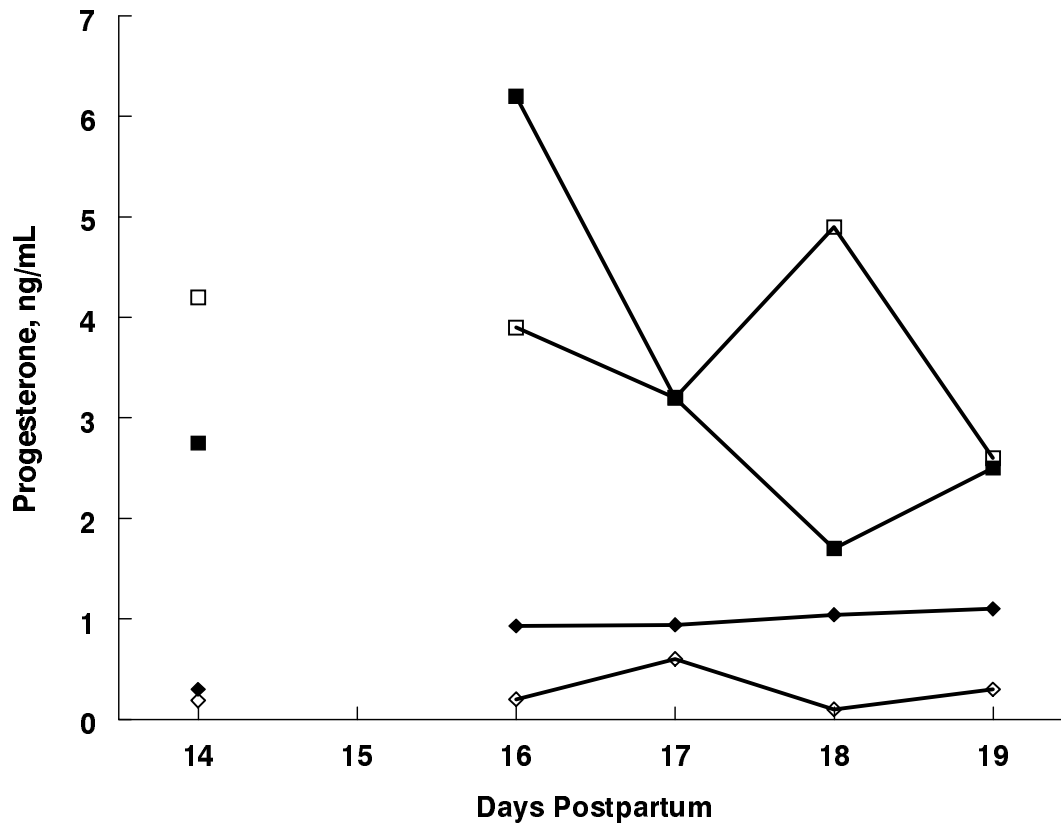


Figure 6. The average concentrations of progesterone in vena caval blood from ewes given intrauterine inoculations of bacteria on d 14 postpartum in Experiment 1.

Concentrations of progesterone were greater ($P < .001$; SEM = .1 ng/mL) in ewes receiving progesterone supplementation.

◆ OIL-SHAM; ◇ OIL-OVEX; ■ P4-SHAM; □ P4-OVEX

CHAPTER IV

PROSTAGLANDINS AND PHASE OF THE ESTROUS CYCLE MODULATE THE UTERINE RESPONSE TO INFECTIOUS BACTERIA IN ESTROUS CYCLIC EWES

Introduction

Millions of dollars are lost each year due to the reduced reproductive efficiency caused by nonspecific uterine infections (Arthur *et al.*, 1989; Lewis, 1997). Current estimates of incidence of uterine infections range from 10% to 40% in dairy herds in the United States (Arthur *et al.*, 1989; Lewis, 1997). Impaired or down-regulated neutrophil and lymphocyte activities seems to increase the susceptibility of cows to uterine infections, and bacteria that are found commonly in the environment (*A. pyogenes* and *E. coli*) can then become established in the uterus (Lewis, 1997).

Typically, the uterine immune system is up-regulated until it is exposed to progesterone. After exposure to P₄, the uterine immune system is down-regulated, and the incidence of uterine infections often increases. Progesterone down-regulates the uterine immune system, specifically lymphocyte and neutrophil activities. Under the influence of P₄, lymphocytes have a reduced blastogenic response to mitogens. Also, neutrophil chemotaxis, superoxide anion production, and final destruction of a pathogen are reduced. When P₄ concentrations are low and(or) livestock are in estrus, uterine infections seldom develop. Several factors including dystocia (difficult birth), poor nutritional status of the dam, inadequate hygiene at the time of calving, and retained fetal membranes can predispose a cow and increase her chances of developing a uterine infection.

One of the early signs of a uterine infection is a generalized inflammation of either the endometrium or the uterus (Lewis, 1997). Several arachidonic acid metabolites, the prostaglandins and leukotrienes, act as direct mediators of inflammation (Fantone *et al.*, 1985). Based upon these observations, the effect of arachidonic acid metabolites on leukocytes has attracted considerable attention. Much of this attention has focused on the effects of PGE₂ and the use of PGF_{2α} (or its analogues). Generally, PGE₂ is considered to down-regulate lymphocyte and neutrophil activities, whereas PGF_{2α} is considered to up-regulate the immune system. It is possible that PGF_{2α} up-regulates the immune system because it can decrease P₄ concentrations and cause clearance of uterine infections. Few reports exist that attempt to link the action of arachidonic acid metabolites on lymphocyte and neutrophil activities. Most research in this area has been concerned more with *in vitro* action rather than on the *in vivo* action because prostaglandins have a short half-life in tissue and circulation. Moreover, most studies have failed to describe the link between the *in vitro* lymphocyte and neutrophil activities and the *in vivo* hormonal environments from which they were collected.

Materials and Methods

Experiments 2 and 3.

The objectives of these experiments were to:

- 1) Determine the effect of ovarian hormones in the development of a uterine infection in response to infectious bacteria.

- 2) Monitor the daily *in vitro* changes in lymphocyte proliferation and the development of a uterine infection in response to infectious bacteria.
- 3) Determine the relationship between vena caval concentrations of ovarian steroids and eicosanoids and the response of the uterus after exposure to infectious bacteria.
- 4) Monitor changes in the differential white blood cell counts during the follicular and luteal phases as they relate to the development of a uterine infection.
- 5) Determine, in Experiment 2, *in vitro* changes in lymphocyte proliferation in response to incubation with PGE₂ and(or) indomethacin.
- 6) Determine, in Experiment 3, *in vitro* changes in lymphocyte proliferation in response to incubation with PGF_{2 α} and(or) indomethacin.

General. Experiment 2 was conducted in the winter of 1998, and Experiment 3 was conducted in the winter of 1999. Primiparous and multiparous Dorset, Suffolk, and mixed breed ewes (2 to 9 yr old) from the Virginia Tech Sheep Center were used for this experiment. All ewes were healthy and had no history of uterine infections.

Vasectomized rams were used twice daily to check ewes for signs of estrus. Ewes with at least two consecutive estrous cycles of 14 to 15 d in duration had their estrous cycles synchronized. Feed and water were withheld for at least 24 h before ewes went to surgery.

In vivo Experimental Protocol. Ewes were assigned to randomized treatments in a 2 x 2 factorial arrangement. Stage of the estrous cycle (follicular vs luteal) and uterine inoculation (saline vs bacteria) were the main effects. On either d 0 or 6, all uteri were inoculated with either 35×10^7 cfu of *E. coli* and 56.25×10^7 cfu of *A. pyogenes* in 5 mL of saline or 5 mL of saline. For specific surgical techniques see Appendix A. Final

treatment combinations were follicular-saline, follicular-bacteria, luteal-saline, and luteal-bacteria. There were five ewes per treatment in Experiment 2 and six ewes per treatment in Experiment 3.

On d 7 of the estrous cycle prior to treatments, ewes received one i.m. injection of 15 mg of PGF_{2α} (Lutalyse; Upjohn & Pharmacia; Kalamazoo, MI) to induce estrus. On either d 0 or 6, polyvinyl catheters were placed in the vena cava at a point just anterior to where uteroovarian blood empties into the vena cava (Benoit and Dailey, 1991). By using vena caval catheters, lymphocytes that had filtered through the uterus and presumably had been affected by the uterine environment could be collected. Vena caval blood (12 mL) was collected into sterilized heparinized tubes for 3 d after inoculation (d 1, 2, and 3 for follicular-phase ewes and d 7, 8, and 9 for luteal-phase ewes) for determination of differential white blood cell counts, PGF_{2α}, and PGE₂ concentrations, and for collection of lymphocytes to examine their *in vitro* responses to mitogens and prostaglandin treatments. Another blood sample (12 mL) was collected at the same time for serum collection and placed into sterilized tubes. Serum was separated and stored at -20°C until the P₄ RIA could be performed. All ewes were killed 3 d after inoculation for collection of reproductive tracts. Uteri were collected and flushed with 10 mL of PBS, and the amount of sediment was used to determine whether the uterus was resistant or susceptible to infection. An aliquot (100 μL) of the uterine flushing was cultured on Brain Heart Infusion Agar in a humidified chamber at 37°C for 48 h to determine whether the uterus was contaminated with bacteria. Figure 7 shows a timeline of events for Experiments 2 and 3.

In vitro Experimental Protocol. Vena caval lymphocytes were collected from each ewe for 3 d after inoculation to determine their blastogenic response (previously described) to mitogens (none, Con A, or LPS). Lymphocytes were arranged in a 3 x 2 x 2 factorial arrangement with mitogens, prostaglandin, and indomethacin (INDO) as the main effects. In Experiment 2, PGE₂ was used as the *in vitro* prostaglandin treatment. In Experiment 3, PGF_{2α} was used instead of PGE₂. The final incubation volume included 100 μL (1 x 10⁵ cells) of lymphocyte suspension, 100 μL of respective mitogen suspension (unstimulated, 1 μg/well of Con A, or .5 μg/well of LPS), and respective *in vitro* treatment (none, respective prostaglandin, INDO, or respective prostaglandin + INDO) suspended in 10 μL of 200 proof ethanol. The final concentration of both prostaglandins and indomethacin was 10⁻⁷ M per well.

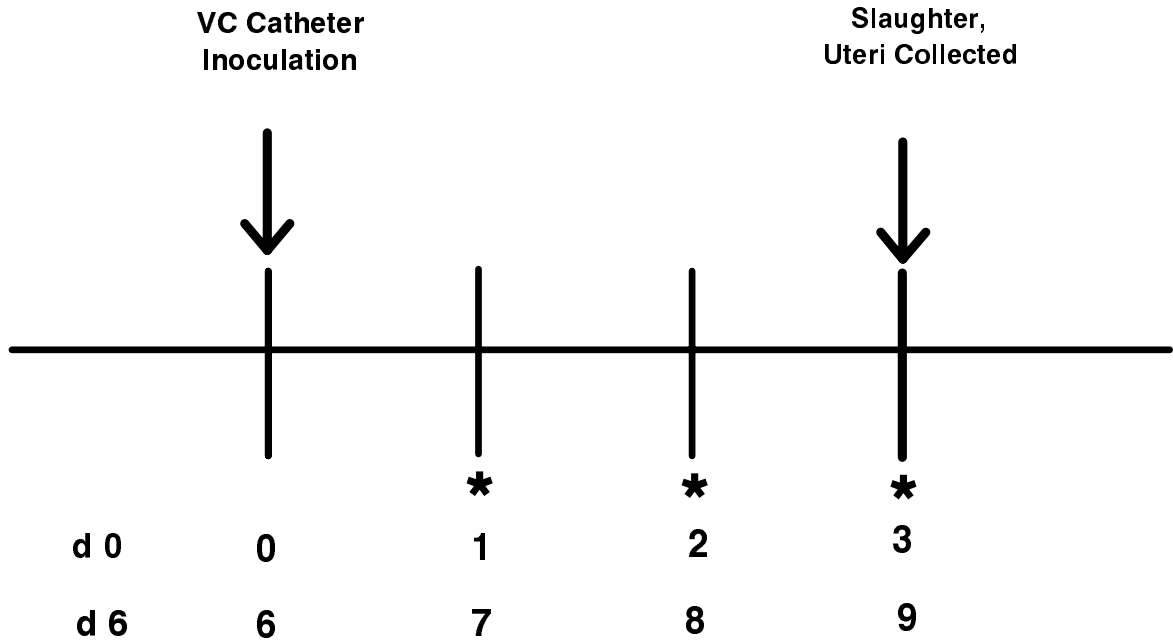


Figure 7. Timeline of Events for Experiments 2 and 3. All ewes were fitted with a vena caval catheter on their respective day of treatment (d 0 for follicular phase ewes or d 6 for luteal phase ewes) and had their uteri inoculated with either saline or bacteria (35×10^7 cfu of *E. coli* and 56.25×10^7 cfu of *A. pyogenes*). Vena caval blood was collected on days marked with stars to quantify serum concentrations of progesterone, plasma concentrations of prostaglandins ($\text{PGF}_{2\alpha}$ and PGE_2), determination of differential WBC counts, and for collection of lymphocytes for submission to a lymphocyte proliferation assay.

Bacterial Culture and Inoculations. We used two bacterial species to induce infections in ewes: *Arcanobacterium pyogenes* and *E. coli*. The strains of these bacteria were collected from a dairy cow at Virginia Tech diagnosed with endometritis, and both strains are β -hemolytic and pathogenic to cows and ewes (Del Vecchio *et al.*, 1992; Ramadan *et al.*, 1997). Both were previously purified and stored in skim-milk-broth medium at -20°C . An aliquot of frozen bacteria was thawed, 10 to 20 μL were placed on Brain Heart Infusion agar (Difco, Detroit, MI), and these were incubated in a humidified chamber at 37°C for 24 h. A single colony from each culture was taken aseptically, transferred to another plate of agar, and incubated for another 24 h. The cultures were washed separately with 10 mL of Brain Heart Infusion broth (Difco, Detroit, MI), transferred into side-arm flasks (Bellco, Vineland, NJ), and held in a shaking 37°C water bath for at least 6 h. Every hour, the flasks were rotated, allowing broth to enter the side arm, and optical densities were measured until it reached the desired turbidity need for inoculation. The appropriate amount of broth culture was transferred into sterile glass tubes, centrifuged at $10 \times g$ for 5 min, and the pellet resuspended in 5 mL of sterile saline.

Surgical Techniques and Lymphocyte Blastogenesis. All surgical techniques including midventral laparotomy, vena caval catheterization, and laparoscopy are described in detail in Appendix A. Lymphocyte separation and blastogenic measurements were performed as described Burrells and Wells (1977). Briefly, vena caval lymphocytes were separated from heparinized whole blood. After centrifugation at $2,800 \times g$ for 15 min at 4°C , the buffy layer was transferred, mixed with 5 mL of filter-sterilized Hank's balanced salt solution (HBSS; Gibco BRL, Grand Island, NY), gently

layered on top of 4 mL of Histopaque (Sigma Chemical), and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte layer separated on top of the Histopaque. The lymphocytes were transferred with sterile Pasteur pipettes into other sterile tubes, washed twice with HBSS, and suspended in complete RPMI-1640 medium (Gibco) supplemented with 2 mM of glutamine, 10% of fetal calf serum (Gibco), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin (Sigma Chemical). The total number of live lymphocytes was determined with the trypan blue exclusion method and suspended to a final concentration of 1×10^6 cells/mL of medium. Two types of mitogens were used in this experiment: Con A and LPS. Concanavalin A ($1 \mu\text{g}/\text{well}$; Sigma Chemical, C-5275) is a nonspecific T-cell stimulator and LPS ($.5 \mu\text{g}/\text{well}$; Sigma Chemical, L-3755) is a B-cell activator. Lymphocytes (1×10^5) were added to all wells in a row in sterilized 96-well culture plates (Beckton Dickenson Labware, Lincoln Park, NJ). Wells 1 to 3 were used to determine basal activity of lymphocytes, Wells 4 to 6 were used to determine T cell activity (cells plus Con A), Wells 7 to 9 were used to determine B cell activity (cells plus LPS), and Wells 10 to 12 were used to determine cell viability at the time of lymphocyte harvest. Each plate was incubated for 48 h in a humidified chamber at 37°C in 5% CO_2 . Sixteen hours before harvesting of cells, $1 \mu\text{Ci}$ of [^3H]thymidine (specific activity of 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in complete RPMI-1640 medium was added to Wells 1 to 9. At the end of the incubation period, lymphocytes were transferred to fiberglass filter paper (Whatman Ltd., Maidstone, England) with the use of a Mini Mash system (Whittaker, Walkersville, MD). Filter paper was cut according to each corresponding well and placed into scintillation vials (Mini poly-Q vials; Beckman, Fullerton, CA). Lymphocytes were dissolved by incubation with 400 μL of .3 M HCl for

30 min at room temperature; 40 μ L of NaOH was added to neutralize the solution. One milliliter of scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was added to each vial, thoroughly shaken, placed into a liquid scintillation spectrophotometer, and radioactive decay was quantified. The total amount of incorporated [3 H]thymidine was expressed in picomoles. This was calculated with the following equation:

[3 H]thymidine incorporation in pmol = cpm \times (100/E) \times [1/(S \times 2.22)], where cpm = counts per minute for each vial, E = efficiency of counting, and S = specific activity of [3 H]thymidine. The efficiency of counting was calculated from the cpm and a tritium quench curve. The quench curve consisted of a set of vials containing a known amount of [3 H]H $_2$ O, equal volume of scintillation cocktail, and increasing amounts of carbon tetrachloride (CCl $_4$; a quenching agent). The counting efficiency was calculated by the division of the measured cpm by the known dpm.

Progesterone Assay. A [125 I]progesterone RIA kit (Diagnostic Products Company, Los Angeles, CA) was used to quantify P $_4$ in all samples after treatment. Samples were assayed in duplicate. The intra- and interassay CV were 5.6 and 12.8%, respectively.

Prostaglandin Assays. Plasma concentrations of PGF $_{2\alpha}$ were determined with a RIA using the procedure of Pexton *et al.*, (1975), and PGE $_2$ concentrations were determined with a RIA using the procedure of Lewis *et al.*, (1978). The intra- and interassay CV for PGF $_{2\alpha}$ were 4.4 and 10.3%, respectively. The intra- and interassay CV for PGE $_2$ were 11.2% and 13.1%, respectively.

Statistical Analyses. The MIXED procedures of the Statistical Analysis System (SAS, 1996) were used to analyze the data. Hormonal data (P $_4$, PGE $_2$, and PGF $_{2\alpha}$) and differential white blood cell counts were analyzed as a split-plot design. Phase of the

estrous cycle (follicular vs luteal) and uterine inoculation (saline vs bacteria) were the main effects in the models. The initial model used to analyze the data included the following independent variables: phase, inoculation, phase x inoculation, ewe nested within phase x inoculation, time, time x phase, time x inoculation, time x phase x inoculation, and residual. The error term for the main plot was ewe nested within phase x inoculation, and the residual was the error term for the subplot. The initial model used to analyze the *in vitro* blastogenic responses to mitogens and *in vitro* treatments was a split-split-split-plot design. The model included the same independent variables as the model for hormonal data in addition to two split plots that included terms for the mitogens (unstimulated, Con A, or LPS), the individual well treatments (control, prostaglandin treatment, INDO, and prostaglandin + INDO), all appropriate interactions, and their error terms. The Least-Squares Mean, PDIFF, and STDERR options in SAS was used to compare the responses of each variable when the MIXED procedure indicated a significant effect of phase (follicular vs luteal) and(or) uterine inoculation (saline vs bacteria).

Results of Experiment 2

Development of Uterine Infections.

All ewes inoculated with *A. pyogenes* and *E. coli* on d 6 developed uterine infections, whereas none of the ewes inoculated with bacteria on d 0 or with saline on d 0 or 7 developed uterine infections.

Lymphocyte Blastogenic Activity.

Phase of the estrous cycle tended to affect ($P = .07$) the unstimulated and Con A-, and LPS-stimulated lymphocyte blastogenesis. The average for the unstimulated and Con A-, and LPS-stimulated incorporation [^3H]thymidine for ewes inoculated during the luteal phase tended ($P = .08$) to be greater than that for ewes treated during the follicular phase (2.6 vs 4.5 pmol for follicular and luteal phase, respectively; SEM = .5; Figure 8). Terms for mitogen, well treatment, and a mitogen x well treatment interaction were significant ($P < .05$). The Con A-stimulated blastogenesis was increased ($P < .001$) compared with both unstimulated and LPS-stimulated blastogenesis (2.9, 4.5, and 3.2 pmol for unstimulated, Con A, and LPS, respectively; SEM = .5). There was also a uterine inoculation type x mitogen x well treatment interaction ($P = .03$). Concanavalin-A stimulated vena caval lymphocytes that were either treated as controls (5.9 vs 3.1 pmol for saline and bacteria, respectively) or with INDO (6.6 vs 2.8 pmol for saline and bacteria, respectively; SEM = .5) were inhibited ($P < .05$) in ewes inoculated with bacteria. There was a tendency ($P < .1$) for Con A-stimulated vena caval lymphocytes that were either treated with PGE_2 (4.9 vs 3.7 pmol for saline and bacteria, respectively) or $\text{PGE}_2 + \text{INDO}$ (5.5 vs 3.8 pmol for saline and bacteria, respectively; SEM = .5) collected from ewes inoculated with bacteria to be inhibited (Figure 9). Average lymphocyte proliferation from ewes inoculated with saline, when stimulated with Con A, tended to be higher ($P = .09$) when incubated with INDO (4.9 vs 6.6 for PGE_2 and INDO, respectively).

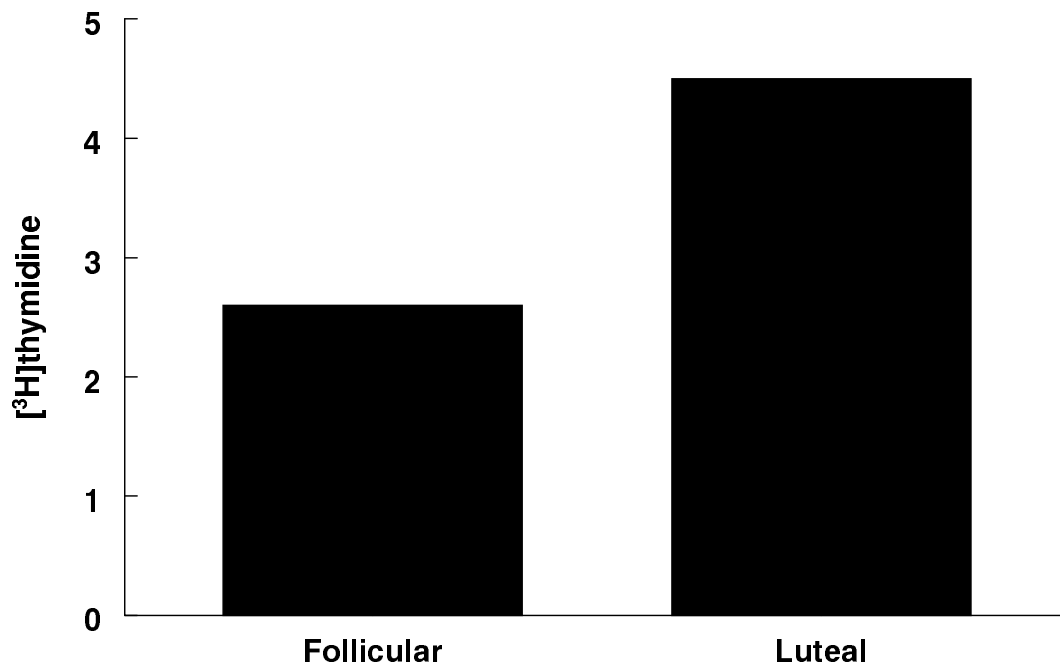


Figure 8. Effect of phase of the estrous cycle on [³H]thymidine incorporation into lymphocytes in Experiment 2. Vena caval blood was collected from ewes inoculated with either saline or bacteria during the follicular (d 0) or luteal phase (d 6). Lymphocytes were recovered and incubated *in vitro*. The average lymphocyte blastogenic response to Con A for ewes inoculated during the luteal phase tended ($P = .08$; SEM = .5) to be greater than that for ewes treated during the follicular phase.

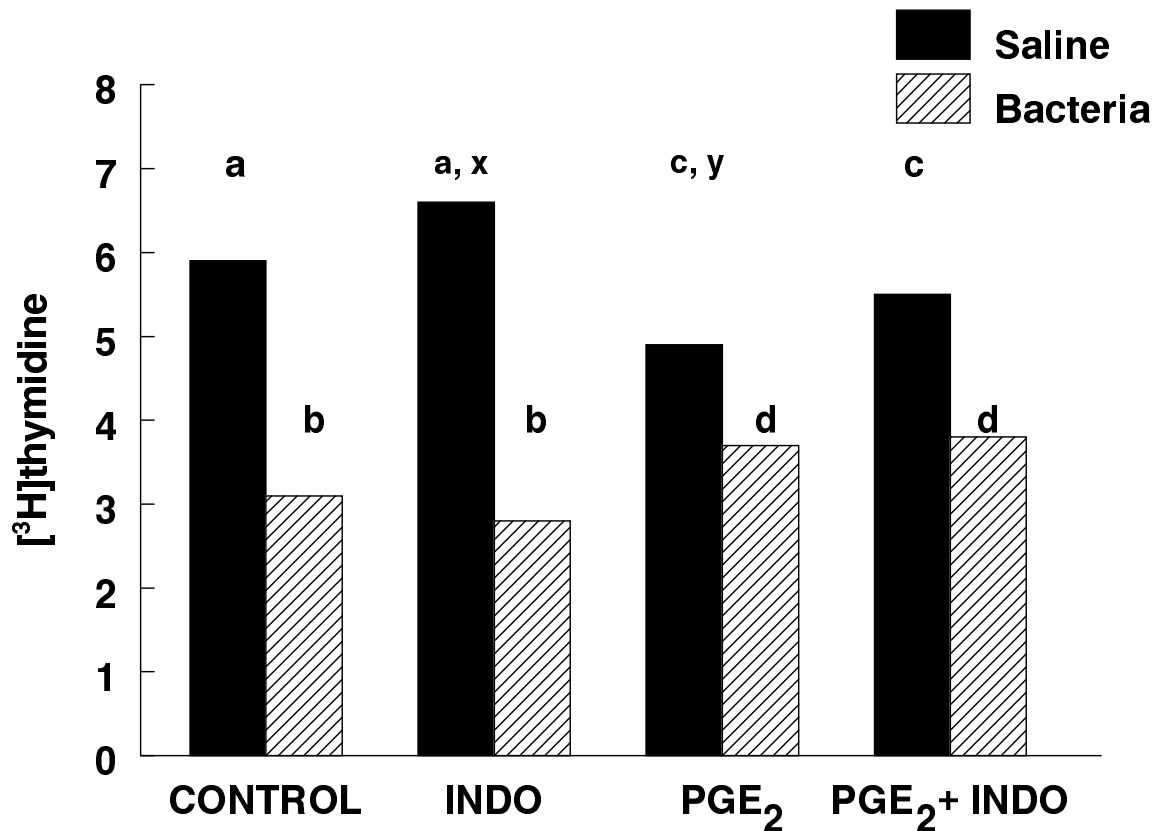


Figure 9. Effect of bacterial inoculation on *in vitro* treatments concanavalin A-stimulated lymphocyte proliferation in Experiment 2. Vena caval blood was collected from follicular and luteal phase ewes that received either intrauterine inoculations of saline or bacteria. Lymphocytes were recovered and incubated *in vitro*. The interaction between uterine inoculation type and mitogen and well treatment was significant ($P < .01$; SEM = .5). Concanavalin-A stimulated vena caval lymphocytes that were either treated as controls or with INDO were inhibited ($P < .05$) in ewes inoculated with bacteria. There was a tendency ($P < .1$; SEM = .5) for Con A stimulated vena caval lymphocytes that were either treated with PGE₂ or PGE₂ + INDO collected from ewes inoculated with bacteria to be inhibited. Also, Con A-stimulated lymphocyte proliferation from ewes inoculated with saline tended to be higher ($P = .09$) when incubated with INDO. ^{a,b} Means with unlike superscripts differ ($P < .05$). ^{c,d,x,y} Means with unlike superscripts tend to differ ($P < .1$).

Prostaglandin and Steroid Data.

Phase of the estrous cycle, inoculation type, and day had no significant effect on either PGF_{2α} or PGE₂ vena caval concentrations in this experiment (Figure 10). The average concentration of PGE₂ in follicular phase ewes was 123 pg/mL, whereas the average was 167 pg/mL for luteal phase ewes. The average concentration of PGE₂ in ewes inoculated with saline was 155 pg/mL, and the average was 136 pg/mL for ewes inoculated with bacteria. The average concentration of PGF_{2α} in follicular phase ewes was 60 pg/mL, and the average was 69 pg/mL for luteal phase ewes. The average concentration of PGF_{2α} in ewes inoculated with saline was 65 pg/mL, and the average was 64 pg/mL for ewes inoculated with bacteria.

Progesterone concentrations were greater ($P < .0001$) in luteal phase ewes (3.4 ng/mL) than in follicular phase ewes (.44 ng/mL). Progesterone concentrations in ewes treated during the luteal phase fluctuated and averaged approximately 3.4 ng/mL of vena caval blood. Ewes treated during the follicular phase with saline had minimal P₄ concentrations until 3 d after inoculation when average concentrations increased from .4 to .7 ng/mL of vena caval blood. Ewes treated during the follicular phase with bacteria had minimal P₄ concentrations on all days of the experiment; P₄ averaged .4 ng/mL. Ewes treated during the luteal phase with saline had increased P₄ from 1.4 to 2.5 ng/mL of vena caval blood from 1 to 3 d after inoculation. In contrast, ewes treated during the luteal phase with bacteria had decreasing P₄ concentrations from 6.2 to 3.3 ng/mL from 1 to 3 d after inoculation (Figure 11).

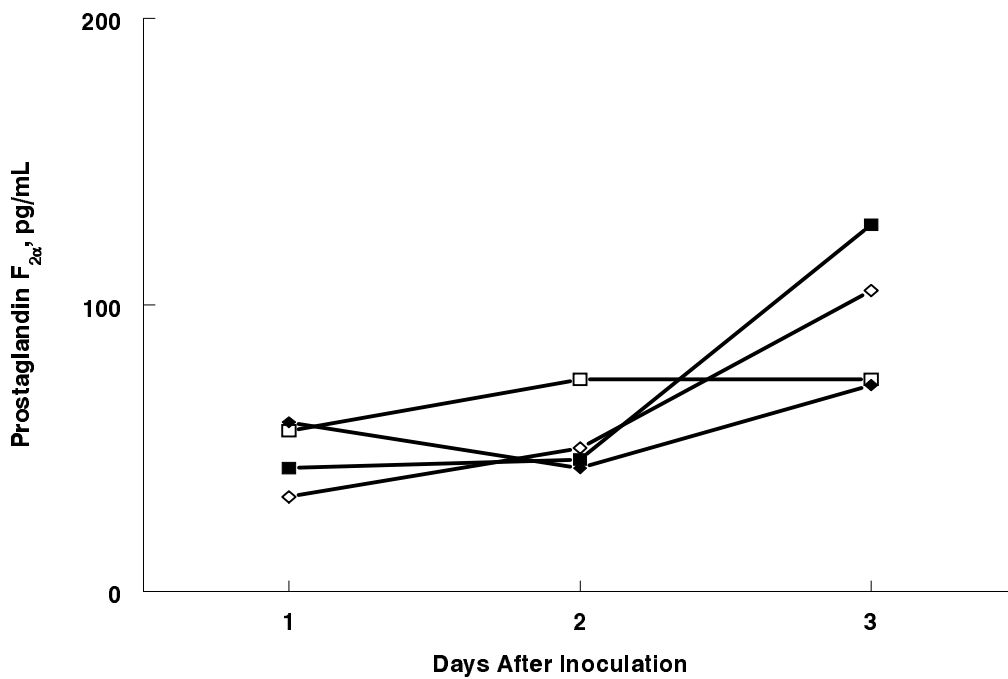
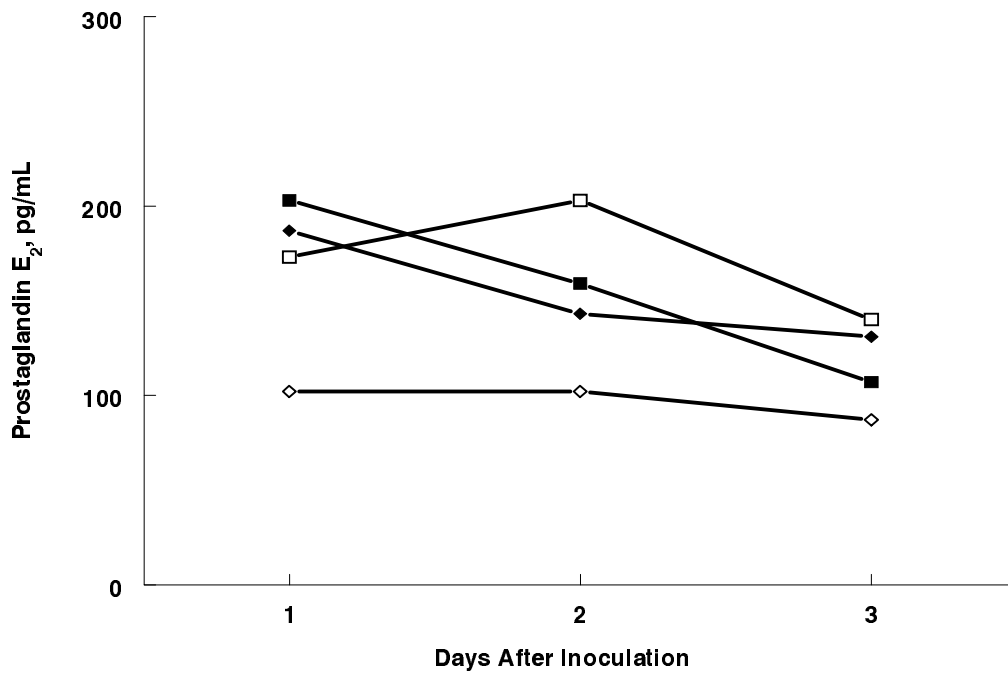


Figure 10. Concentrations of prostaglandins in vena caval blood collected from ewes given intrauterine inoculations of saline or bacteria on d 0 or d 6 of the estrous cycle in Experiment 2.

PGE₂ SEM = 12 pg/mL; PGF_{2α} SEM = 10 pg/mL

◆ Follicular, Saline; ◇ Follicular, Bacteria; ■ Luteal, Saline; □ Luteal, Bacteria

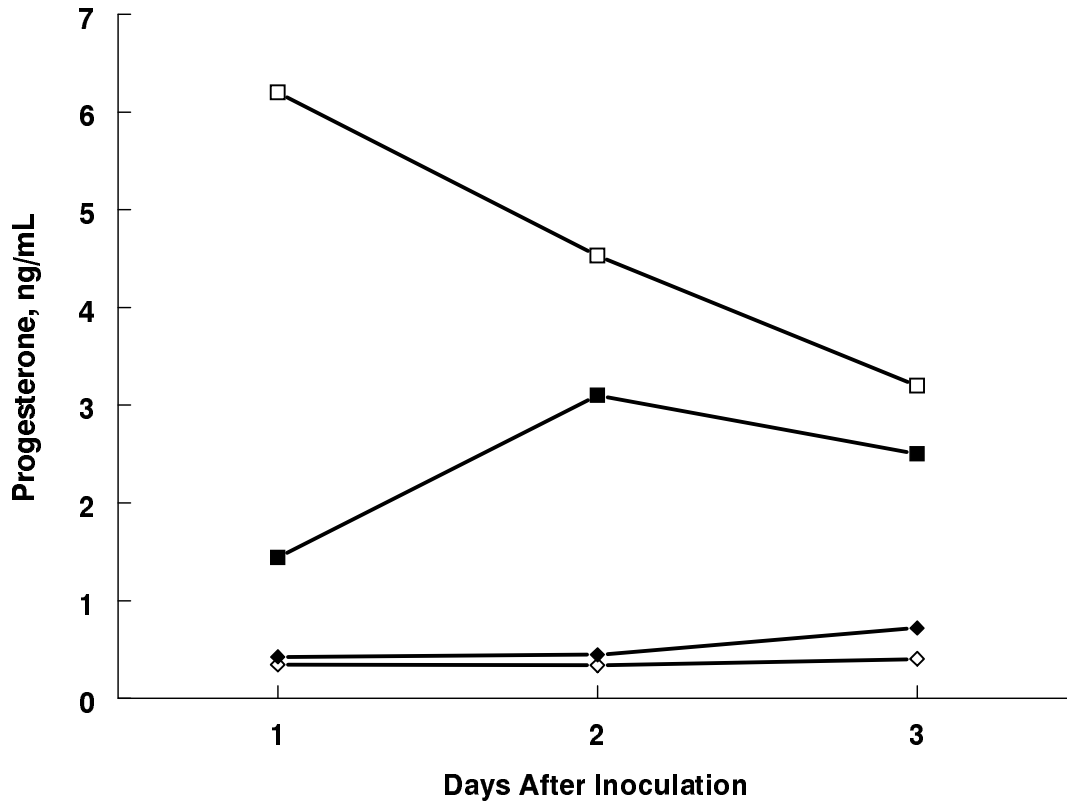


Figure 11. Concentrations of progesterone in vena caval blood collected from ewes given intrauterine inoculations of saline or bacteria on d 0 or d 6 of the estrous cycle in Experiment 2.

Concentrations of progesterone were greater ($P < .0001$) in ewes during the luteal phase than the follicular phase, and was affected by bacterial inoculation ($P = .05$; SEM = .2 ng/mL).

◆ Follicular, Saline; ◇ Follicular, Bacteria; ■ Luteal, Saline; □ Luteal, Bacteria

Differential White Blood Cell Counts.

For lymphocytes, neutrophils, and eosinophils, there was no effect of phase, inoculation type, or their interaction ($P > .1$). Day affected ($P < .05$) the number of lymphocytes and eosinophils and tended to affect ($P = .06$) the number of neutrophils. Monocytes tended to be fewer in number in ewes inoculated with bacteria ($P = .07$; 8 vs 5 for bacteria and saline inoculation, respectively). The average number of eosinophils 1 d after inoculation was 2 and increased to 9 at 3 d after inoculation. Neutrophils averaged 48 at 1 d after inoculation, decreased to 40 at 2 d after inoculation, and decreased further to 36 at 3 d after inoculation.

The phase x day interaction tended to affect ($P = .07$) the number of lymphocytes. Ewes treated in the follicular phase had 28, 41, and 37 lymphocytes for 1, 2, and 3 d after inoculation, whereas ewes treated in the luteal phase had 14, 34, and 52 lymphocytes at 1, 2, and 3 d after inoculation. Figure 12 shows the WBC data.

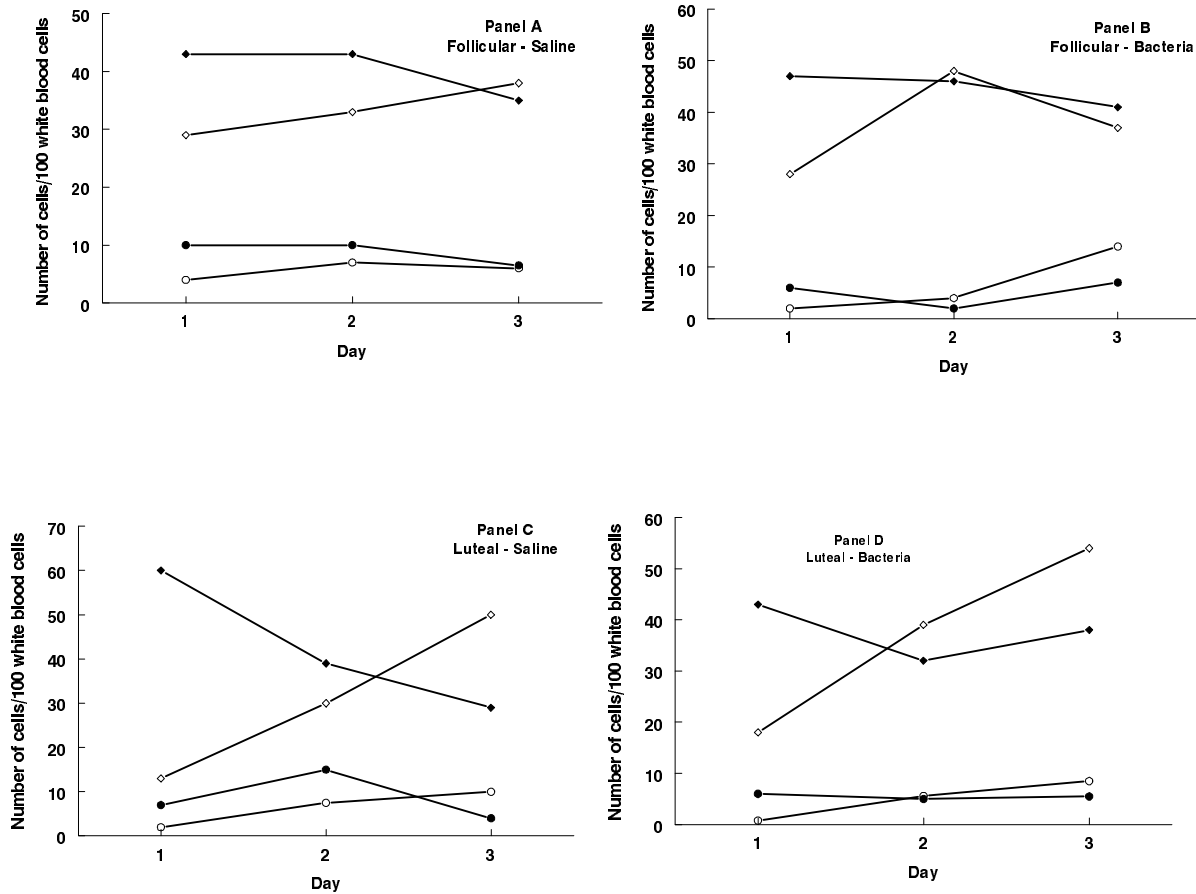


Figure 12. The effect of treatments on the average number of different types of WBC in Exp. 2. Ewes were assigned to randomized treatments, estrus was synchronized, and ewes received intrauterine inoculations (saline or bacteria) on d 0 or 6 of the estrous cycle. Different types of WBC (lymphocytes, neutrophils, eosinophils, and monocytes) were counted out of 100 WBC from a thin smear stained with Wright's stain. Treatments are defined in each panel. Time after inoculation ($P < .05$) affected lymphocyte and eosinophil numbers, bacterial inoculation tended ($P = .06$) to affect monocyte numbers, and phase of the cycle and time after inoculation tended ($P = .07$) to affect the number of lymphocytes.

Neutrophils (SEM = 2); lymphocytes (SEM = 2.4); monocytes (SEM = .9); eosinophils (SEM = .7)

◆ = Neutrophils; ◇ = Lymphocytes; ● = Monocytes; ○ = Eosinophils

Results of Experiment 3

Development of Uterine Infections.

All ewes inoculated with *A. pyogenes* and *E. coli* on d 6 developed uterine infections, but none of the ewes inoculated with bacteria on d 0 or with saline on d 0 or 6 developed uterine infections.

Lymphocyte Blastogenic Activity.

There was a tendency ($P = .07$) for a phase x inoculation interaction for unstimulated and Con A-, and LPS-stimulated lymphocyte blastogenesis. The average lymphocyte blastogenesis for follicular-saline, follicular-bacteria, luteal-saline, and luteal-bacteria treated ewes was 1.6, 2.1, 2.0, and .9 pmol, respectively; SEM = .5). Concanavalin A-stimulated vena caval lymphocytes had greater ($P < .0001$) incorporation of [^3H]thymidine than unstimulated and LPS-stimulated lymphocytes (.5, 3.6, and .8 for unstimulated, Con A, and LPS, respectively; SEM = .5). Concanavalin A-stimulated blastogenesis was less ($P < .05$) for ewes inoculated during the luteal phase than for ewes in the follicular phase (3.1 vs 4.1 pmol; Figure 13). Blastogenesis in response to culturing lymphocytes with $\text{PGF}_{2\alpha}$ and Con A was greater ($P < .01$) for follicular-phase ewes than for luteal-phase ewes (2.71 vs 3.54 pmol; SEM = .5), but INDO had no effect on unstimulated or mitogen-stimulated blastogenesis (Figure 14).

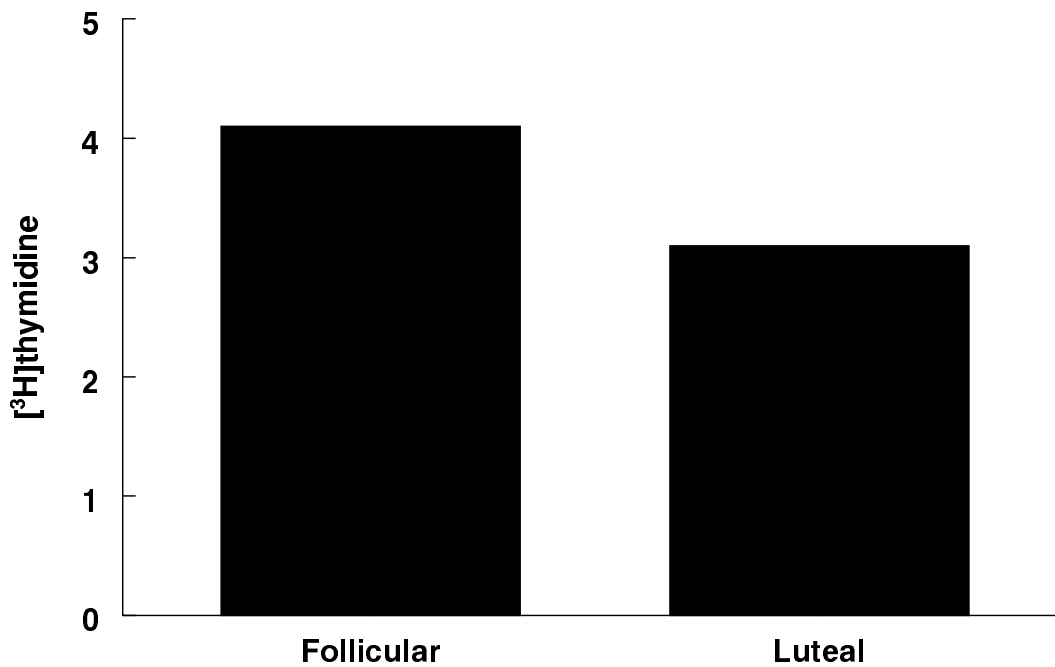


Figure 13. Effect of phase of the estrous cycle on [³H]thymidine incorporation into lymphocytes in Experiment 3. Vena caval blood was collected from ewes inoculated with either saline or bacteria during the follicular (d 0) or luteal phase (d 6). Lymphocytes were recovered and incubated *in vitro*. The average lymphocyte blastogenic response to Con A for ewes inoculated during the follicular phase was greater ($P < .05$; SEM = .5) than that for ewes treated during the luteal phase

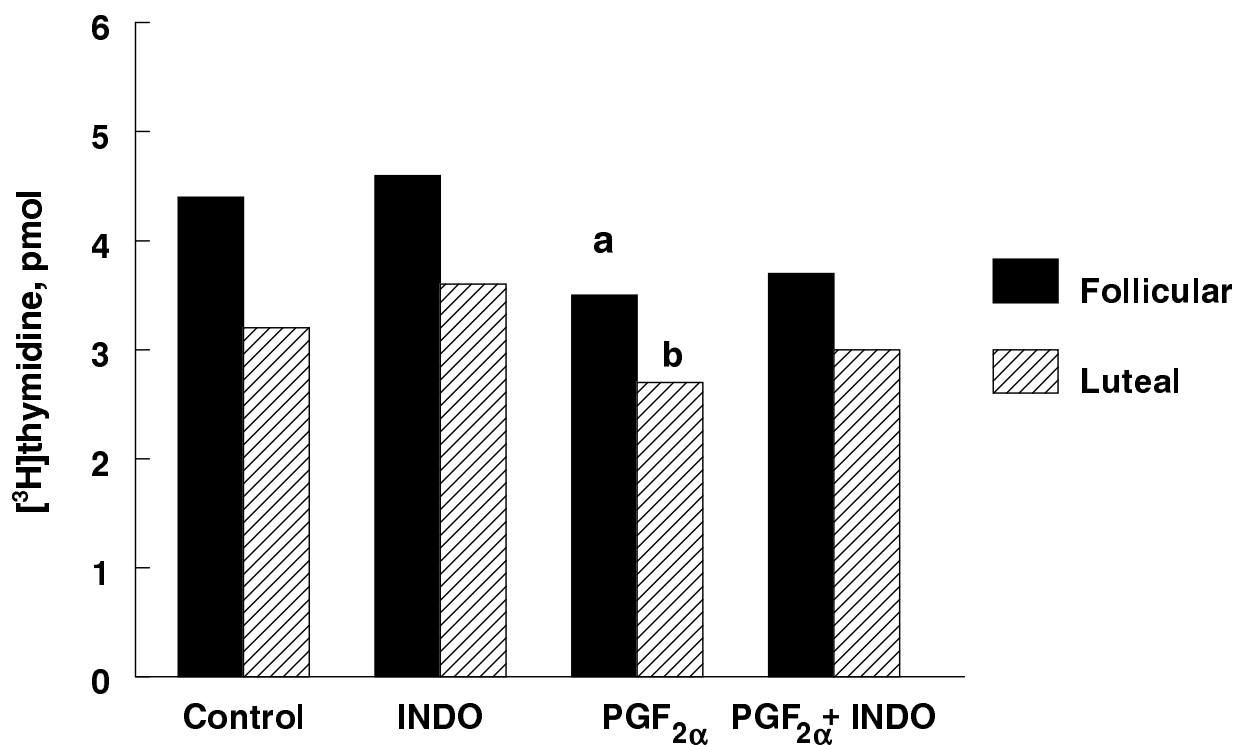


Figure 14. Effect of concanavalin A-stimulated lymphocyte proliferation on *in vitro* treatments Experiment 3. Vena caval blood was collected from follicular and luteal phase ewes that received either intrauterine inoculations of saline or bacteria. Lymphocytes were recovered and incubated *in vitro*. The interaction between phase of the estrous cycle and mitogen and uterine inoculation type interaction was significant ($P < .01$; SEM = .5). In follicular phase ewes, Con A-stimulated vena caval lymphocytes that were treated with PGF_{2α} incorporated greater ($P < .05$) amounts of [3H]thymidine than ewes inoculated during their luteal phase. ^{a,b} Means with like superscripts differ ($P < .05$).

Prostaglandin and Steroid Data.

Phase of the estrous cycle, inoculation type, day, and their interactions had no significant effect on vena cava PGE₂ concentrations in this experiment (Figure 15). Phase of the estrous cycle, day, and their interactions had no significant effect on vena cava PGF_{2α} concentrations in this experiment (Figure 15). Inoculation type tended ($P = .09$) to influence vena caval PGF_{2α} concentrations (33 vs 40 pg/mL for saline and bacteria treated ewes). The average concentration of PGE₂ in follicular phase ewes was 110 pg/mL, and the average was 116 pg/mL for luteal phase ewes. The average concentration of PGE₂ in ewes inoculated with saline was 125 pg/mL, and the average was 102 pg/mL for ewes inoculated with bacteria. The average concentration of PGF_{2α} in follicular phase ewes was 35 pg/mL, and the average was 38 pg/mL for luteal phase ewes. The average concentration of PGF_{2α} in ewes inoculated with saline was 33 pg/mL, whereas the average was 40 pg/mL for ewes inoculated with bacteria.

Progesterone concentrations were greater ($P < .001$) in luteal-phase ewes (6.5 ng/mL) than in follicular-phase ewes (1.28 ng/mL). Progesterone concentrations in ewes treated during the luteal phase fluctuated and averaged approximately 6.5 ng/mL of vena caval blood. Ewes treated during the follicular phase with saline had minimal P₄ concentrations until 3 d after inoculation when P₄ increased from .6 to 2.1 ng/mL of vena caval blood. Ewes treated during the follicular phase with bacteria had minimal P₄ concentrations until 3 d after inoculation when P₄ increased from .9 to 1.4 ng/mL of vena caval blood. Progesterone in ewes treated during the luteal phase with saline decreased from 7.7 to 4.3 ng/mL of vena caval blood from 1 to 3 d after inoculation. In

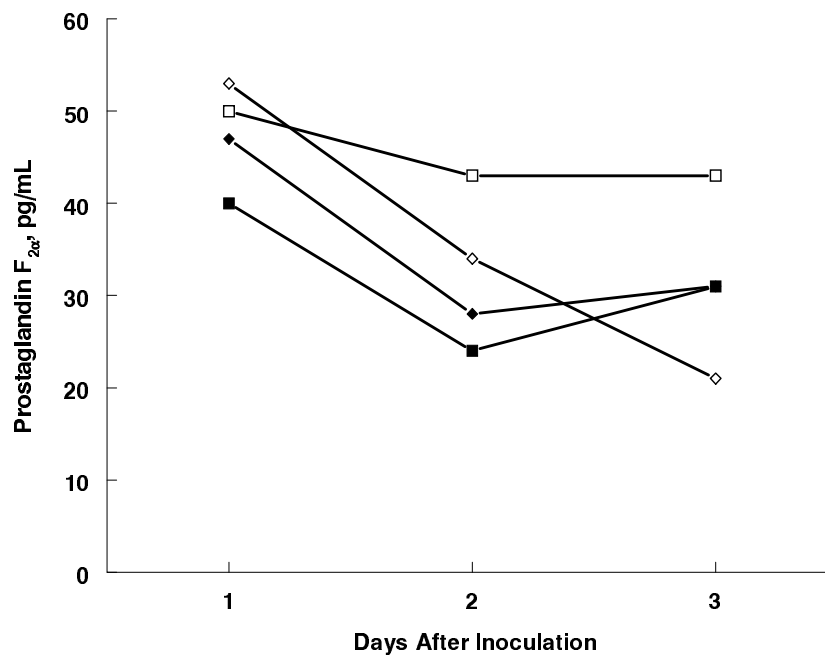
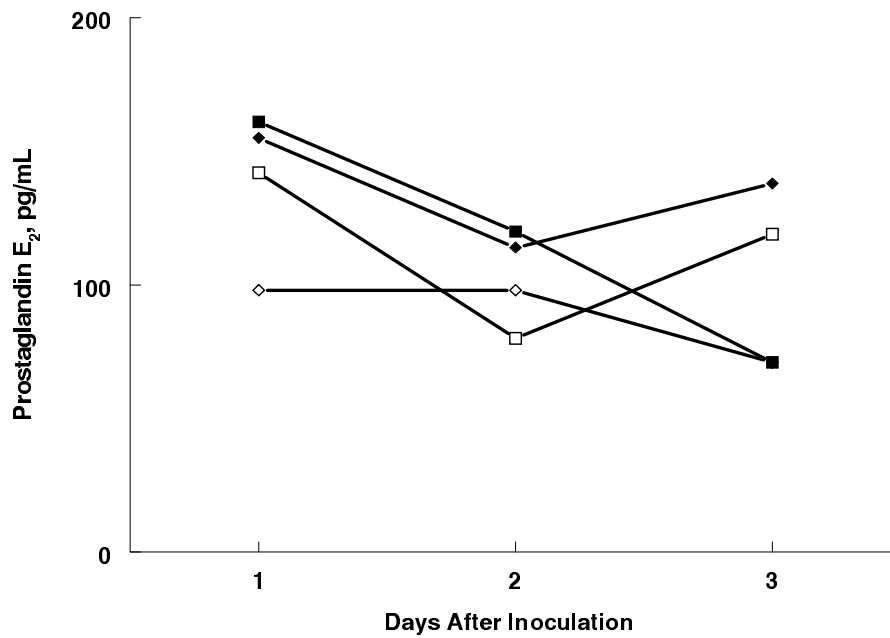


Figure 15. Concentrations of prostaglandins in vena caval blood collected from ewes given intrauterine inoculations of saline or bacteria on d 0 or d 6 of the estrous cycle in Experiment 2.

Concentrations of PGF_{2α} tended to be greater ($P = .09$; SEM = 3 pg/mL) in ewes who had their uteri inoculated with bacteria. PGE₂ SEM = 11 pg/mL;

◆ Follicular, Saline; ◇ Follicular, bacteria; ■ Luteal, Saline; □ Luteal, Bacteria

contrast, ewes treated during the luteal phase with bacteria had increasing P₄ concentrations from 5.7 to 9.1 ng/mL from 1 to 3 d after inoculation (Figure 16).

Differential White Blood Cell Counts.

Phase of the estrous cycle tended to affect ($P = .06$) the number of vena caval neutrophils. Ewes treated during the luteal phase had increased numbers of neutrophils. There was a phase x inoculation interaction ($P < .01$) for the number of neutrophils and lymphocytes. Average number of neutrophils in ewes inoculated in the follicular phase with saline or bacteria was 48 and 44, respectively. Neutrophils in ewes inoculated in the luteal phase with saline or bacteria was 45 and 55, respectively. Lymphocytes in ewes inoculated in the follicular phase with saline or bacteria was 28 and 34, respectively. Lymphocytes in ewes inoculated in the luteal phase with saline or bacteria was 32 and 23, respectively.

Time affected ($P < .05$) the numbers of lymphocytes, neutrophils, monocytes, and eosinophils. Neutrophils decreased from 58 to 40 from 1 to 3 d after inoculation. Lymphocytes increased from 26 to 33 from 1 to 3 d after inoculation. Monocytes increased from 12 to 18 from 1 to 3 d after inoculation. Eosinophils increased from 2 to 7 from 1 to 3 d after inoculation (Figure 17).

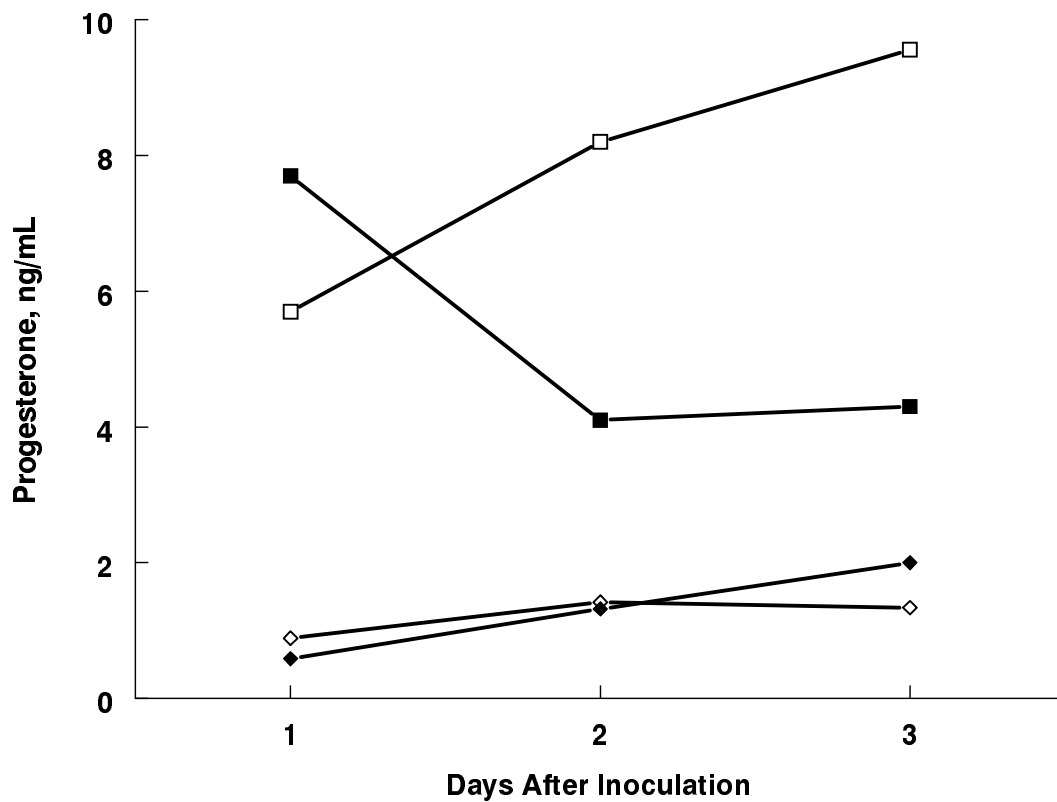


Figure 16. Concentrations of progesterone in vena caval blood collected from ewes given intrauterine inoculations of saline or bacteria on d 0 or d 6 of the estrous cycle in Experiment 3.

Concentrations of progesterone were greater ($P < .001$; SEM = .5 ng/mL) in ewes during the luteal phase than during the follicular phase.

◆ Follicular, Saline; ◇ Follicular, Bacteria; ■ Luteal, Saline; □ Luteal, Bacteria

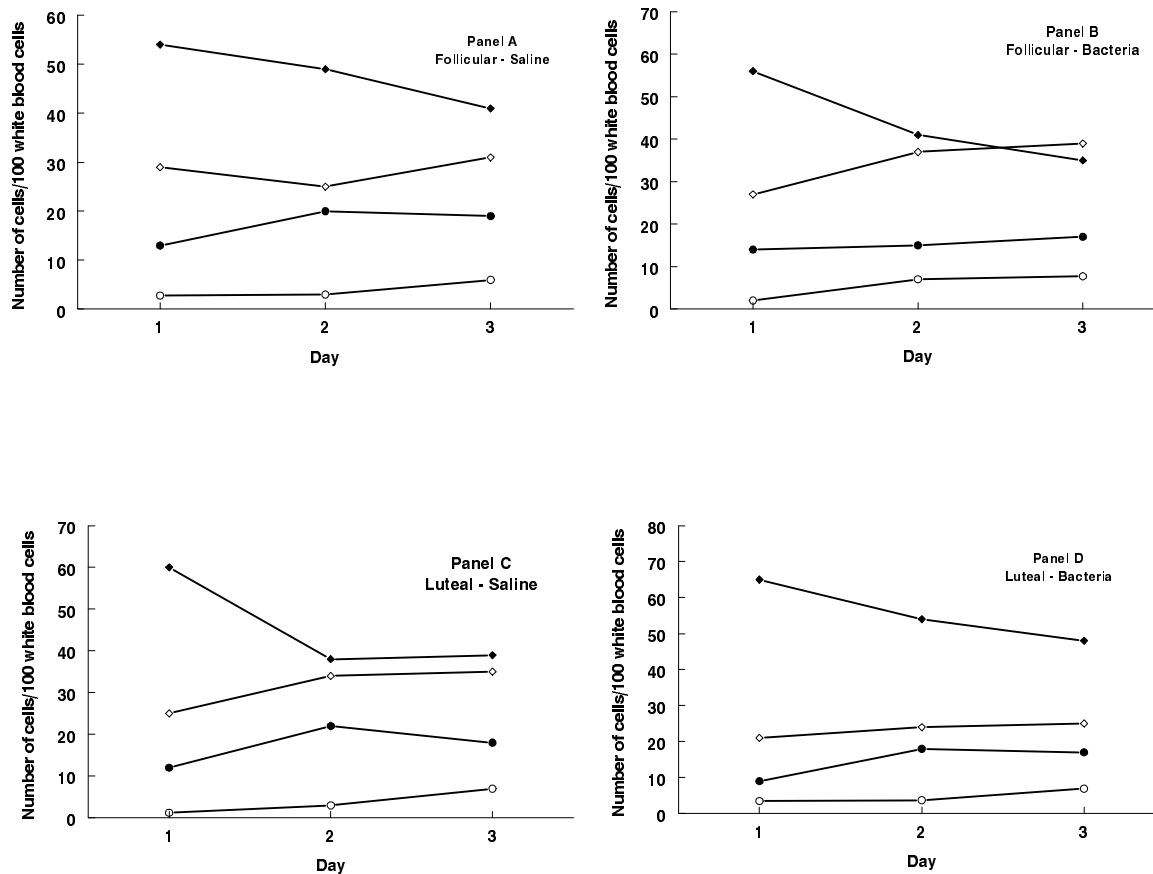


Figure 17. The effect of treatments on the average number of different types of WBC in Exp. 2. Ewes were assigned to randomized treatments, estrus was synchronized, and ewes received intrauterine inoculations (saline or bacteria) on d 0 or 6 of the estrous cycle. Different types of WBC (lymphocytes, neutrophils, eosinophils, and monocytes) were counted out of 100 WBC from a thin smear stained with Wright's stain. Treatments are defined in each panel. Phase of the estrous cycle tended ($P = .06$) affect the number of neutrophils, phase of the estrous cycle and bacterial inoculation affected ($P < .01$) of neutrophils and lymphocytes, and time after inoculation affected ($P < .05$) the number of all WBC.

Neutrophils (SEM = 1.2); lymphocytes (SEM = 1.1); monocytes (SEM = .8); eosinophils (SEM = .4)

◆ = Neutrophils; ◇ = Lymphocytes; ● = Monocytes; ○ = Eosinophils

CHAPTER V

LUTALYSE CHANGES THE UTERINE RESPONSES TO INFECTIOUS BACTERIA

Introduction

Prostaglandin $F_{2\alpha}$ analogues are increasingly used to treat postpartum uterine infections, especially in cows without CL. The mechanism of how $PGF_{2\alpha}$ analogues facilitate clearing of uterine infections is not completely understood. Prostaglandin $F_{2\alpha}$ probably induces luteolysis in cows with CL, and this reduces P_4 and allows E_2 to increase (Fogwell *et al.*, 1978). These changes in P_4 and E_2 concentrations should up-regulate the immune system and allow the animal to clear the uterine infection. Wade and Lewis (1996) reported that a $PGF_{2\alpha}$ analogue induced the uterus to secrete more $PGF_{2\alpha}$. Exogenous $PGF_{2\alpha}$ may increase endometrial availability of free arachidonic acid, which can be converted to $PGF_{2\alpha}$ through the cyclooxygenase pathway. Free arachidonic acid can also be converted through the lipoxygenase pathway to LTB_4 . Both $PGF_{2\alpha}$ and LTB_4 can act as powerful chemotactic agents, and LTB_4 is a potent activator of neutrophil activity (Nilsson *et al.*, 1991; Hoedemaker *et al.*, 1992). Exogenous $PGF_{2\alpha}$ may also increase uterine release of PGE_2 , which is consistently associated with decreased neutrophil and lymphocyte activity (Nilsson *et al.*, 1991). Uterine PGE_2 typically increases blood flow, and it may enhance diapedesis (Resnik and Brink, 1978).

A generalized inflammation of either the endometrium or the entire uterus is one of the early signs of a uterine infection. Prostaglandins and leukotrienes, arachidonic acid metabolites, act as direct mediators of inflammation (Fantone *et al.*, 1985). Based

upon these observations, the effect of arachidonic acid metabolites on leukocytes (specifically neutrophils) has attracted considerable attention. Much of this attention has focused on the use of $\text{PGF}_{2\alpha}$ (or its analogues), because it can decrease P_4 concentrations, presumably up-regulate the uterine immune system, and cause clearance of uterine infections. However, few have investigated the direct action of arachidonic acid metabolites on the immune cells (specifically on neutrophil and lymphocyte activities). Most research with arachidonic acid metabolites has been performed *in vitro* because of the short half-life of prostaglandins in tissue or in circulation.

Materials and Methods

Experiments 4 and 5.

The objectives for these experiments were to:

- 1) Determine the effect of ovarian hormones on the development of a uterine infection in response to infectious bacteria.
- 2) Monitor the daily *in vitro* changes in lymphocyte proliferation and development of a uterine infection in response to infectious bacteria.
- 3) Determine the relationship between vena caval concentrations of ovarian steroids and eicosanoids and the response of the uterus after exposure to infectious bacteria.
- 4) Monitor changes in the differential white blood cell counts during development of a uterine infection.
- 5) Determine, in Experiment 4, *in vitro* changes in lymphocyte proliferation in response to incubation with PGE_2 and(or) indomethacin.

- 6) Determine, in Experiment 5, *in vitro* changes in lymphocyte proliferation in response to incubation with PGF_{2α} and(or) indomethacin.
- 7) Determine the *in vivo* and *in vitro* effect of administration of Lutalyse on the clearance of a uterine infection.

General. Experiment 4 was conducted in the winter of 1998, and Experiment 5 was conducted in the winter of 1999. Primiparous and multiparous Dorset, Suffolk, and mixed breed ewes (2 to 11 yr old) from the Virginia Tech Sheep Center were used for this experiment. All ewes were healthy and had no history of uterine infections. Vasectomized rams were used twice daily to check ewes for signs of estrus. Ewes with at least two consecutive estrous cycles of 14 to 15 d in duration had their estrous cycles synchronized. All ewes were healthy and had no history of uterine infections. Feed and water were withheld for at least 24 h before ewes went to surgery.

In vivo Experimental Protocol. Ewes (n = 5/treatment in Experiment 4 and n = 6/treatment for Experiment 5) were randomly assigned to one of two treatments. On d 6 of the estrous cycle, vena caval catheters were inserted as in previous experiments, and uteri of all ewes were inoculated with 35×10^7 cfu of *E. coli* and 75×10^7 cfu of *A. pyogenes* in 5 mL of saline. For specific surgical techniques see Appendix A. Three days later, on d 9, ewes received either i.m. injections of 3 mL of saline or 15 mg of Lutalyse (5 mg/mL). Vena caval blood (12 mL) was collected into sterilized heparinized tubes on d 7, 8, 9, 10, and 11 for determination of differential white blood cell counts, PGF_{2α} and PGE₂ concentrations, and for collection of lymphocytes to examine their *in vitro* responses to mitogens and prostaglandin treatments. An additional blood sample (12 mL) was collected at the same time for serum collection and placed into sterilized

tubes. Serum was separated and stored at -20°C until the P₄ RIA could be performed. All ewes were killed 2 d after i.m. injections for collection of reproductive tracts. Uteri were collected and flushed with 10 mL of PBS, and the amount of sediment was used to determine whether the uterus was resistant or susceptible to infection. An aliquot (100 µL) of the uterine flushing was cultured on Brain Heart Infusion Agar in a humidified chamber at 37°C for 48 h to determine whether the uterus was contaminated with bacteria. See Figure 18 for a timeline of events for Experiments 4 and 5.

In vitro Experimental Protocol. Vena caval lymphocytes were collected from each ewe for 3 d after inoculation to determine their blastogenic response (previously described) to mitogens (none, Con A, or LPS). Lymphocytes were arranged in a 3 x 2 x 2 factorial arrangement with mitogens, prostaglandin treatment, and indomethacin (INDO) as the main effects. In Experiment 4, PGE₂ was used as the *in vitro* prostaglandin treatment, and, in Experiment 5, PGF_{2α} was used instead of PGE₂. The final incubation volume included 100 µL (1 x 10⁵ cells) of lymphocyte suspension, 100 µL of respective mitogen suspension (unstimulated, 1 µg/well of Con A, or .5 µg/well of LPS), and respective *in vitro* treatment (none, respective prostaglandin, INDO, or respective prostaglandin + INDO) suspended in 10 µL of 200 proof ethanol. The final concentration of both prostaglandins and indomethacin was 10⁻⁷ M per well.

Progesterone Assay. A [¹²⁵I]progesterone RIA kit (Diagnostic Products Company, Los Angeles, CA) was used to quantify P₄ in all samples after treatment. Samples were assayed in duplicate. The intra-assay CV were 9.8%, respectively.

Prostaglandin Assays. Plasma concentrations of PGF_{2α} were determined with a RIA using the procedure of Pexton *et al.* (1975), and PGE₂ concentrations were

determined with a RIA using the procedure of Lewis *et al.* (1978). The intra- and interassay CV for PGF_{2α} were 4.1 and 18.9%, respectively. The intra- and interassay CV for PGE₂ were 6.3% and 28%, respectively.

Statistical Analyses. The MIXED procedures of the Statistical Analysis System (SAS, 1996) were used to analyze the data. Hormonal data (P₄, PGE₂, and PGF_{2α}) and differential white blood cell counts were analyzed as a split-plot design. The initial model used to analyze the data included the following independent variables: ewe treatment (saline or Lutalyse), ewe nested within ewe treatment, time, time X ewe treatment, and residual. The error term for the main plot was ewe nested within ewe treatment, and residual was the error term for the subplot. The initial model used to analyze the *in vitro* blastogenic responses to mitogens and *in vitro* treatments was a split-split-split-plot design. The model included the same independent variables as the hormonal data, in addition to two split plots that included terms for the mitogens (unstimulated, Con A, or LPS), the individual well treatments (control, prostaglandin treatment, INDO, and prostaglandin + INDO), all appropriate interactions, and their error terms. The Least-Squares Mean, PDIFF, and STDERR options in SAS were used to compare response of each variable when the MIXED procedure indicated a significant effect of any variable in the model.

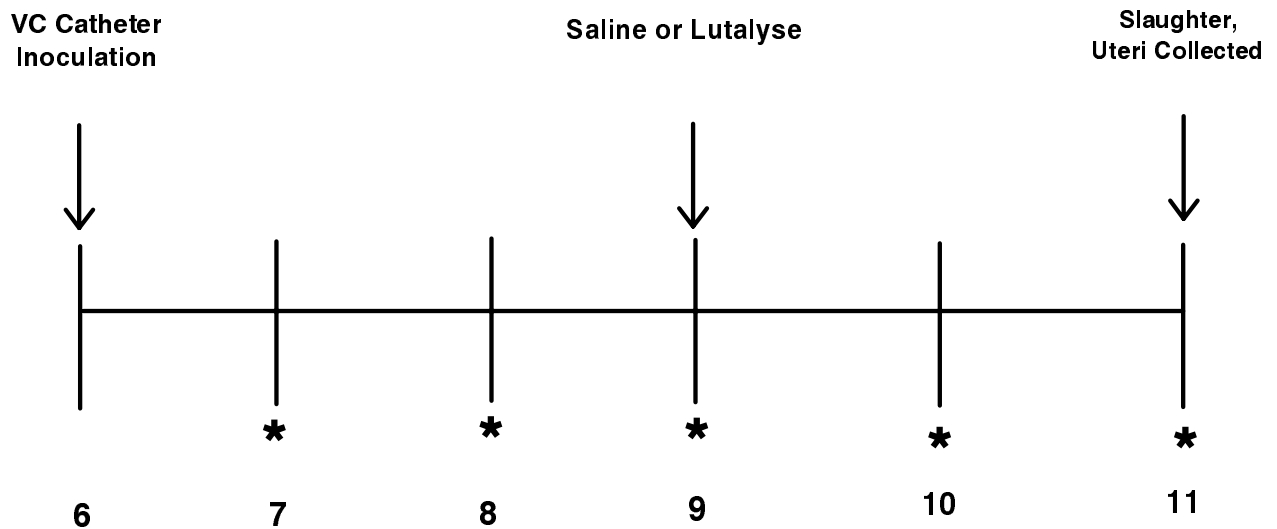


Figure 18. Timeline of Events for Experiments 4 and 5. All ewes were fitted with a vena caval catheter and had their uteri inoculated with bacteria (with 35×10^7 cfu of *E. coli* and 75×10^7 cfu of *A. pyogenes*) on d 6 of the estrous cycle (d 0 = estrus). On d 9, ewes received either an i.m. injection of saline or 15 mg of Lutalyse. Vena caval blood was collected on days marked with stars to quantify serum concentrations of progesterone, plasma concentrations of prostaglandins ($\text{PGF}_{2\alpha}$ and PGE_2), determination of differential WBC counts, and for collection of lymphocytes for submission to a lymphocyte proliferation assay.

Results of Experiment 4

Development of Uterine Infections.

All ewes inoculated with *A. pyogenes* and *E. coli* on d 6 and treated with saline on d 9 developed uterine infections, whereas none of the ewes inoculated with bacteria and treated with Lutalyse on d 9 had signs of a uterine infection.

Lymphocyte Blastogenic Activity.

Mitogens affected ($P < .001$) the blastogenic activity of lymphocytes, but there was no effect of the well treatment or no *in vitro* interactions. Concanavalin A-stimulated vena caval lymphocytes had greater ($P < .0001$) incorporation of [³H]thymidine than did unstimulated and LPS-stimulated lymphocytes (2.8, 5.2, and 3.4 for unstimulated, Con A, and LPS, respectively). The average lymphocyte blastogenesis for saline-treated ewes was 3.4 pmol and for Lutalyse-treated ewes was 4.1 pmol. There were no differences in blastogenic response when lymphocytes were cultured with PGE₂, INDO, or the combination (4.0, 3.4, 3.4, and 3.8 pmol for control, PGE₂, INDO, and PGE₂ + INDO, respectively).

Prostaglandin and Steroid Data.

Neither treatment, day, nor their interaction affected vena caval PGE₂ or PGF_{2α} concentrations in this experiment (Figure 19). The average concentration of PGE₂ in saline-treated ewes was 136 and 162 pg/mL before and after treatment, respectively. The average concentration of PGF_{2α} in saline-treated ewes was 67 and 53 pg/mL

before and after treatment. The average concentration of PGE₂ in Lutalyse-treated ewes was 134 and 175 pg/mL before and after treatment. The average concentration of PGF_{2α} in Lutalyse-treated ewes was 67 and 82 pg/mL before and after treatment.

Progesterone concentrations were less ($P < .001$) after treatment with Lutalyse (.9 vs 5.8 ng/mL for Lutalyse- and saline-treated ewes, respectively). Both treatment groups reached an average of 5.5 ng/mL of P₄. After treatment with saline, P₄ concentrations decreased to 2.2 ng/mL on d 11. This observation was expected, because the average estrous cycle length in our flock is 14 to 15 d. Thus, ewes may have been undergoing “natural” luteolysis. After treatment with Lutalyse, P₄ concentrations decreased to below .5 ng/mL on d 11 (Figure 20).

Differential White Blood Cell Counts.

The interaction of Lutalyse and time affected ($P = .04$) the numbers of neutrophils. Treatment with Lutalyse tended ($P = .08$) to decrease neutrophils (33, 31, and 30/100 WBC on d 9, 10, and 11, respectively). There were no other differences observed with lymphocyte, monocyte, or eosinophil numbers (Figure 21).

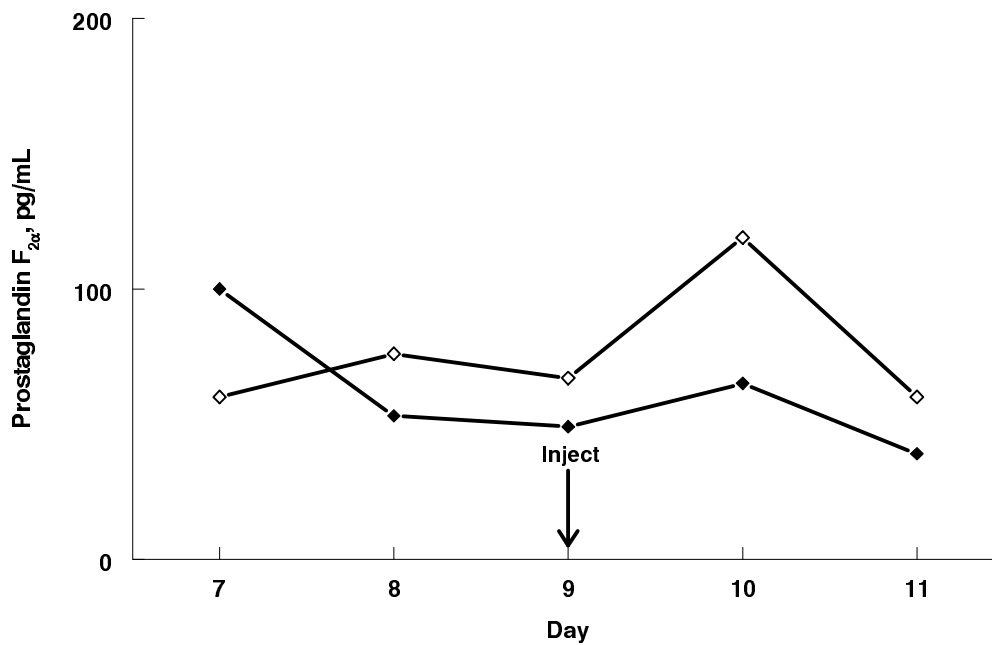
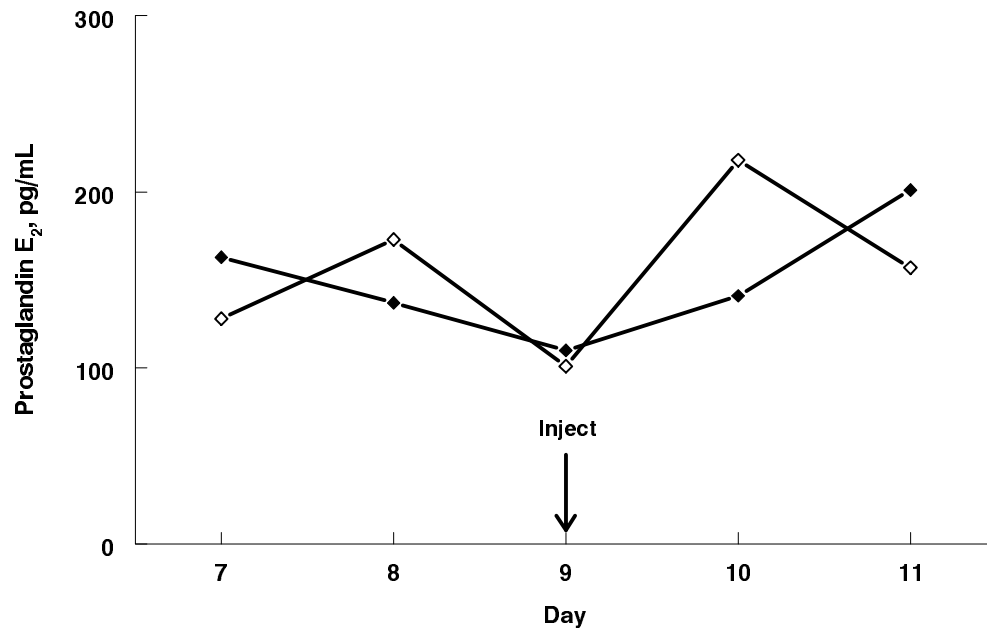


Figure 19. Concentrations of prostaglandins in vena caval blood collected from ewes given intrauterine inoculations of bacteria on d 6 of the estrous cycle and administered either saline or 15 mg of Lutalyse on d 9 of the estrous cycle in Experiment 4.

PGE₂ SEM = 17 pg/mL; PGF_{2α} SEM = 10 pg/mL; Arrow indicates when ewes received their respective i.m. treatments.

◆ Saline; ◇ Lutalyse

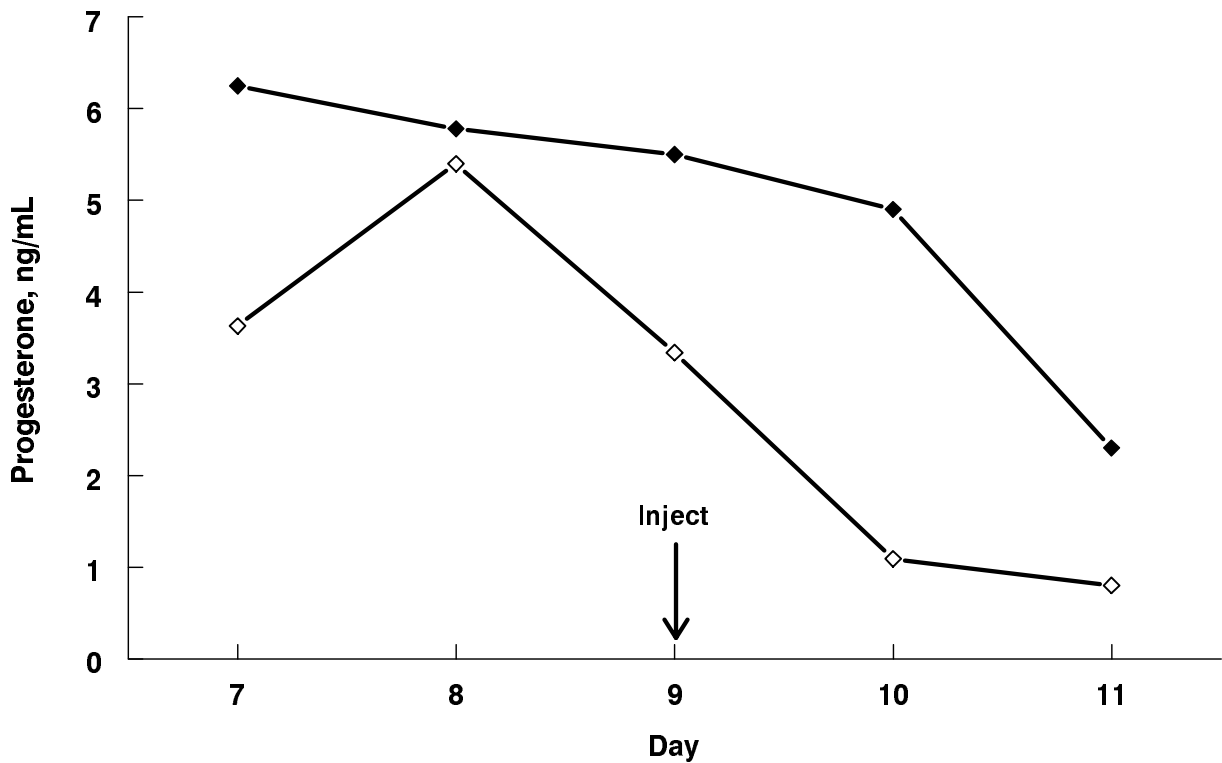


Figure 20. Concentrations of progesterone in vena caval blood collected from ewes given intrauterine inoculations of bacteria on d 6 of the estrous cycle and administered either saline or 15 mg of Lutalyse on d 9 of the estrous cycle in Experiment 4. Concentrations of progesterone was less ($P < .05$; SEM = .5 ng/mL) in ewes after they were treated with Lutalyse. Arrow indicates when ewes received their respective i.m. treatments.
 ◆ Saline; ◇ Lutalyse

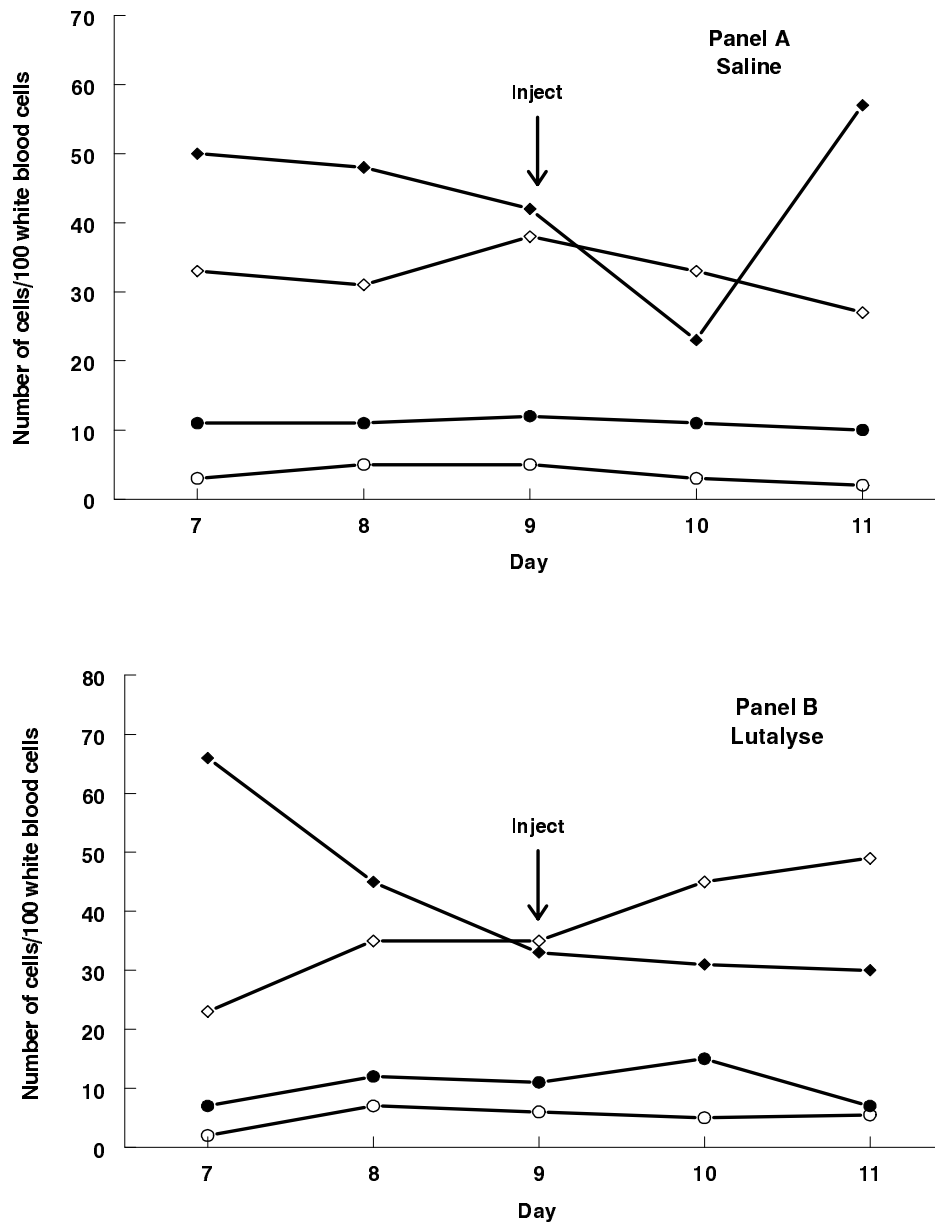


Figure 21. The effect of treatments on the average number of different types of WBC in Exp. 4. Ewes were assigned to randomized treatments, estrus was synchronized, and all ewes received bacterial intrauterine inoculations on d 6. Ewes received either saline or 15 mg of Lutalyse on d 9 of the estrous cycle. Different types of WBC (neutrophils, lymphocytes, monocytes, and eosinophils) were counted out of 100 WBC from a thin smear stained with Wright's stain. Treatments are defined in each panel. Neutrophils (SEM = 2.1); lymphocytes (SEM = 2.4); monocytes (SEM = 1.2); eosinophils (SEM = .5).

◆ = Neutrophils; ◇ = Lymphocytes; ● = Monocytes; ○ = Eosinophils

Results of Experiment 5

Development of Uterine Infections.

All ewes inoculated with *A. pyogenes* and *E. coli* on d 6 and treated with saline on d 9 developed uterine infections, whereas none of the ewes inoculated with bacteria and treated with Lutalyse on d 9 had signs of a uterine infection.

Lymphocyte Blastogenic Activity.

Mitogens affected ($P < .001$) the blastogenic activity of lymphocytes, but there was no effect of the well treatment and no interactions. Concanavalin A-stimulated vena caval lymphocytes had greater ($P < .0001$) incorporation of [³H]thymidine than unstimulated and LPS-stimulated lymphocytes (.5, 4.1, and .8 for unstimulated, Con A, and LPS, respectively). The average lymphocyte blastogenesis for saline-treated ewes was 1.87 pmol and for Lutalyse-treated ewes was 1.73 pmol. There were no differences in blastogenic response when lymphocytes were cultured with PGF_{2α}, INDO, or the combination (1.9, 1.9, 1.8, and 1.6 pmol for control, PGF_{2α}, INDO, and PGF_{2α} + INDO, respectively).

Prostaglandin and Steroid Data.

Neither treatment, day, nor their interaction affected vena caval PGE₂ or PGF_{2α} concentrations in this experiment (Figure 22). The average concentration of PGE₂ in saline-treated ewes was 91 and 82 pg/mL before and after treatment. The average concentration of PGF_{2α} in saline-treated was 52 and 80 pg/mL before and after treatment. The average concentration of PGE₂ in Lutalyse-treated was 108 and 127

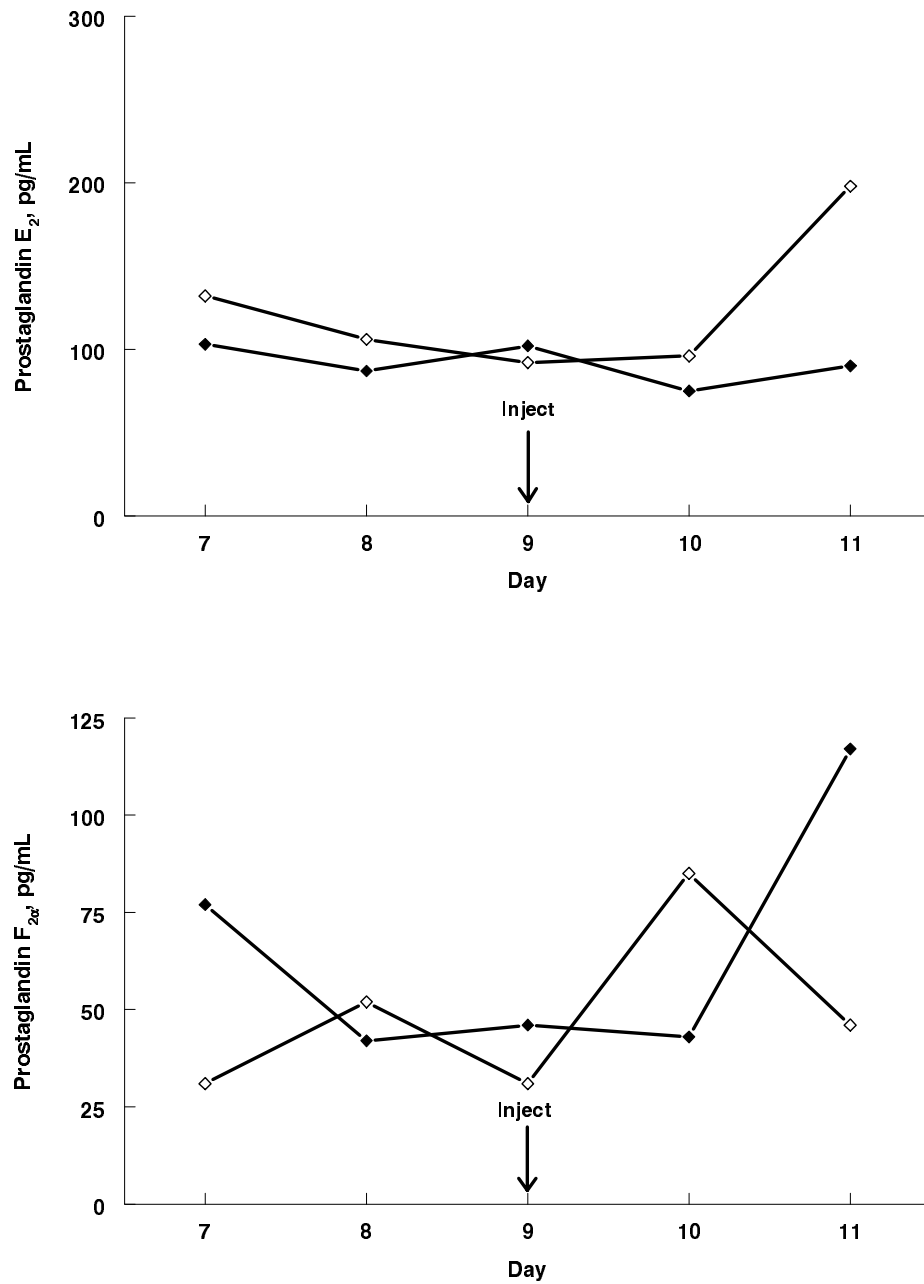


Figure 22. Concentrations of prostaglandins in vena caval blood collected from ewes given intrauterine inoculations of bacteria on d 6 of the estrous cycle and administered either saline or 15 mg of Lutalyse on d 9 of the estrous cycle in Experiment 5.

PGE₂ SEM = 6 pg/mL; PGF_{2α} SEM = 6 pg/mL

Arrow indicates when ewes received their respective i.m. treatments.

◆ Saline; ◇ Lutalyse

pg/mL before and after treatment. The average concentration of $\text{PGF}_{2\alpha}$ in Lutalyse-treated was 49 and 66 pg/mL before and after treatment.

Progesterone concentrations were less ($P < .001$) after treatment with Lutalyse (1.6 vs 9.1 ng/mL for Lutalyse- and saline-treated ewes, respectively). Both treatment groups reached an average of 9 ng/mL of P_4 . After treatment with Lutalyse, P_4 concentrations decreased from 9.1 to 1.6 ng/mL on d 11 (Figure 23).

Differential White Blood Cell Counts.

Time affected ($P < .05$) the numbers of neutrophils and monocytes and tended to affect ($P = .06$) the numbers of eosinophils. Treatment with Lutalyse tended ($P = .06$) to increase neutrophils (48, 54, and 42/100 WBC on d 9, 10, and 11, respectively).

Neutrophils tended ($P = .06$) to be higher in Lutalyse-treated ewes (44 vs 53 for saline- and Lutalyse-treated ewes, respectively). As a result, lymphocyte numbers tended ($P = .07$) to be less in Lutalyse-treated ewes (35 vs 27). The numbers of eosinophils tended to increase from d 7 to d 9 (1, 3, and 5/100 WBC; Figure 24).

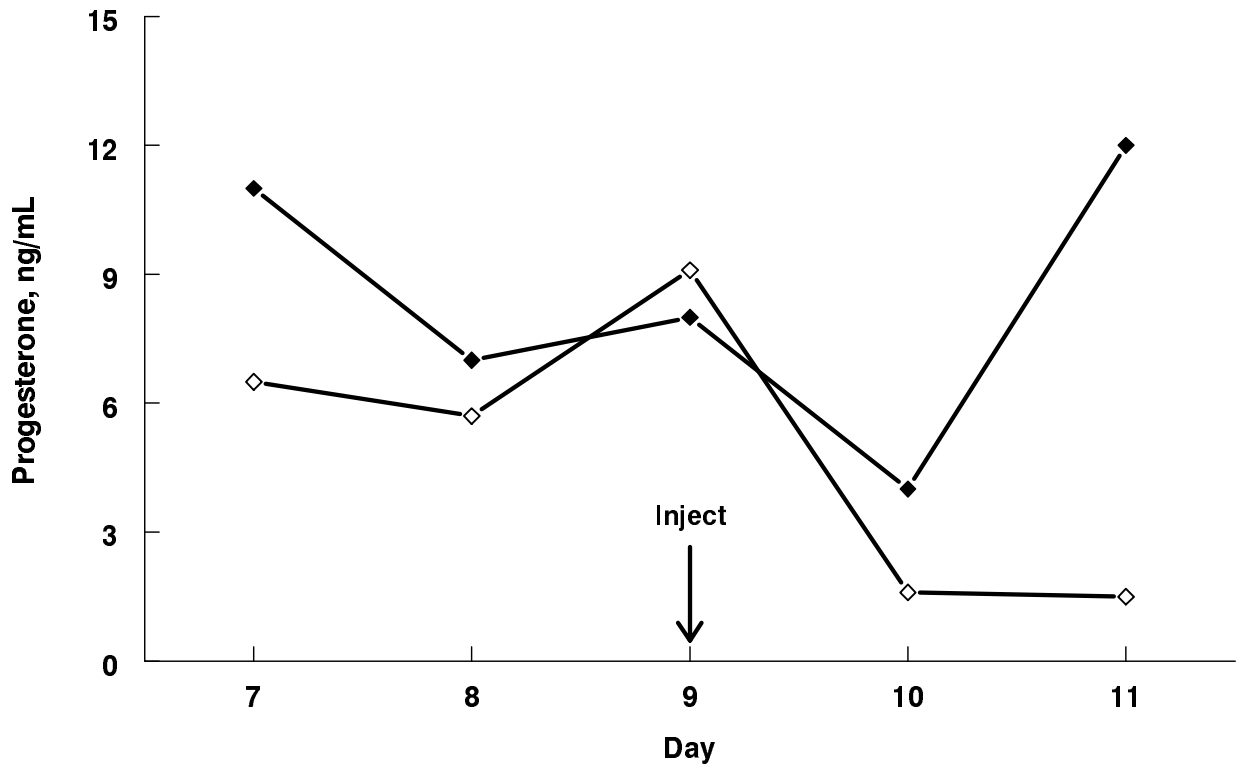


Figure 23. Concentrations of progesterone in vena caval blood collected from ewes given intrauterine inoculations of bacteria on d 6 of the estrous cycle and administered either saline or 15 mg of Lutalyse on d 9 of the estrous cycle in Experiment 5. Concentrations of progesterone was less ($P < .05$; SEM = .8 ng/mL) in ewes after they were treated with Lutalyse. Arrow indicates when ewes received their respective i.m. treatments.

◆ Saline; ◇ Lutalyse

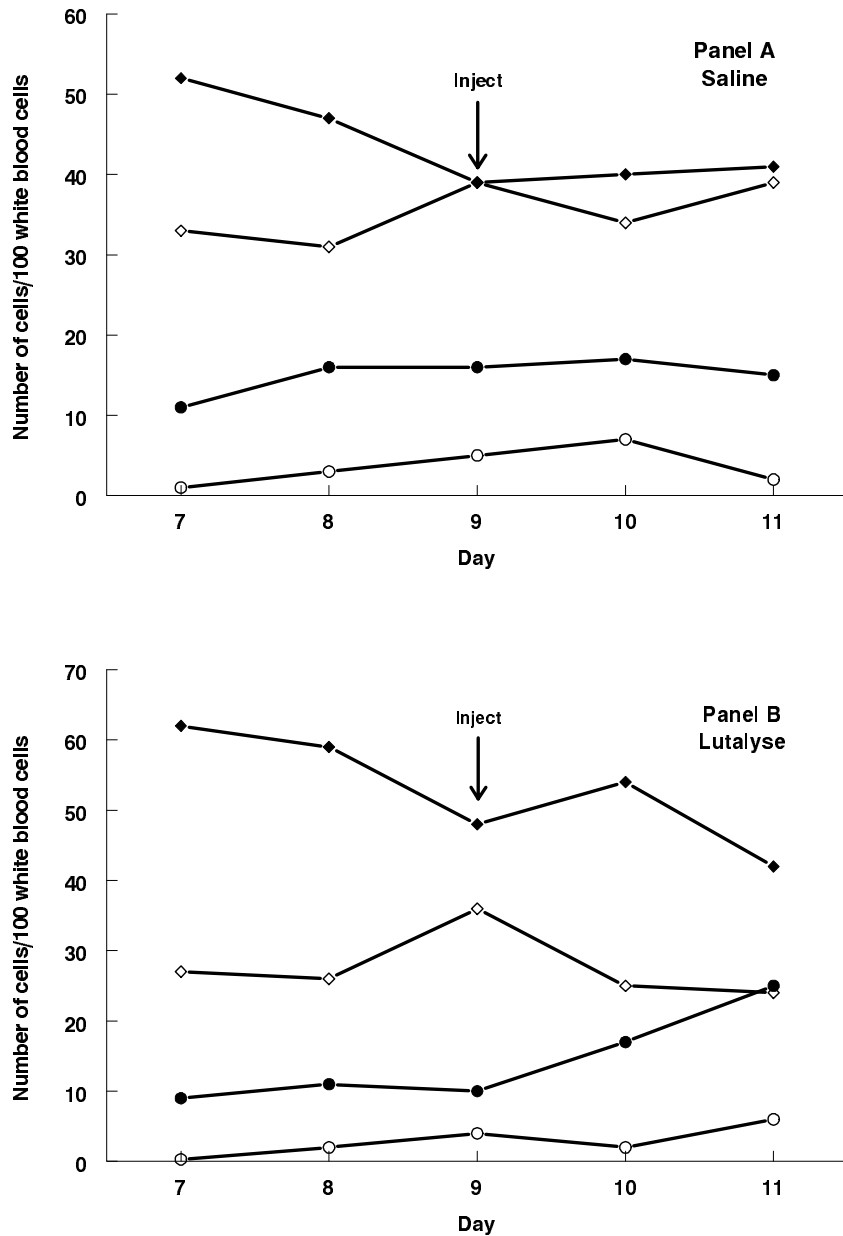


Figure 24. The effect of treatments on the average number of different types of WBC in Exp. 5. Ewes were assigned to randomized treatments, estrus was synchronized, and all ewes received bacterial intrauterine inoculations on d 6. Ewes received either saline or 15 mg of Lutalyse on d 9 of the estrous cycle. Different types of WBC (neutrophils, lymphocytes, monocytes, and eosinophils) were counted out of 100 WBC from a thin smear stained with Wright's stain. Treatments are defined in each panel. Neutrophils (SEM = 1.7); lymphocytes (SEM = 1.2); monocytes (SEM = 1.1); eosinophils (SEM = .5)

◆ = Neutrophils; ◇ = Lymphocytes; ● = Monocytes; ○ = Eosinophils

CHAPTER VI

Discussion

For an animal to avoid or manage an infection, the immune system must receive and send proper signals to mount a successful immune response. If one or more of those signals are reduced or blocked, the immune system cannot properly manage potential pathogens. Results from the last 50 yr and the experiments described in this dissertation suggest that livestock under the influence of P₄ are more likely to develop uterine infections, whereas animals without significant P₄ concentrations are less likely to develop uterine infections.

In this discussion, I will refer to the hormonal environment instead of stage of the estrous cycle because of the use of postpartum animals in Exp. 1. In all five experiments, the hormonal environment had the most modulatory effect on the uterus and on the development of a uterine infection in response to a bacterial challenge. All ewes under the influence of P₄ developed infections, but none of the ewes in the absence of P₄ developed infections. Several other researchers have also reported that cows, ewes, pigs, and rabbits (Rowson *et al.*, 1953; Broome *et al.*, 1959; Hawk *et al.*, 1961; Wulster-Radcliffe, 2000) under the influence of P₄ will develop uterine infections when exposed to infectious bacteria.

Progesterone has been hypothesized to directly or indirectly affect immune cell activities. Directly, lymphoid tissue has receptors for P₄ (Fujii-Hanamoto *et al.*, 1985; Sakabe *et al.*, 1986). Indirectly, P₄ induces the formation of many uterine proteins that can inhibit lymphocyte blastogenesis and neutrophil activity. For example, crude uterine flushings collected from ewes on d 4 of the estrous cycle inhibited lymphocyte

blastogenesis in response to phytohemagglutinin (a T cell specific mitogen; Segerson *et al.*, 1981). Ramadan *et al.* (1997) found two protein fractions (molecular weights of \geq 100 kDa and approximately 12.7 kDa) in uterine flushings collected on d 0 or 7 of the estrous cycle, or on 18 d after ovariectomy. The heavier fraction from d 0 had a stimulatory component, but the major effects of the fractions were inhibitory in response to phytohemagglutinin. Liu *et al.* (1999) suggested that ovine serpin (a P₄-induced uterine protein) can interact with lymphocytes by binding to a cell surface molecule and inhibiting lymphocyte activation.

In Exp. 1, P₄ treatment increased P₄ concentrations. The OVEX-P₄ ewes had lower blastogenic responses than in ewes in all other treatment groups. Lymphocyte blastogenesis was less in luteal phase ewes than in follicular phase ewes in Exp. 3. However, Lymphocyte proliferation was greater for ewes in their luteal phase than in ewes in their follicular phase in Exp. 2. It may be possible that lymphocytes collected from ewes in their follicular phase in Exp. 3 may have been fully activated and could not respond to the stimulation by mitogens. On the other hand, lymphocytes collected from ewes in their follicular phase in Exp. 2 may have a greater propensity to proliferate in response to mitogens than lymphocytes collected from ewes in their luteal phase indicating that these lymphocytes are down-regulated during the luteal phase. It would seem from these experiments and others (Ramadan *et al.*, 1997) that lymphocytes that are in a P₄-dominated hormonal environment have a reduced ability to divide in response to mitogens.

Concanavalin A-stimulated lymphocytes incubated with PGE₂ (Exp. 1 and 2) had an overall decreased blastogenic response. This decreased response was not due to

the differences in live cells, because the viability was comparable between the well treatments when it was determined before the [³H]thymidine was added and just before harvest of the lymphocytes. Several mechanisms may be responsible for this observation. Prostaglandin E₂ is associated with an increase in intracellular cAMP; PGE₂ seems to exert part of its effect through the cAMP-PKA system. Moreover, increased concentrations of cAMP in immune cells have been shown to decrease their activity (Geertsma *et al.*, 1994). However, cAMP and PKA inhibitors and PGE₂ receptor blockers can reduce the effects of PGE₂ (Geertsma *et al.*, 1994).

Prostaglandin E₂ can also inhibit T-cell activation following hemorrhagic injuries or bacterial sepsis. Choudry *et al.* (1995) showed an inhibition of T-cell mobilization and proliferation in response to Con A in septic rats inoculated with *E. coli* and *Bacteriodes fragilis*. Concentrations of PGE₂ increased after inoculation with bacteria, and T-cell mobilization was decreased. Intraperitoneal injection of indomethacin prevented the depression in T cell mobilization and proliferation in response to Con A (Choudry *et al.*, 1995).

Slama *et al.* (1991) reported that cows that had their uteri infused with 20 µg of 16,16-dimethyl PGE₂ (dPGE₂; a long-acting PGE₂ analogue) once a day from d 10 to 16 after parturition had reduced uterine involution (measured as uterine tone, length, and weight). Treatment with dPGE₂ also inhibited lymphocyte blastogenesis in response to Con A, phytohemagglutinin, and pokeweed mitogen (stimulates both T and B cells), and it increased the incidence and severity of uterine infections. The uterine infections were specifically caused by *A. pyogenes*. Thus, *in vivo* infusion of a PGE₂ analogue can cause a reduction in immune function and uterine involution and may cause an increase

in the incidence of *A. pyogenes* uterine infections. However, careful interpretation must be made, because the half-life of PGE₂ is < 20 s (Kindahl, 1981), whereas the half-life of dPGE₂ is much longer, probably hours.

Prostaglandin E₂ also inhibits IL-2 production from T lymphocytes, and it can inhibit the formation of the receptor for IL-2 (Choudry *et al.*, 1999). Interleukin-2 is a cytokine that is involved with T cell growth and proper antigen presentation and is important for differentiation that promotes cytotoxic T cell and B cell activity.

Lymphocytes from ewes in the follicular phase and incubated with PGF_{2α} (Exp. 3) had an increased blastogenic response to Con A. Prostaglandin F_{2α} exerts cellular responses through the cGMP-PKC system and is associated with a reduction in intracellular concentrations of cAMP (Parker, 1984). Decreased cAMP concentrations are consistently seen in active immune cells (Valitutti *et al.*, 1993). Because cGMP has opposite effects of cAMP, it is possible that the cAMP:cGMP ratio in lymphoid tissues (i. e., ratios of PGE₂:PGF_{2α}) may be critical for a proper immune response.

Results from this study suggest that the distribution of the various types of WBC in vena caval blood was affected by the phase of the estrous cycle, bacterial inoculation, and time. Generally, as a uterine infection progressed over time, numbers of lymphocytes increased whereas the number of neutrophils decreased. Ewes inoculated with bacteria on d 6 developed infections and the number of neutrophils decreased with time after inoculation. It is possible that neutrophils are being sequestered in the uterus in order to fight the infection. Hoedemaker *et al.* (1992) and Cai *et al.* (1994) suggested that the chemotactic and phagocytic ability of the neutrophil influences the ability of the uterus to resist or clear an infection. Ramadan *et al.* (1997)

observed the same pattern and suggested that this reduction in numbers of vena caval neutrophils may reflect their movement into the uterus to engulf and destroy the pathogens. However, the differences in the arterial and venous exchange of WBC across the uterus and(or) the endometrium is not known.

The increase in numbers of lymphocytes in vena caval blood in ewes inoculated with bacteria on d 6 may reflect the recruitment and passage of those lymphocytes through the uterus. Also, it has been suggested that older animals (i.e., more exposure to bacteria) are more resistant to uterine infections than are younger animals. Thus, it is possible that some form of active immunity may have developed naturally. Wulster-Radcliffe (2000) conducted an experiment to determine whether if antibodies to specific bacteria would develop in response to multiple exposures of bacteria. The uteri of nulliparous luteal phase ewes were inoculated with either saline or *A. pyogenes* and *E. coli* (the same strains used for this dissertation) on d 6 of the first synchronized estrous cycle, and vena caval blood was collected on d 6 through 12 of the estrous cycle. Ewes were administered a PGF_{2α} analogue on d 12 to ensure clearance of infections and rested for an estrous cycle. Estrus was again synchronized, ewes had their uteri inoculated with saline or bacteria on d 6 of the third estrous cycle, and vena caval blood was collected on d 6 through 12. Uteri were collected at slaughter on d 12 following the second inoculation. Subsequent bacterial challenges increased the percentage of neutrophils and decreased the percentage of lymphocytes. Uterine infections were detected in all ewes receiving bacterial inoculations during the second inoculation period at the time of slaughter. Ewes that were exposed to the bacteria twice had less severe infections than ewes that were only exposed once. Multiple exposures to

bacterial challenge enhanced lymphocyte blastogenic responses to Con A and LPS. Moreover, antibody titers were increased in ewes that were exposed to the bacteria twice. The author suggested that decreased severity of uterine infections in ewes exposed to the bacteria twice might indicate that multiple exposures enhance the immune response to bacteria in sheep possibly due to the development of antibodies to the bacteria.

An inherent weakness in using differential WBC counts is that only 100 randomly selected cells are counted. This technique only indicates a change in the percentage of WBC and not a change in the absolute number in circulation. Previous work in our laboratory (Hunter *et al.*, 1999, unpublished data) indicates that the observation of neutrophils decreasing over time (as soon as 2 h after inoculation) in ewes inoculated with bacteria, oyster glycogen, and *A. pyogenes* supernatant is due to chemotaxis to the uterus. Additionally, it seems unlikely that this change is due to an increase in lymphocyte numbers because mitosis takes an average of 24 h to complete.

Injections of Lutalyse (Exp. 4 and 5) decreased concentrations of P_4 and allowed the clearance of uterine infections. This observation could simply be due to the removal of the immunosuppressive effects of P_4 and up-regulation of the uterine immune system to aid in the clearance. Wade and Lewis (1996) reported that Lutalyse (a $PGF_{2\alpha}$ analogue) induces the uterus to secrete more $PGF_{2\alpha}$. Prostaglandin $F_{2\alpha}$ analogues probably increase the availability of free arachidonic acid, which is converted to $PGF_{2\alpha}$. Also, free arachidonic acid may be converted to LTB_4 through the lipoxygenase pathway. Both $PGF_{2\alpha}$ and LTB_4 can act as chemotactic agents, and LTB_4 is a potent activator of neutrophil activity (Nilsson *et al.*, 1991; Hoedemaker *et al.*, 1992).

Exogenous $\text{PGF}_{2\alpha}$ may also increase uterine release of PGE_2 , which is consistently associated with decreased neutrophil and lymphocyte proliferation (Nilsson *et al.*, 1991). However, uterine PGE_2 typically increases blood flow, and it may enhance diapedesis (Resnik and Brink, 1978). Thus, the “proper” $\text{PGF}_{2\alpha}:\text{PGE}_2$ ratio must be maintained to ensure that the immune cells are activated but can also move across blood vessels to the site of infection.

Injection of Lutalyse caused a decrease in the percentage of neutrophils in vena caval blood, whereas the percentage of lymphocytes increased. This observation would suggest that ultimate elimination of a uterine infection relies more heavily on the function of neutrophils rather than lymphocyte proliferation. It has been hypothesized that $\text{PGF}_{2\alpha}$ analogues increase arachidonic acid availability, which is converted to $\text{PGF}_{2\alpha}$ by cyclooxygenase and(or) LTB_4 by lipoxygenase. Both of these products have been associated with an increase in neutrophil chemotaxis and bacterial destruction (Nilsson *et al.*, 1991; Hoedemaker *et al.*, 1992). Removal of the immunosuppressive effects of P_4 , increases in chemotactic signals, and enhanced neutrophil chemotaxis may be the mechanism by which $\text{PGF}_{2\alpha}$ analogues cause an increase in vena caval neutrophils. Increased numbers as well as enhanced neutrophil activity may allow for the clearance of the uterine infection.

It has been hypothesized that clearance of a uterine infection is strictly dependent on the ability of the uterus to contract and eliminate the infection through the vagina, rather than through immune cell involvement. With the use of a $\text{PGF}_{2\alpha}$ analogue, it is thought that because of the decrease in P_4 and increase in E_2 , the cervix begins to dilate and allow the flow of pus and exudates from the uterus for clearance of

an infection. Nikolakopoulos and Watson (1999) inoculated horse uteri with bacteria and began treatment with clenbuterol (a β_2 agonist that inhibits myometrial activity) 5 d after inoculation. Only mares treated with clenbuterol accumulated intrauterine fluid. The authors suggested that uterine contractility is important for the clearance of uterine fluid, but not for bacterial elimination. Due to the convoluted nature of the cervix in ewes, (Dun, 1955), a “leakage” from the uterus seems to be a less likely mechanism of uterine infection clearance. No ewes in any of these experiments exhibited vaginal discharge; thus, most of the pus and exudate must have been reabsorbed by the uterus.

In conclusion, culturing lymphocytes with PGE_2 inhibited blastogenic responses, but culturing lymphocytes with $PGF_{2\alpha}$ enhanced blastogenic responses. When indomethacin was cultured with lymphocytes, there was a consistent numerical (not statistical) increase in lymphocyte blastogenesis. Because indomethacin is a cyclooxygenase inhibitor, there is a possibility that arachidonic acid was converted to LTB_4 . Leukotriene B_4 is associated with improved immune cell activity, including neutrophil chemotaxis and activation. Injection of Lutalyse reduced P_4 and allowed clearance of uterine infections. *In vivo* treatment with P_4 and *in vitro* treatment with PGE_2 seem to have a negative effect on uterine immunity in postpartum and cyclic ewes. However, stage of the estrous cycle as well as prostaglandins seem to have major modulatory effects on uterine immune activity.

Implications

Nonspecific uterine infections are a serious problem to cattle all over the world. The uterine immune system is up-regulated until it is exposed to progesterone; then the

uterine immune system is down-regulated. Down-regulation of the uterine immune system may predispose cows to uterine infections. Injection of $\text{PGF}_{2\alpha}$ analogues decreases P_4 concentrations and allows for clearance of uterine infections. Manipulating arachidonic acid metabolites may provide us with a prevention or treatment plan for uterine infections. Knowledge of the mechanisms that govern the regulation of the uterine immune system may help us develop treatment and (or) prevention strategies that are superior to those that are currently available.

CHAPTER VII

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Appendix A

Surgical Procedures

Laparoscopic Procedures

1. All ewes were housed and denied access to feed and water for 24 h before the laparoscopic surgeries.
2. Ewes were anesthetized with an initial combination of ketamine (150 mg) and xylazine (50 mg). The ketamine and xylazine were administered i.v. until ewes were considered to have reached a surgical plane of anesthesia.
3. Wool was removed from around the udder and from the 12 to 18 cm of the belly proximal to the udder.
4. The surgical area was scrubbed with antiseptic soap.
5. The ewes were restrained in a laparoscopic cradle, and the hind legs were lifted to present the animals at a 45° angle.
6. Two small incisions were made through the skin to the left and right of the midline 2 cm below the teats.
7. A 7 mm diameter trocar and cannula were inserted into the peritoneal cavity through an incision left of the midline.
8. A 5 mm diameter trocar and cannula were inserted into the peritoneal cavity through an incision right of the midline.
9. The peritoneal cavity was inflated with CO₂.
10. The trocars were removed from each cannula.
11. The laparoscope was placed in the 7 mm cannula, and a probe was placed in the 5 mm cannula. The interior of the abdomen could then be viewed through the laparoscope.
12. A 17 g aspiration needle was placed in the cannula that held the probe. The needle was inserted in to the uterus. A 20 mL syringe was attached to the leuc lock hub of the needle and the appropriate amount of bacteria or saline was injected via the needle into the uterus.
13. The laparoscope and the needle were removed.
14. The bellies of the ewes were depressed to remove excess CO₂.

15. The cannulae were removed, and incisions were stapled or sewn.
16. The animals were allowed to remain undisturbed in pens for 2 to 3 h after the procedure.

Midventral Laparotomy

1. Ewes were restricted from feed for 36 h and from water for 24 h before surgery.
2. Ewes were anesthetized with sodium pentobarbital (Sigma Chemical Co., St. Louis, MO) in sterile isotonic saline (65 mg of sodium pentobarbital/mL). The sodium pentobarbital was administered i.v. until ewes were considered to have reached a surgical plane of anesthesia.
3. Anesthetized ewes were placed on a stainless steel surgical table. The ewes were placed in a dorsal recumbency position, and the surgical field was sheared.
4. The surgical field encompassed approximately 20 cm anterior to the udder or to the most caudal mammary gland and 15 cm in both directions from the midline. The surgical field was scrubbed at least three times with an antiseptic soap. Following the final scrub, a 10% iodine solution was applied to the surgical area.
5. Aseptic surgical techniques were used throughout all procedures. A sterile disposable drape was placed over the surgical field, and a longitudinal opening was cut directly anterior to the udder or most caudal mammary gland.
6. A 10 cm skin incision was made with a scalpel to one side of the midline in an attempt to avoid severing the mammary vein.
7. The subcutaneous layer of tissue was blunt dissected from the body wall.
8. At the midline, a scalpel was pushed through the body wall at the most anterior portion of the incision. Forceps were inserted into the hole created by the scalpel and used to lift the body wall as it was cut with the scalpel. The body wall incision was slightly shorter than the skin incision.
9. The uterus was located using fingers and carefully lifted to the surface in an attempt not to damage the uterus or the ovaries.
10. The ovaries were examined and described.

Umbilical tape was used to tie off the uterine ovarian blood supply and the ovaries were excised.

Vena Caval Catheters

1. Ewes were restricted from feed and water for a minimum of 12 h before surgery.
2. Ewes were anesthetized with a combination of xylazine (50 mg) and (sodium pentobarbital (Sigma Chemical Co., St. Louis, MO) in sterile isotonic saline (65 mg of sodium pentobarbital/mL). The sodium pentobarbital was administered i.v. until ewes were considered to have reached a surgical plane of anesthesia.
3. The saphenous vein dorsal to the hock was exposed.
4. A 90-cm long polyvinyl catheter (i.d. .42, o.d. .74 mm, ICO-Rally Corp., Palo Alto, CA) was inserted into each vein through a small incision and then passed through the saphenous vein into the vena cava.
5. The catheters were marked at 45, 50, 55, and 60 cm from the external end.
6. One 4-mL blood sample was taken at each marked position on one of the catheters as it was passed up the vein.

Catheter placement was determined according to progesterone concentrations depending on the time of the cycle.

Appendix B

Development and Validation of Neutrophil Activity Assays

Neutrophil Separation. To measure neutrophil activities, neutrophils must be separated from other leukocytes because some leukocytes, especially eosinophils, can mimic the activity of neutrophils. A system was developed to purify neutrophils (see appendix A for complete details) based on the separation technique of Roth and Kaeberle (1981). Briefly, whole blood (10 mL) was collected from the jugular vein of wethers and placed into 16 x 150 mm test tubes containing 1 mL of acid-citrate-dextrose anticoagulant solution. Blood tubes were centrifuged at 1,000 x g for 20 min at 4°C. The plasma, buffy layer, and the top one-fourth were removed and discarded. The erythrocytes were lysed with two volumes of hypotonic phosphate-buffered distilled water (.0132 M, pH 7.2) followed 50 s later with one volume of phosphate-buffered (.0132 M, pH 7.2) 2.7% saline. The mixture was centrifuged at 300 x g for 10 min at room temperature. The supernatant was removed and the pellet was resuspended in 5 mL of phosphate-buffered saline (.015 M, pH 7.2). The mixture was carefully layered onto 4 mL of Ficoll-Hypaque (Histopaque; Sigma Chemicals, St. Louis, MO) and centrifuged at 500 x g for 45 min at room temperature. The eosinophils should form a layer at the interphase, and the neutrophils should form a pellet at the bottom of the tube. The supernatant was removed and the pellet washed twice with 5 mL of .015 M PBS and centrifuged at 250 x g for 10 min at room temperature. If the pellet had excessive amounts of red blood cells, the pellet was incubated with 1 mL of Tris buffer (pH 7.2) for 10 min at 37°C and was centrifuged at 250 x g for 10 min at room temperature. The pellet was resuspended in 1 mL of Medium 199 (Gibco, Grand Island,

NY), supplemented with 20 mM of HEPES buffer, 10% FCS (Sigma Chemical), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin (Sigma Chemical). The cells were counted using a hemocytometer, and viability was determined using the trypan blue exclusion test. The final concentration of live cells was adjusted to 5×10^7 cells/mL of medium. The purity of neutrophils ranged from 92 to 98% (average = 95%). The major contaminant in the preparation was eosinophils.

Chemotaxis Assay. After the uterus is contaminated with bacteria, inflammation occurs, and(or) bacteria release chemoattractant molecules that attract neutrophils to the site of the infection (chemotaxis). In order to measure chemotactic ability of neutrophils, one can measure chemotaxis *in vitro*. The most common chemotaxis assay is the “under agarose” technique in which three wells are made in agarose gels either on a general glass microscope slide or in a petri dish. Generally, 3 wells are made in the agarose. The center well is filled with neutrophils, and the other side wells are filled with either a control chemical or a chemoattractant, commonly f-Met-Leu-Phe or opsonized zymosan. After incubation, comparisons can be made between the cells migrating toward the chemoattractant and the cells moving away from the chemoattractant. A typical plate of the chemotaxis assay after incubation can be seen in Figure 25.

Roth and Kaeberle (1981) used an aliquot of neutrophils at a final concentration of 5×10^7 cells/mL (there was no mention of exact cell numbers) and determined the chemotactic ability of neutrophils. To determine the appropriate cell number to use in this assay, 125,000, 250,000 500,000, and 750,000 cells/well were used. Agarose plates were prepared as described by Dr. Jim Roth and coworkers (personal communication; Iowa State University). Briefly, Medium 199 supplemented with 20 mM

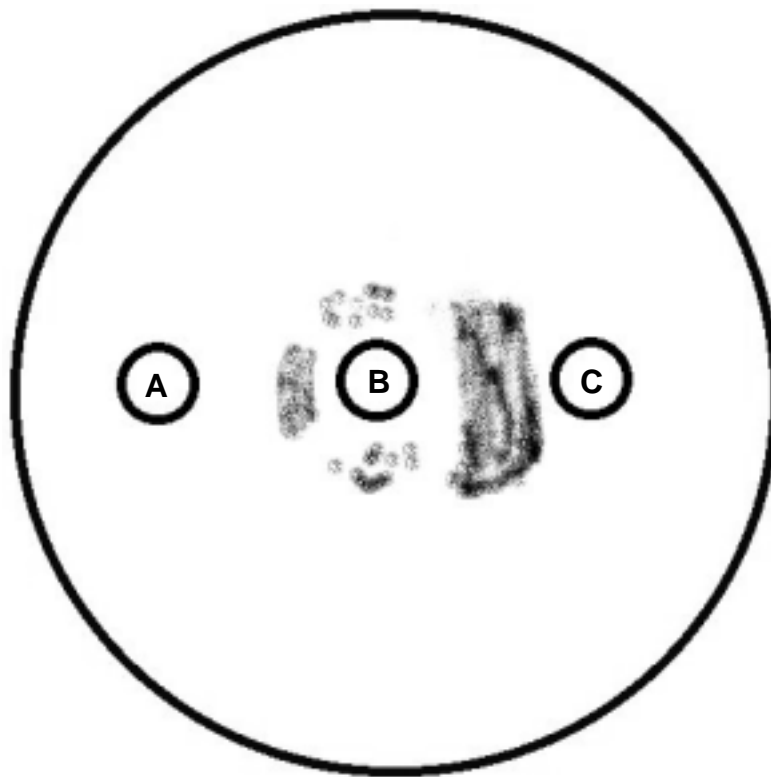


Figure 25. A typical developed petri dish after neutrophil chemotaxis. Well A = control serum, Well B = neutrophils, Well C = opsonized zymosan.

HEPES buffer was boiled with Indubiose A37 agar (Biosepra; Marlborough, MA) at a final concentration of .8%. After boiling, the agar was allowed to cool to 48°C, and the agar was supplemented with 10% FCS, 100 IU/mL penicillin, and 100 µg/mL of streptomycin. Agar (6 mL) was placed into 60 x 15 mm petri dishes, allowed to completely cool, and stored at 4°C. Three wells (3 mm in diameter) were cut into the agar in a straight line and were at least 3.5 mm away from one another. Neutrophils were added to the center well, zymosan-activated ovine serum was added to a side well, and control serum was added to the remaining well. Plates were incubated at 37°C for 18 h on a level surface, the plate was flooded with 8% glutaraldehyde for 30 min, the agar was removed, and the cells were stained with Wright's stain. The distance of the leading edge of the neutrophils migrating toward the chemoattractant (zymosan activated serum) was measured if possible. The final cell concentration that could be measured and gave consistent results was 750,000 cells/well.

Nitroblue Tetrazolium Reduction Assay. Once the neutrophils are called to the site of infection and initiate the respiratory burst, a release of superoxide anions ($\cdot\text{O}_2^-$) occurs. Current techniques to measure the quantitative amounts of $\cdot\text{O}_2^-$ include oxygen consumption, chemiluminescence, and increased reduction of NBT. We decided to use the NBT reduction method (Roth and Kaeberle, 1981) due to its ease and repeatability. The overall reaction of the respiratory burst is $2\text{O}_2 + \text{NADPH} \rightarrow 2\cdot\text{O}_2^- + \text{NADP}$ (Gabig and Babior, 1981). Reduction of NBT is accomplished by the reaction $2\cdot\text{O}_2^- + \text{NBT} \rightarrow \text{O}_2 + \text{formazan}$. Soluble NBT is transformed into an insoluble precipitate within the neutrophils to the blue-black formazan. Formazan can be seen microscopically and can be quantified spectrophotometrically after extraction of cells with hot pyridine.

A standard reaction mixture included neutrophils, .4 mg of NBT, and preopsonized zymosan in a total volume of 1 mL. In this assay, preopsonized zymosan was phagocytized and caused a release of $\cdot\text{O}_2^-$ due to the respiratory burst. To determine the best assay conditions for our laboratory, the number of neutrophils (1.25×10^6 , 2.5×10^6 , 5×10^6 , and 7.5×10^6 cells) and the amount of zymosan (0, 1, 5, 10, 20, and 40 mg) were varied. Samples were assayed in triplicate. All components of the reaction were added to 16 x 100 mm siliconized borosilicate tubes, except neutrophils, and were allowed to equilibrate in a 37°C shaking water bath for 15 min. Zymosan was deleted from the reaction mixture to determine resting NBT reduction. The neutrophils were added and allowed to incubate for 5 min. To stop the reaction, 5 mL of cold 1 mM N-ethylmaleimide in saline was added. Tubes were centrifuged at 500 x g for 10 min and the supernatant was aspirated (taking special care not to disturb the pellet) and discarded. The pellet was resuspended in 5 mL of pyridine (Sigma Chemical; P-3776), sonicated (to break up the pellet), and heated in a boiling water bath for 10 min in a fume hood. The tubes were capped and centrifuged at 500 x g for 10 min. Tubes were wiped clean with tissue paper, and the optical density (OD) was read at 580 nm with a spectrophotometer.

Results showed that as few as 1.25×10^6 cells/tube could be used for this assay. Also, there seemed to be a sigmoidal response curve to the amount of zymosan added in the reaction mixture (Figure 26). The optimal dose of zymosan seemed to be 10 mg/tube; there appeared to be an inhibition of NBT reduction at the dose of 20 mg/tube.

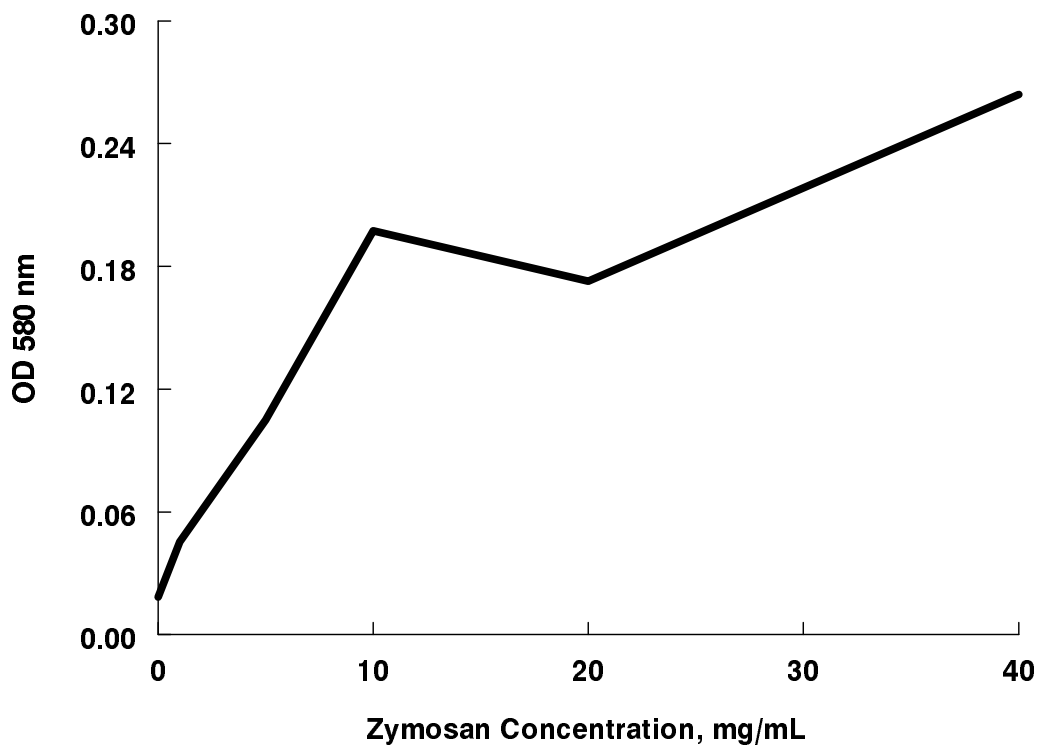


Figure 26. Effect of varying the concentration of zymosan used in the NBT reduction assay.

Thus, we decided to use 1.25×10^6 neutrophils, 10 mg of zymosan, and .4 mg of NBT in a total volume of 1 mL for this assay. Much to our dismay, our ability to collect neutrophils from whole vena caval blood after the uteri of ewes were inoculated with either bacteria or chemotactic agents (Hunter *et al.*, 1999, unpublished results) was very poor. Thus, that portion of the experiments had to be removed and our concentration shifted to the lymphocytes.

Neutrophil Isolation Procedure

1. Transfer 10 mL of blood in 16 x 150 test tube filled with 1 mL acid-citrate-dextrose solution and mix gently.
2. Immediately spin the blood and centrifuge at 1000 x g for 20 min.
3. Remove plasma, buffy coat, and top one-fourth of the packed red cells.
4. Add two volumes of cold phosphate buffered (0.0132 M, pH 7.2) deionized water.
5. After 30 s, add one volume of cold phosphate buffered (0.0132 M, pH 7.2) 2.7% NaCl.
6. Centrifuge at 300 x g for 10 min.
7. Remove supernatant, resuspend cells in 2 mL of .015 M phosphate-buffered saline, and repeat Steps 4 to 6 one time.
8. Resuspend pellet in 5 mL of 0.015 M phosphate buffered saline and centrifuge at 250 x g for 10 min.
9. Layer mixture onto 4 mL of Ficoll-Hypaque and centrifuge at 500 x g for 30 min with the brake **OFF**.
10. Aspirate supernatant and wash the pellet with 5 mL of .015 M PBS and centrifuge at 250 x g for 10 min at room temperature with the brake on.
11. Wash the pellet twice with 5 mL of PBS and centrifuge at 250 x g for 10 min at room temperature with the brake on.
12. Resuspend in 1 mL of respective media for the assay you are doing (Medium 199 for chemotaxis or EBSS for NBT reduction assay).

13. Count the cells using a hemacytometer and the trypan blue exclusion method.
14. For the chemotaxis assay, suspend cells to a final concentration of 1.5×10^8 cells/mL in Medium 199 (Gibco, Inc., Gaithersburg, MD; Catalog # 31100-035).
15. For the NBT reduction assay, suspend cell to a final concentration of 1.25×10^7 cells/mL in Earle's Balanced Salt Solution.

Acid-citrate-dextrose anticoagulant solution

	1X	2X	2X	2X
Citric acid monohydrate	3.27g	6.54g	.654g	3.27g
Sodium citrate	25.4g	50.8g	5.08g	25.4g
Sodium phosphate monobasic	2.22g	4.44g	.444g	2.22g
Dextrose (monohydrate)	25.56g	51.12g	5.112	25.56g
Sterile saline	1 L	1 L	100 mL	500mL
EDTA*	.3 g	.3 g	.03 g	.15 g

*Supplemented with EDTA at 300 µg/mL.

From the 2X solution, 1mL of solution per 10 mL of blood

Medium 199

Reconstitute 1 package with 1 liter of water
 Add 2 g sodium bicarbonate
 Adjust pH to 7.2
 Filter sterilize with .2 µm filter paper.

Mixing the assay media

HEPES	.4766 g
Fetal calf serum	10 mL
Pen/Strep	1 mL
Medium 199	89 mL
Total	100 mL

Filter sterilize with .45 µm filter paper.

This media contains 20 mM HEPES, 10% FCS, 100 IU penicillin/mL, and 100 µg of streptomycin/mL.

Phosphate Buffered Stock Solutions

.2 M Sodium phosphate monobasic

1.38 g in 50 mL distilled water or
2.76 g in 100 mL distilled water

.2 M Sodium phosphate dibasic

1.42 g in 50 mL distilled water or
2.82 g in 100 mL distilled water

Phosphate Buffered Solutions

.0132 M phosphate buffered distilled water

.2 M sodium phosphate monobasic	26 mL
.2 M sodium phosphate dibasic	40 mL
Distilled water	934 mL
Total	1,000 mL

.0132 M phosphate buffered 2.7% saline

.2 M sodium phosphate monobasic	26 mL
.2 M sodium phosphate dibasic	40 mL
Sodium chloride	27 g
Distilled water	934 mL
Total	1,000 mL

.015 M phosphate buffered saline

.2 M sodium phosphate monobasic	29.95 mL
.2 M sodium phosphate dibasic	45.75 mL
Sodium chloride	9 g
Distilled water	925 mL
Total	1,000 mL

Preparation of Preopsonized Zymosan

1. Suspend zymosan at a concentration of 10 mg/mL (1g/100mL) in cold Earle's balanced salt solution by homogenization for 1 min.
2. Stir at room temperature for 1 h.
3. Aliquot (10 mL) in 15 mL conical tubes and freeze.
4. Thaw and homogenize prior to use.

Preparation of Zymosan-activated Serum

1. Add 100 mL of fresh ovine serum¹ and stir for 1 h at room temperature.
2. Centrifuge at 250 x g at room temperature.
3. Collect supernatant and aliquot in 1.5 mL microcentrifuge conical tubes.

¹Serum should be collected from a wether or an OVEX ewe. If this is not possible, charcoal strip the serum to remove steroids.

Earle's Balanced Salt Solution

Reconstitute 1 package with 1 L of water
Add 2.2 g sodium bicarbonate
Adjust pH to 7.2
Filter sterilize with .45 µm filter paper.

EBSS	89 mL
Lamb Serum	10 mL
Pen/Strep	1 mL
Total	100 mL

Filter sterilize with .45 µm filter paper.

This media contains 20 mM HEPES, 10% FCS, 100 IU penicillin/mL, and 100 µg of streptomycin/mL.

Preparation of Plates and Procedures for Chemotaxis Assay

Medium 199	89 mL
HEPES	.4766 g
Indubiose A37	0.8 g
Total	100 mL

1. Boil the media until it turns clear.
2. Take off hot plate, let cool to 52°C, and add 10 mL of fetal calf serum, 1 mL of Pen/Strep (final concentration of 100 IU penicillin/mL and 100 µg streptomycin/mL while stirring).
3. Begin pouring 6 mL of media-agar mix into 60 X 15 mm petri dishes (MOVE FAST).
4. Follow with a brief flaming with propane to remove any bubbles on the plate. Allow the plates to completely cool. This media contains 0.8% agar. This will prepare about 15 plates.
5. After plates have cooled, cut 3 3 mm holes in diameter in the agar. The holes must be at least 3.5 mm away from one another.
6. Incubate in humidified 37°C incubator with 5 % CO₂ on a flat surface for 18 hours.
7. After 18 h, remove from incubator, and flood (5 mL) with 8% glutaraldehyde, and incubate at room temperature for 1 h.
8. Pour liquid off and **carefully** remove agar from the plate by turning upside down and gently scrape the agar off using a wooden stick or tongue depressor.
9. **Gently** wash plate with deionized distilled water and flood plate with 1% gentian violet (or 1% crystal violet) and incubate for 10 min at room temp.
10. Pour the dye off and **gently** wash the plate with deionized distilled water and allow plate dry at room temperature.

Procedures for NitroBlue Tetrazolium (NBT) Reduction Assay

Assay in triplicate in 16 x 100 mm silicon-coated glass test tubes.

Standard Reaction Mixture

NBT solution (2 mg/ml)	200 µL
Preopsonized zymosan in EBSS*	100 µL
EBSS	500 µL
Total	800 µL

*For determination of resting NBT reduction, add all components except zymosan.

1. Allow to reaction mixture to equilibrate in a 37°C shaking water bath for 15 min.

2. Add 200 μL (1.25×10^6 cells) from the neutrophil stock (6.25×10^6 cells/mL).
3. Incubate in 37°C water bath for 5 min.
4. Add 5 mL of cold 1 mM N-ethylmaleimide in saline to stop the reaction.
5. Centrifuge at $500 \times g$ for 10 min.
6. Aspirate supernatant and discard. (Avoid aspirating pellet in bottom of tube).
7. Resuspend pellet in 5 mL of pyridine in a fume hood.
8. Sonicate until pellet is broken up and heat in boiling water bath for 10 min in a fume hood.
9. Cap tube with stoppers and centrifuge at $500 \times g$ for 10 min.
10. Clean tubes with tissue paper and read OD at 580 nm in a spectrophotometer using a pyridine blank.

Silicon Coated Glass Tubes

1. Wear gloves.
2. Add 750 μL of SigmaCote (Sigma Chemicals, St. Louis, MO) to 16 x 100 mm borosilicate tube.
3. Vortex three times for 1 min waiting 5 min between vortexes.
4. Pour the extra SigmaCote back into the bottle for use at a later date.
5. Allow to dry in a fume hood overnight. Inspect tubes for complete dryness.

Vita

Richard Seals was born on July 28, 1972 in Morristown, Tennessee. He attended Hamblen County public schools in Morristown, where he graduated from Morristown-Hamblen High School East in June of 1990. Richard attended Walters State Community College in the summer of 1990 and then transferred to the University of Tennessee at Knoxville in the fall of 1992 majoring in Animal Science. In the spring of 1994, Richard graduated from the University of Tennessee with a B.S. degree and began working on a M. S. degree in the Animal Science department in the fall of 1994 under the direction of Dr. F. Neal Schrick. In the summer of 1996, he graduated with a M.S. degree in Animal Science with a concentration in reproductive physiology. Richard accepted a Ph. D. position at Virginia Polytechnic Institute and State University under the direction of Dr. Greg Lewis to begin in the fall of 1996. He graduated in the spring of 2000 with a Ph. D. degree in Animal Science with a concentration in reproductive physiology.