THE MECHANISM OF ACTION OF EXOGENOUS $PGF_{2\alpha}$ IN CLEARANCE OF NONSPECIFIC UTERINE INFECTIONS IN SHEEP AND PIGS

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ABSTRACT

Six experiments were conducted to determine the mechanism of action of exogenous $PGF_{2\alpha}$ on the clearance of uterine infections in sheep and pigs. The first two experiments were designed to characterize the uterine immune response to bacterial infection under progesterone dominance in pigs. The uterine immune response to infections seems to change with parity. This is probably an artifact of increased number of bacterial exposures; therefore, the third experiment was designed to evaluate the uterine immune response to multiple intrauterine bacterial inoculations. Experiments 4, 5, and 6 were designed to evaluate the effects of endogenous and exogenous $PGF_{2\alpha}$ on the uterine immune response to uterine infections in sheep and pigs. Injections with Lutalyse ($PGF_{2\alpha}$ analogue) during the luteal phase in sheep causes luteolysis; therefore, it impossible to evaluate the effects of Lutalyse independently of luteolysis. In order to cause an endogenous release of $PGF_{2\alpha}$ without causing luteolysis in sheep a $PGF_{2\alpha}$ secretagogue (oxytocin) was used in Exp. 5. And in Exp. 6, we were able to evaluate the effects of Lutalyse independently of luteolysis using pigs as a model. From these six experiments we concluded that during periods of estrogen dominance, the uterine immune system is up-regulated, and therefore, infections do not develop after intrauterine inoculation with bacteria, during periods of progesterone dominance, the uterine immune system is down-regulated, and, therefore, infections develop after intrauterine inoculation with bacteria, and stimulation of the uterus with $PGF_{2\alpha}$ or oxytocin independently of luteolysis up-regulates the uterine immune.

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ABBREVIATIONS

The following abbreviations were used throughout this dissertation:

A. pyogenes	*Arcanobacterium pyogenes*
Ab	Antibody
Ag	Antigen
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. coli	*Escherichia coli*
E_2	Estrogen or estrodiol-17β
EIA	Enzymeimmunoassay
GnRH	Gonadotropin releasing hormone
hr	Hour(s)
Ig	Immunoglobulin
i.m.	Intramuscular
IP_3	Inositol triphosphate
LTB	Leukotriene
LH	Luteinizing hormone
LPS	Liposaccharides
min	minute(s)
P_4	Progesterone
PBS	Phosphate buffered saline
PG	Prostaglandin
PKA	Protein Kinase A
PKC	Protein Kinase C
RIA	Radioimmunoassay
S	second(s)
SEM	Standard error of the mean
TX	Thromboxane
wk	week(s)
WBC	White blood cell
yr	year(s)

Chapter I

INTRODUCTION

Although uterine infections are a major contributor to reproductive problems in most livestock species, the repercussions of uterine infections are commonly ignored. The scientific literature contains thousands of publications with some reference to uterine infections; however, most of these publications are more relevant to humans or companion animals than to livestock. Uterine disorders, including nonspecific uterine infections, reduce the reproductive efficiency of livestock and the profit potential for associated farms. Because of intensive management, the incidence of uterine infections is most accurately reported in dairy and swine herds. Accurate estimates of the incidence of uterine infections in beef cattle herds and in sheep flocks are unavailable. However, it is reasonable to believe that under similar intensive management schemes the estimates would be similar to the estimates for dairy cattle. In some dairy herds, 40% of the postpartum dairy cows may be diagnosed with uterine infections (Arthur et al, 1989; Lewis, 1997). It is impossible to estimate the number of infections that remain undetected. Uterine infections often reduce reproductive efficiency and increase apparent (i.e., diagnosis and treatment) and hidden (i.e., reductions in feed consumption, reductions in milk or meat production, and increases in culling rates) herd health costs.

The term uterine infection indicates that the uterus is contaminated with multiplying pathogenic organisms. After a uterine infection is detected, three criteria are used to characterize the type of uterine infection: 1) clinical signs; 2) degree of severity; and 3) time postpartum or time of the estrous cycle (Youngquist and Little, 1988; Lewis, 1997). Subclinical uterine infections often go undiagnosed and uncharacterized, but may result in significant uterine damage ad lasting reproductive effects. The term subclinical uterine infection indicates that the

uterus is contaminated with pathogenic organisms that may or may not be multiplying, but that there are no clinical signs of the infection. The most common bacterial species associated with uterine infections in livestock is *A. pyogenes,* an opportunistic bacterium that causes various degrees of uterine infections alone or in conjunction with other bacterial species.

Most commonly, uterine infections develop during the postpartum period. Changes in the hormonal and immune environment before and after parturition contribute to the establishment of infections. The postpartum period has been defined as the interval from parturition to complete uterine involution and has been broken into three time periods: 1) puerperal period (the time period after calving until the pituitary replenishes LH stores, and therefore, responds to GnRH release form the hypothalamus); 2) intermediate period (from the end of the puerperal period until the first postpartum ovulation); and 3) postovulatory period (from the end of the intermediate period until uterine involution is complete) (Olson et al., 1986). During the puerperal period, most species develop mild nonpathological endometritis, which is characteristic of the initiation and early stages of uterine involution. During the intermediate period, the pathogenic organisms are reduced or eliminated. We use postpartum and cyclic populations of livestock to model the puerperal, intermediate, and postovulatory periods. These experimental models allow us to study the uterine response to exogenous pathogenic bacteria under physiological conditions similar to those before, during, and after uterine involution.

Treatment of uterine infections varies with the severity of the infection. Traditionally, uterine infections are treated via intrauterine infusion of various antimicrobial-antibacterial compounds or uterine lavage; however, these treatments are largely ineffective and may be harmful (Paisley et al., 1986; Hussain and Daniel, 1991; Gilbert and Schwark, 1992). Currently, the most common treatment for uterine infections in the United States is administration of

Lutalyse (Pharmacia & Upjohn; Kalamazoo, MI) or other $PGF_{2\alpha}$-analogues at various intervals and doses (Olson et al., 1986; Youngquist and Little, 1998). However, the mechanism of action of Lutalyse in the treatment of uterine infections has not been clearly defined. Pyometra usually occurs in livestock with an active CL. Lutalyse causes luteolysis and reduces progesterone concentrations. Regression of the CL and the decreased progesterone concentrations permit the uterus to resolve the infection (Paisley et al., 1986; Youngquist and Little, 1988). This partial mechanism of action is probably correct for ruminants with an active CL, but it does not account for the therapeutic response following administration of Lutalyse to cows with uterine infections but without an active CL. It also does not account for therapeutic effects of Lutalyse administration in the absence of luteolysis. A second suggestion is that the effects of Lutalyse are mediated through changes in uterine motility. Lutalyse briefly increases uterine motility in some cows, and this could increase the rate at which a uterine infection is expelled. Although both of these mechanisms are mediated through changes in the local prostanoid environment, they ignore the possibility that the alterations in uterine prostanoid environment affect the uterine immune response.

Impaired or down-regulated lymphocyte and neutrophil activity before and(or) after parturition increases the susceptibility of livestock to uterine infections. When systemic or local lymphocyte and neutrophil function is suppressed, benign bacteria such as the nonpathogenic organisms associated with “normal” uterine involution become established in the uterus, proliferate, and cause uterine infections. Changes in the immune function are mediated by changes in leukocyte function and population. Impaired or down-regulated leukocyte function increases the susceptibility to uterine infections. Prostaglandins and other eicosanoids regulate many leukocyte functions, in particular lymphocyte and neutrophil functions. In addition to

regulation via arachidonic acid metabolites, neutrophil and lymphocyte functions are regulated by steroid concentration (i.e., progesterone down-regulates lymphocyte and neutrophil function and various cytokines).

For half a century, livestock producers and veterinarians have used antibiotics to control infections and as growth promoters in livestock. These practices have lead to healthier animals and an increased food supply. However, antibiotic supplementation has also increased the incidence of bacterial resistance to antibiotics. The only apparent way to reduce antibiotic resistance is to reduce the use of antibiotics. Understanding the physiological conditions that make livestock more susceptible to uterine infections should allow us to devise protocols that dramatically reduce the incidence of uterine infections and that increase our success of treatment, therefore decreasing the unnecessary use of antibiotics. Understanding the physiological mechanisms associated with generation, maintenance, and clearance of uterine infections should yield alternative treatments for uterine infections (i.e., treatment with $PGF_{2\alpha}$ or one of its analogues).

The experiments described throughout this dissertation were conducted with sheep and pigs as models to study the interactions between exogenous and endogenous prostaglandins on the uterine immune response to bacterial infection. Typically, we use sheep in our laboratory as a convenient and an inexpensive model for studying uterine infections in cattle. However, because the ruminant CL is responsive to Lutalyse it is difficult to use a ruminant model to distinguish between the effects of luteolysis and changes in prostaglandin concentrations per se on uterine infections. Before d 12, the CL in pigs are unresponsive to Lutalyse; therefore, pigs allow us to further separate out the general and independent actions of steroids and prostaglandins on the clearance of uterine infections. This dissertation and the associated experiments were designed to

study the in vivo and in vitro effects of $PGF_{2\alpha}$ on the uterine immune system and, consequently, the clearance of uterine infections in sheep and pigs.

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Chapter II

QUESTIONS AND OBJECTIVES

The scientific literature contains thousands of articles with some reference to uterine infections or to the uterine immune system; however, most of this literature pertains to humans and companion animals, but not to livestock. In addition, the focus of this literature is the dual function of the uterine immune system: enhanced protection against infection and tolerance of the fetal allograft. Until recently, the influence of the uterine immune system on maintenance of reproductive health in livestock has been overlooked.

The overall objective of this chapter is to briefly describe the research conducted in our laboratory and the connections between previous research and the research described in this dissertation. A series of questions and objectives are posed. The questions and objectives are posed in the order that seems logical for evaluating portions the overall model. The chapters and experiments that address these questions and objectives are described here. The questions and objectives are described again in the appropriate chapters. The answers to the questions are described in detail in Chapter VIII.

Earlier research conducted in our laboratory characterized the relationship between changes in immune cell response to bacterial challenge and the hormonal changes in the estrous cycle of sheep (Ramadan et al., 1997). In particular, during the follicular phase, when estrogen is the dominant ovarian hormone, ewes are resistant to uterine infections, whereas, during the luteal phase when progesterone is the dominant ovarian hormone, ewes develop uterine infections after intrauterine exposure to infectious bacteria. Changes in susceptibility to uterine infections throughout the estrous cycle explain why uterine infections are usually only maintained through one estrous cycle in cattle and sheep. After luteal regression, most uterine infections are cleared.

It is not known whether clearance of these infections is related to the concomitant decrease in progesterone during luteolysis, an increase in endogenous $PGF_{2\alpha}$, or a combination of the two events.

Although sheep provide an inexpensive, ruminant model to study "typical" uterine infections and clearance of these infections, there are several physiological limitations associated with this model. For example, it is difficult to separate the effects associated with luteolysis, including changing prostaglandin profiles, from the effects of decreased progesterone concentrations after luteolysis begins. To overcome these limitations, it was necessary to create a new model in a different species. Because an exogenous $PGF_{2\alpha}$ analogue can be administered to pigs before d 12 of the estrous cycle without causing luteolysis, pigs would seem to be an excellent model for investigating the effects of $PGF_{2\alpha}$ on the uterine immune system without causing luteolysis or a decrease in progesterone. In other words, the effects of $PGF_{2\alpha}$ on the uterus would not be confounded with the effects of $PGF_{2\alpha}$ on luteal regression. However, before we could investigate the role of $PGF_{2\alpha}$ in the clearance of uterine infections in pigs, we had to determine whether the uterine response to bacterial challenge was similar to that in sheep. Thus, experiments were designed to test the following questions.

Question 1 (unpublished data; Chapter IV, Exp. 1 and 2; Chapter VII, Exp. 6): Do the bacterial isolates that we use in sheep cause uterine infections in female pigs?

Question 2 (unpublished dose response curve): What is the optimal dose of *A. pyogenes* and *E. coli* to cause an infection in female pigs similar to the infections that we generate in sheep?

Question 3 (Chapter IV, Exp. 1 and 2): Do female pigs provide a good model for detailed studies of uterine infections?

Question 4 (Chapter IV, Exp. 1 and 2): Using female pigs, can we consistently produce animals that are susceptible to or resistant to infections?

Question 5 (Chapter IV, Exp. 1): Does the uterine immune cell response to infectious bacteria differ during the follicular and luteal phases of the estrous cycle in female pigs?

Question 6 (Chapter IV, Exp. 1 and 2): If there are differences in the uterine immune cell response to infectious bacteria during the follicular and luteal phases of the estrous cycle, are these changes steroid hormone dependent?

Question 7 (Chapter IV, Exp. 2): Does progesterone mediate the changes in uterine immune cell response to infectious bacteria during the estrous cycle in female pigs?

Question 7a (Chapter IV, Exp. 2): Do exogenous and endogenous progesterone increase susceptibility to uterine infections via the same mechanism?

Question 7b (Chapter IV, Exp. 2): If female pigs are ovariectomized during the follicular phase, a period when the uterus is not susceptible to infection, do they maintain their resistance to uterine infections?

Before we could perform experiments to further define the mode of action of exogenous and endogenous $PGF_{2\alpha}$ on the clearance of uterine infections, it was necessary to address some discrepancies in our sheep model. Previous research indicated that the uterine immune cell response, as measured by the severity of the induced uterine infections, differed greatly between individual ewes receiving the same treatments. Under normal circumstances, we use a variety of ages and parities distributed among treatments. This caused us to believe that the differences in response might be related to parity. In particular, parity could represent the number of times an animal was “naturally” exposed to the bacteria that most commonly cause uterine infections. For example, livestock are exposed to numerous infectious bacteria at the time of mating, at parturition, and during uterine involution. Therefore, parity would be representative of the number of times the uterus was “naturally inoculated” with infectious bacteria. This was not relevant to our pig model, because for each study, either all gilts or all sows of similar parity were used. Relevance of this question would also decrease if we used bacterial isolates that were not common to the area; however, the bacteria for our experiments are isolates of indigenous bacteria. Extreme differences in the number of exposures to the same or similar bacteria would

cause differences in the immune cell response. Thus, an experiment was designed to test the following questions.

> **Question 8 (Chapter V, Exp. 3):** Does the uterine immune cell response change after multiple exposures to infectious bacteria in nulliparous ewes?
>
> **Question 9 (Chapter V, Exp. 3):** If the uterine immune cell response changes after multiple exposures to infectious bacteria, is it quantifiable?

Typically, we measure changes in uterine immune cell response in terms of the presence or absence of uterine infections, changes in differential white blood cell counts, changes in lymphocyte blastogenesis, and changes in endocrine profile. These techniques allow us to measure changes that occur several days after the induction of an infection. To study the immediate response of the uterine immune system, it is necessary to study elements of the innate immune system. The innate immune responses would be less likely to differ between animals with different bacterial exposure frequencies. The most logical approach would be to study neutrophil function. Initially, we wanted to evaluate three measures of neutrophil activity (i.e., chemotaxis, ingestion, and superoxide production); however, because of limited success in isolating neutrophils, we were unable to accurately characterize neutrophil function.

Following validation of the animal models described above, we designed experiments to examine the role of exogenous and endogenous $PGF_{2\alpha}$ on the clearance of uterine infections. In the United States, Lutalyse (Pharmacia & Upjohn; Kalamazoo, MI) and other $PGF_{2\alpha}$ products seem to be the treatment of choice for uterine infections that occur during the luteal phase (Gilbert and Schwark, 1992). The decrease in progesterone and the concomitant increase in estradiol following treatment with Lutalyse should up-regulate the immune system and permit an animal to clear a uterine infection. However, there is evidence that there are beneficial effects of exogenous $PGF_{2\alpha}$ of treating animals with uterine infections that do not have active corpora lutea

(Gilbert and Schwark, 1992). This suggests that the beneficial effects of treatment with Lutalyse may be mediated by several mechanisms. Lutalyse induces the uterus to secrete $PGF_{2\alpha}$ (Wade and Lewis, 1996). Therefore, the administration of exogenous $PGF_{2\alpha}$ may increase the endometrial availability of free arachidonic acid, which would then be converted into $PGF_{2\alpha}$ via the cyclooxygenase pathway or LTB_4 via the lipooxygenase pathway. Both $PGF_{2\alpha}$ and LTB_4 can be powerful immune modulators. If this is the case, compounds that cause an endogenous release of $PGF_{2\alpha}$ may be used as treatments for uterine infections. The use of Lutalyse as a treatment superimposed on the cyclic sheep and pig models should give us the opportunity to better define the mechanisms of action of exogenous $PGF_{2\alpha}$ in the treatment of uterine infections. Therefore, experiments were designed to answer the following questions in sheep and pigs.

Question 10 (Chapter VI, Exp. 4): Does Lutalyse increase the rate of clearance of uterine infections?

Question 11 (Chapter VII, Exp. 6): Does Lutalyse increase the rate of clearance of uterine infections alone or in conjunction with luteolysis?

Question 12 (Chapter VI, Exp. 4): Does Lutalyse increase the rate of clearance of uterine infections alone or in conjunction with an endogenous increase in $PGF_{2\alpha}$?

Rather than switching models, another way to address the role of $PGF_{2\alpha}$ in the clearance of uterine infections is to use a substance that would cause an endogenous release of $PGF_{2\alpha}$, without causing a decrease in progesterone or luteolysis. Therefore, an experiment using oxytocin as a $PGF_{2\alpha}$ secretogue in sheep was designed to answer the following questions.

Question 13 (Chapter VI, Exp. 5): Does oxytocin increase the rate of clearance of uterine infections in sheep?

Question 14 (Chapter VI, Exp. 5): Does oxytocin increase the rate of clearance of uterine infections via changes in prostaglandin secretion?

Throughout these experiments, the answers to these questions were augmented by several in vitro treatments that were superimposed on in vivo primed cells. In particular, we were able to more closely examine the effects of prostaglandins on individual immune cells.

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Chapter III

REVIEW OF LITERATURE

Reproductive Immunology

The field of reproductive immunology evolved over the last 100 yr as reproductive physiology and immunology fields converged. Reproductive immunology deals with the immunological factors in reproduction, the immunological processes that are involved in the maintenance of reproductive systems, and with the advent and implementation of immunological techniques that allow scientists to further elucidate reproductive processes. The field of experimental reproductive immunology emerged from combining the knowledge of reproduction with noninfectious immunology and is closely associated with the fields of transplantation immunology and immunogenetics.

The fields of immunology and reproduction share one of their oldest questions. Stated by immunologists the question is asked: How is the fetal allograft accepted by the maternal immune system? Stated by reproductive physiologists the question is asked: How is pregnancy maintained?

Historically, reproductive immunologists have been focused on 1) the immunogenicity and antigenic structure of gametes and other components of the reproductive tract, 2) the role of immunological factors in reproduction, 3) the uterus as an immunoprivileged site, and 4) the immunological regulation of the maternal-fetal interface. Metchnikoff may well be considered the father of modern reproductive immunology. In 1899, while studying the role of macrophages in the resorption of spermatozoa introduced into the peritoneal cavity of guinea pigs and rabbits, Metchnikoff established that the injection of xenogenic sperm renders their serum “spermotoxic.” Metchnikoff’s work has been elaborated over the last 100 yr in an attempt to

determine antigenic properties of spermatozoa that decrease fertility. Determination of the antigenicity of spermatozoa in the female tract could lead to techniques that would decrease infertility and(or) could lead to the production of new "birth control" techniques. Medawar (1953) proposed one of the most notable and memorable reproductive immunology theories for which he later received a Noble Prize. Medawar (1953) proposed the concept of the fetal allograft to explain the relationship between the maternal immune system and the fetoplacental unit. His theory perpetuated the study of the uterus as an immunoprivileged site. We now know that much of Medawar's theory was incorrect. The uterus is not an immunoprivileged site, and the presence of a conceptus/fetus is not indicative of a typical allograft.

Currently, the largest growing segment of reproductive immunology is the application of knowledge of the immune system's involvement in nonpathogenic physiological mechanisms to the organs of the reproductive tract. The experiments described in this dissertation further characterize the unique immune involvement in the maintenance of reproductive health in the presence and in the absence of pathogenic invasion in livestock.

Economic Consequences and Prevalence of Uterine Infections in Domestic Livestock

In female livestock, reproductive efficiency is the major determinant of lifetime productivity. Reproductive disorders are a major reason for decreased reproductive performance in all livestock and is particularly well documented in dairy cattle. Endometritis accounts for approximately 20% of the reproductive disorders in dairy cattle (Coleman et al., 1985). In some herds, 40% of the postpartum dairy cows may be diagnosed with and treated for uterine infections (Arthur et al., 1989; Lewis, 1997). Uterine infections and the treatment of these infections increases herd health costs, reduces feed consumption, causes a reduction in milk production, and forces cows that would otherwise remain in the herd to be culled (Lewis, 1997).

A 1986 report indicated that the cost to producers for each lactating dairy cow with a uterine infection was $106 (Bartlet et al., 1986). Similar estimates for the cost of uterine infections are not available for other livestock species. Intensive reproductive management allows for estimates of the costs of uterine infections in dairy cattle. Even though reproductive performance is just as important in sheep and beef cattle, current management of these animals is not conducive to estimating costs of a particular reproductive disorder. The fact that uterine infections often resolve themselves also makes it more difficult to estimate cost in a range species. It seems reasonable, after accounting for management system, that the estimates for occurrence of uterine infections would be similar across ruminant species. However, the incidence of nonspecific uterine infections seems to be less in sheep than in dairy cows. It is not known whether the decreased incidence of uterine infections in sheep is indicative of physiological differences between the species or whether it is an artifact of reduced detection.

It would seem that confinement housing systems, such as the systems to maintain swine, would provide the most accurate estimates of the incidence and cost of uterine infections; however, these estimates have either not been made or are unavailable. It is possible to determine the number of reproductive culls within a given swine herd; however, it is impossible to itemize the reproductive disorders associated with these culls. Itemizing reproductive culls in the swine industry would increase the difficulty of removing the culls from the herd and decrease the possible cull income. The incidence of disease related to uterine infections has not been reported by the swine industry. Severe metritis is recognized as a sign of the lactation disorder PHS formerly known as MMA. The number of sows or gilts affected during a farrowing is highly dependent on environment and management system. From 10 to 100% of the gilts or sows are affected during a farrowing (Martineau et al., 1999). The accuracy of these estimates is

questionable, because neither the severity of the infections nor the time postpartum is documented. The incidence of uterine infections in dairy cattle is directly related to management system; this probably applies across species.

In the past, the public has not perceived uterine infections as a problem in the swine industry. However, this does not mean that there is not a high incidence of uterine infections, only that it is unreported. As larger facilities are built and locations for these facilities change, uterine infections will certainly become, if they are not already, a large problem. As environmental laws are passed in the United States, large companies are moving swine production to countries with less stringent environmental laws. Many swine production companies are building facilities in South America. In South America, increases in temperature and humidity will increase the incidence of *E. coli* and *A. pyogenes* based infections including uterine infections.

Even in the dairy industry, the predictions and measures of incidence and cost of uterine infections is largely inaccurate. The incidence of uterine infections varies greatly between studies. This variation makes it impossible to use these studies as predictors of incidence and cost. Variation among studies of incidence rates is not surprising. Many researchers (Curtis et al., 1985; Markusfeld, 1987) do not describe diagnostic methods, classification of the uterine infections, the postpartum period during which infections were detected, the parity of the cows, the general characteristics of the cow, or the herd management practices (Lewis, 1997). For example (Markusfeld, 1987), 36% of the cows examined between d 5 and 14 postpartum in 7 herds were diagnosed with metritis. The average across the herds ranged from 24.8 to 51.3%. In another study (Curtis et al., 1985), 7.8 % of cows in 31 herds that were examined once during the first 30 d postpartum were diagnosed with metritis. The range of incidence across herds was not

reported, and metritis classification included endometritis, metritis, and pyometra. The incidence of endometritis is greater during the first 14 d postpartum than during d 29 to 35 postpartum (Griffen et al., 1974). The incidence of severe endometritis, which often progresses to pyometra (Arthur et al., 1989), increases at the time that most cows enter the first postpartum ovulatory period (Fonseca et al., 1983). By d 43 to 49 postpartum, the incidence of severe endometritis decreases to approximately 8% (Griffen et al., 1974). Incidence of metritis continues to decrease during consecutive 21-d intervals from calving (Erb et al., 1984). In order to predict the incidence and cost of uterine infections within a herd several, criteria must be established and reported including 1) formal definitions of uterine infections, 2) examination period postpartum, and 3) description of cows and herd management practices.

Economically, uterine infections have the greatest impact during the postpartum period. Even though the endocrine and immune systems foster an environment that is susceptible to uterine infections, the reported incidence of uterine infections is low during the luteal phase of the estrous cycle. Decreases in reported incidence during the luteal phase as compared to postpartum is either an artifact of insufficient detection or decreased exposure to bacteria.

Immune System and the Immune Response to Bacterial Pathogens

Introduction. Complex cellular interactions, cytokine signals, and spatial structures regulate the vertebrate immune response to bacterial pathogens. In the interest of brevity, the review of the immune response to pathogens in this dissertation will focus on the immune response to bacterial pathogens similar to the organisms that we use to induce uterine infections in livestock.

Leukocytes mediate five basic immune responses that combat bacterial infection (Roitt et al., 1998): 1) Ab neutralization of toxins or enzymes; 2) the killing of bacteria by Ab,

complement, and various enzymes involved in cell lysis; 3) opsonization (i.e., coating of foreign particles with Ab or complement to facilitate phagocytosis) resulting in phagocytosis and destruction; 4) the intracellular destuction of bacteria by activated macrophages; and 5) direct killing of bacteria by cytotoxic T lymphocytes and natural killer cells. The importance of each of these processes depends on the type, structure, and disease-causing mechanisms of the particular bacterial species.

Innate Versus Acquired Immune Responses. The immune system is divided into two branches (Tizard, 1996): 1) innate and 2) acquired. The innate immune system provides immediate defense against microorganisms by directing the engulfment and breakdown of microbial cells and the secretion of cytokines and chemokines that enhance antibacterial and antiviral cellular responses. The detection mechanisms associated with the innate immune system are preset (Fearon, 1997). Acquired immunity is mediated by a system of B and T lymphocytes, which express a distinct Ag receptor (Roitt et al., 1998). Antigen increases the production of specific lymphocytes and the induction of immune memory.

The innate and acquired branches of the immune system contribute to the two types of immune responses: 1) humoral and 2) cell-mediated. In general, the type and action of lymphocytes defines a response as humoral or cell-mediated. Bone-marrow dependent cells (B lymphocytes) and their secretions (Ig) mediate humoral responses. Thymus-dependent cells (T lymphocytes) and their secretions mediate cell-mediated responses. However, there are several mechanisms that do not conform to rule. For example, there are several cell-mediated responses that are not directly linked to the actions of T lymphocytes (i.e., phagocytosis and natural killer cell activity). It is difficult to separate T lymphocyte-dependent cell-mediated responses from T lymphocyte-independent cell-mediated responses from humoral responses. The cells involved in

many cell-mediated responses signal the induction of Ab production, and Ab may act as an essential link governing some cell-mediated responses.

Leukocytes. A variety of cells and their products mediate the immune response. Leukocytes play a central role in governing the actions of the immune system; however, other cell types participate. Cells of the immune system arise from pluripotent stem cells through two main lines of differentiation: 1) lymphoid (produces lymphocytes) and 2) myeloid (i.e., produces phagocytes).

The two main subsets of lymphocytes are T and B. Initial development of B and T lymphocytes occurs in the bone marrow. The B lymphocytes remain in the bone marrow to mature. Maturation of T lymphocytes occurs in the thymus. Maturation suggests that the B or T lymphocytes "acquire" the ability to recognize specific Ag through the development of specific cell surface Ag receptors. A third population of lymphocytes also develops in the bone barrow. Large granular lymphocytes or natural killer cells do not express Ag receptors and are functionally distinguished from B and T lymphocytes by their ability to lyse certain tumor cell lines (Timonen et al., 1982; Lotzova and Ades., 1989).

The T lymphocyte Ag receptor provides another method of classifying T lymphocytes. There are two defined types of T lymphocyte receptors: 1) $\alpha\beta$, and 2) $\delta\gamma$. The $\alpha\beta$ T lymphocytes are divided into CD4 and CD8 subsets depending on expression of cell markers. The CD4 and CD8 T lymphocytes are functionally defined as T helper and T cytotoxic cells, respectively. The T helper cells or CD4 are further subdivided into the classes of T_H1 and T_H2 on the basis of their cytokine profile. The $\delta\gamma$ T lymphocytes are also referred to as large granular lymphocytes or natural killer cells. Natural killer cells mature in the bone marrow (Haller and Wigzell, 1979). In the presence of a bacterial pathogen, lymphocytes have three distinct reactions: 1) B

lymphocytes secrete Ab that can bind to the pathogen and signal degradation of the pathogen by macrophages and other cells, 2) cytotoxic T lymphocytes develop the ability to directly recognize and kill infected cells, and 3) helper T cells recognize the pathogen and secrete cytokines that stimulate growth and responsiveness of B lymphocytes, T lymphocytes, and macrophages.

The phagocytes are divided into two families: 1) monocytes/macrophages and 2) polymorphonuclear granulocytes (neutrophils, basophils, and eosinophils). Selective phagocytes have two main functions: 1) to remove particulate Ag and 2) to present Ag. Macrophage precursors are released into circulation as monocytes. These immature cells acquire macrophage characteristics as they migrate from circulation into the tissues. Neutrophils are released from the bone marrow as fully mature cells. Populations of monocytes/macrophages and neutrophils are heterogeneous (Brown et al., 1991; Pryjma et al., 1992). Defining surface phenotype allowed researchers to define several neutrophil subpopulations (Brown et al., 1991). Phenotypic heterogeneity between monocyte populations is associated with differential functional responses of the populations to stimuli (Pryjma et a., 1992), and it may reflect the continuum of maturation within the circulating monocyte population (van Furth and Sluter, 1986; Golemboski et al., 1990). Eosinophils are involved primarily in killing parasites, and therefore, are not given much attention in this review. In tissue, basophils promote inflammation; however, their relative numbers are so low in the circulation of sheep and pigs that it is not believed that they are of consequence to the uterine immune response to infection. In addition to the lymphocytes and phagocytes, there are a number of accessory cells that mediate immune cell function (i.e., platelets, mast cells, and endothelial cells).

Mechanism of Immune Response to Bacterial Pathogens. When bacteria traverse the physical barriers and invade the body, they must be recognized as foreign. This information is conveyed to the humoral and cell-mediated response systems. Nonspecific cells (i.e., macrophages) and specific cells (i.e., B lymphocytes) engulf the bacterial Ag, process it, and present it to T lymphocytes. Antibody-producing cells and cells conferring cell-mediated immunity will respond to the processed Ag. During the primary immune response, B lymphocytes secrete IgM. After T lymphocytes recognize Ag, they divide producing T lymphocytes that "attack" the Ag. Specialized subsets of T lymphocytes "attack" Ag and signal an increase in B lymphocyte proliferation, differentiation, and Ig formation. Specific Ig attaches to and immobilizes Ag. The immune system "remembers" these events. Following a second exposure to the same Ag, the immune response will be faster and more efficient. The secondary immune response will involve the secretion of IgG.

The rest of this review explains the general model for defense against bacterial infection in greater detail. It is necessary to first present the entire model to ensure that the following mechanisms are viewed as parts of the whole, rather than as individual defense units.

Physical Barriers Against Bacterial Invasion. The first line of defense against pathogenic bacteria consists of simple physical barriers to the entry and establishment of infection. The skin and exposed epithelial surfaces have innate protective systems that limit the entry of bacterial pathogens. Intact skin is impenetrable to most bacteria. In addition, fatty acids produced by the skin are toxic to many organisms (Lacy and Lord, 1981). Exposed epithelial surfaces are cleansed (i.e., ciliary action in the trachea or urine expulsion in the urinary tract). Changes in environmental pH destroy many pathogenic bacteria (i.e., the acidic environment in the vagina)

(Boskey et al., 1999). Indigenous nonpathogenic bacteria may secrete substances that limit the growth of pathogenic bacteria (Tizzard et al., 1996).

The cervix is the major barrier to the entry of pathogens into the uterus. At estrus, the cervix is "relaxed" in sheep and pigs. In sheep, spermatozoa are ejaculated into the vagina and traverse the cervix and enter the uterus (Senger, 1998). In pigs, the glans penis is inserted directly into the cervix (Senger, 1998). Spermatozoa move through the anterior portion of the cervix into the uterus (Senger, 1998). Semen (i.e., from natural or artificial insemination) and the male reproductive tract (i.e., during natural insemination) are sources of bacterial contamination (Reed, 1969; Waltz et al., 1968). However, the uterus in sheep is almost never affected at estrus (Ramadan et al., 1997). We are unsure of the extent to which the uterus in pigs may be affected. Residual bacteria from exposure at estrus may cause uterine infections in pigs during the luteal phase (Scofield et al., 1974). Expressed in a teleological form, the uterine immune system is up-regulated at estrus to combat an increase in bacterial exposure at estrus. The physical barriers in sheep and pigs are more difficult to traverse during the luteal phase (i.e., the diameter of the cervix and uterine motility decrease) when the uterine immune system is susceptible to uterine infection (Scofield et al., 1974; Khalifa et al., 1992; Ramadan et al., 1997).

If pathogenic bacteria traverse the initial physical barriers, the remainder of the immune defense system is related to the structure of the bacterial pathogen and, hence, the immunological mechanisms to which they are susceptible. Immunity against bacterial pathogens is directly related to bacterial surface structure. In the case of *A. pyogenes* and *E. coli* bacterial infections, the induction of immunity is against Gram-negative and Gram-positive bacteria. The outer lipid bilayer of Gram-negative organisms is susceptible to mechanisms that lyse membranes including

the complement system and certain cytotoxic cells. In contrast, destruction of Gram-positive bacteria usually involves uptake by phagocytes.

Antibody Independent Cell-mediated Response to Bacterial Pathogens. If pathogenic bacteria enter tissues, initially an innate, cell-mediated immune response is triggered. Numerous bacterial components are recognized without relying on Ag specific receptors of B and T lymphocytes. For instance, there is a complex pathway that recognizes LPS and destroys the bacterial products. Lipopolysaccharide released from Gram-negative bacteria such as *E. coli* binds to soluble CD14 and to lipoprotein particles in the plasma (Wurfel et al., 1994). A lipid transfer protein called LPS-binding protein catalyzes these reactions. Binding to the lipoprotein particle neutralizes LPS, and binding soluble CD14 leads to cell activation. The CD14 also exists as a GP1-linked membrane protein on neutrophils and macrophages. Lipopolysaccharide is transferred from the soluble CD14-LPS complex to the membrane bound form. The membrane bound CD14-LPS complex in association with other membrane bound factors transduces signals that cause increased expression of integrins and increased secretion of TNF-α and IL-1 (Wurfel et al., 1994). These cytokines activate endothelial cells and initiate the acute phase response in the liver. Some other conserved microbial products, including the teiochic acid structures attached to *A. pyogenes*, may be recognized and handled in a similar mechanism.

Much of the initial defensive reaction to bacterial pathogens depends on recognition of common bacterial components by receptors that are not Ag specific. Molecules present in the serum and receptors on immune cells recognize several bacterial components. Recognition may result in the activation of one or several pathways and cell types. Numerous bacterial components cause chemotaxis of phagocytes toward the site of infection either by initiating the cascade of events associated with alternate complement pathway or by serving as chemotactic

agents themselves. Another mechanism that is independent of T lymphocytes or Ab responses during the initial stages of bacterial infection is the release of cytokines and chemokines from macrophages and an assortment of other cells. Among the cytokines released by macrophages in response to bacterial pathogens are TNF-α and IL-12. Release of these cytokines from macrophages signals the recruitment of more leukocytes, activates phagocytic cells in the infected tissue, and provides signals that determine the type of T lymphocyte response that will develop.

Ultimately, phagocytosis kills most bacteria. In general, macrophage- and neutrophil-induced phagocytosis is considered an Ab-independent cell-mediated event; however, after the initial immune response, phagocytosis is often enhanced by Ab-dependent cell-mediated events. Neutrophil- and macrophage- induced phagocytosis is divided into four steps: 1) chemotaxis, 2) adherence, 3) ingestion, and 4) digestion. Bacterial components, complement products, and cytokines and chemokines attract phagocytes.

The function of neutrophils is to capture and destroy foreign material. Neutrophils are found in circulation and in tissues (van Furth and Sluiter, 1986). Adhesiveness of the endothelial cells forming the blood vessel walls signals neutrophils to leave circulation and move toward the site of infection. During the initial phase of the inflammatory response, specific cell surface adhesion molecules control the relationship between neutrophils and vascular endothelial cells (Ley et al., 1991). Cell surface molecules (i.e., selectins) are expressed early in inflammation and permit neutrophils to roll along the vascular wall (Ley et al., 1991). With additional activation signals, L-selectin molecules are lost from the cell surface and Mac-1 adhesion molecules are expressed (Kishimoto et al., 1989). The Mac-1 adhesion molecules attach neutrophils firmly to the vascular wall via the intracellular adhesion molecule ICAM that is present on endothelial

cells (Kishimoto et al., 1989). Attachment is required for neutrophils to migrate through the endothelial cell layer to extravascular sites of pathogenic challenge (Kishimoto et al., 1989). Directed migration is called neutrophil chemotaxis. A variety of chemical signals functionally activates and acts as chemotactic agents for neutrophils. For example, receptor mediated activation can occur with the chemotactic peptide FMLP (Rao et al., 1989); LTB_4 (Kriesle and Parker, 1983; Bomalaski et al., 1990); C5a (Van Epps et a., 1990), IFN-γ (Morrison et al., 1987; Hansen and Finebloom, 1990), and platelet activating factor (Gomez-Cambronero et al., 1991). If a neutrophil encounters a foreign particle, it must bind to it in order to engulf and destroy it. Adherence/binding does not occur spontaneously. In general, the cell and pathogen are negatively charged, and therefore, repel each other. Coating the negatively charged particle with positively charged proteins neutralizes the particle. Positively charged coatings include protein products of the complement pathway and Ab. Molecules that coat pathogens are called opsonins, and the process is called opsonization. Pathogens coated with these proteins will bind to neutrophils. Once bound firmly to the neutrophil surface, a pathogen is drawn into the cell. As the cytoplasm engulfs the particle, it becomes enclosed in a vacuole. Following engulfment, neutrophils attack and destroy targeted cells through the production of oxygen radicals and nitric oxide and through the release of various enzymes stored in intracellular granules (Korchak et al., 1984; Ignarro, 1990; Spitzer et al., 1994).

A variety of neuroendocrine, hepatic, and immune products can modulate neutrophil activity. For example, estrogens and dexamethasone suppress neutrophil activity, suggesting a mechanism for the increased susceptibility to infection during estrogen dominated periods (Lomas et al., 1991; Styrt and Sugarman, 1991).

Phagocytosis by macrophages is similar to phagocytosis by neutophils. However macrophages are not only attracted to bacterial products and products of complement activation, but also to molecules released from damaged cells and tissues. For example, dying neutrophils release elastase and collagenase, which serve as monocyte chemotactic factors. Macrophages destroy invading organisms by oxidative and nonoxidative mechanisms.

A variety of endogenous substances modulate macrophage activity. Platelet activating factor stimulates TNF-α production in alveolar macrophages (Dubois et al., 1989). Prostaglandin E_2 regulates macrophage TNF-α expression and provides a negative signal for macrophage activation (Kunkel et al., 1988), and glucocorticoids reduce macrophage and cytokine production (Zanker et al., 1990). Leukotriene B_4 induces selective oncogene transcription (Stankova and Rola-Pleszczynski et al., 1992) and modulates macrophage responsiveness to a variety of cytokines (Stankova et al., 1993).

Natural killer cells are defined as a third population of lymphocytes that has the capacity to lyse tumors without prior sensitization (Timonen et al., 1982) and without recognizing MHC Ag on the target cell (Lotzova and Ades, 1989). Natural killer cells are responsible for immune surveillance against virus-infected cells (Santoli et al., 1978; Bishop et al., 1984) and tumor cells (Kawase et al., 1982). To a lesser extent, natural killer cells are involved in the innate immune response against bacterial pathogens. Natural killer cells can directly cause the death of several types of bacteria. Gram-negative bacterial species are particularly susceptible to the effects of natural killer cells.

Cell-mediated cytotoxicity is divided into three stages: 1) target recognition and binding, 2) transmission of cytotoxic products, and 3) death and lysis of the target cell. Natural killer cells bind to targets initiating a cascade of second messenger production. Second messengers signal

degranulation and release of proteolytic enzymes responsible for the cytotoxic effects of natural killer cells (Atkinson et al., 1990; Ting et al., 1992). The granular contents of natural killer cells includes proteoglycans and arylsulfatases (i.e., serine proteases and perforin) (Liu et al., 1986; Amoscato et al., 1991; Yagita et al., 1992). After the natural killer cells attack, the target cells undergo apoptosis. Other cells of the immune system including macrophages and T lymphocytes produce cytokines that modulate natural killer cell activity. In response to IL-2, natural killer cell activity increases, and the natural killer cells acquire the ability to kill cells that are resistant to "spontaneous" natural killer cell cytotoxicity. The cytokine IFN-α can further enhance the effects of IL-2 (Bergmann et al., 1990). In addition to the IL-2, substances such as superoxide anion, platelet activating factor, and PG modulate natural killer cell activity (Bloom et al., 1990; Shau et al., 1993).

The helper and cytotoxic responses of T lymphocytes are similar. Both responses rely on the secretions of specific cytokines and proteins. The T helper lymhocytes secrete cytokines that stimulate T and B lymphocyte growth in response to pathogens. Antigen-presenting cells present processed Ag to T helper lymphocytes. These cells recognize particular epitopes and, thus, select those as targets. They then select and activate the appropriate effector mechanisms. Not all T helper lymphocytes secrete the same cytokines. The different subsets of T helper lymphocytes are based on their secretory profile. Different subsets of T helper lymphocytes orchestrate the response to different types of infection and modulate the various types of cellular cooperation. Cytotoxic lymphocytes respond to pathogens in much the same way as natural killer cells; however, initial binding is through the MHC complex. Cytotoxic lymphocytes secrete proteases (i.e., perforin) or activate the Fas ligand system and apoptosis to kill pathogens. Cytotoxic T lymphocytes recognize processed Ag presented on the target cell by MHC class I molecules.

Antibody Dependent Immune Responses to Bacteria. The function of specific immunogolobulins and, therefore, of the humoral immune system is to attach and immobolize Ag in situ and generate memory. Following immobilization, several accessory systems are signaling to aid in the destruction and removal of the Ag (i.e., complement system). In order to accomplish these functions, activation of B lymphocytes and generation of large amounts of Ab must occur. Circulating Ab recognizes Ag in the serum and tissue fluids. Depending on the species, there are five classes of Ab (i.e., IgG, IgA, IgM, IgD, and IgE). Imunoglobulins consist of two light chains and two heavy chains. The heavy chains differ between classes. The chains are folded into discrete regions called domains. There are two domains in the light chain and four or five in the heavy chain depending on class. All Ab are bifunctional. They exhibit several effector functions in addition to Ag binding (i.e., complement activation and cell binding). The Ag and number of exposures to the Ag signal the production of the appropriate Ig class. Class switching is important in the maturation of the immune response: for example, the primary versus secondary immune response to Ag. Following initial exposure to an Ag, a primary immune response involving the secretion of IgM is mounted. If the immune system is exposed to the same Ag a second time, a greater response is mounted involving the secretion of IgG.

Immune activation to stimulate Ab production involves interaction between T lymphocytes and Ag-presenting cells, and later between primed T lymphocytes and B lymphocytes (Roitt et al., 1998). The interaction between B and T lymphocytes is bidirectional. The B lymphocytes present Ag to T lymphocytes and receive signals from the T lymphocytes that initiate division and differentiation of B lymphocytes. The specific interaction is between the MHC class II-Ag complex and the T lymphocyte receptor. Interactions between LFA-3 and CD2 and between ICAM-1 or ICAM-3 and LFA-1 augment the interaction between B and T

lymphocytes (Watts and DeBenedette, 1999). During B and T lymphocyte interactions, T lymphocytes secrete a number of cytokines that have direct effects on the B lymphocytes. These include IL-2 (induces proliferation of B and T lymphocytes), IL-4 (induces B lymphocyte activation and proliferation), IL-5 (activates B lymphocytes depending on species), and IL-6 (signals B cell differentiation). Antigen on Ag-presenting cells activates B lymphocytes in the presence of IL-4 and IL-1. The presence of these cytokines causes expression of IL-2 and other cytokines that impact differentiation, proliferation, and Ab secretion.

The response to most Ag depends on the interaction between T and B lymphocytes. However, there are Ag capable of activating B lymphocytes without interaction with T lymphocytes. The T lymphocyte-independent Ag are large polymeric molecules with repeating antigenic determinant. In high concentrations, T lymphoyte independent Ag may cause polyclonal B lymphocyte activation (Roitt et al., 1998). In low concentrations, they activate B lymphocytes specific for themselves (Roitt et al., 1998). Many T lymphocyte-independent Ag are particularly resistant to degradation and most are microbial in origin (i.e., the liopopolysaccharide component of *E. coli*). Polymeric structures of T lymphocyte-independent Ag cross link to B lymphocyte receptors inducing activation of the B lymphocytes. The primary Ab response is weaker to T lymphocyte-independent Ag than to T lymphocyte-dependent Ag . The secondary response to T lymphocyte-independent Ag resembles the primary response, whereas the secondary response to T lymphocyte-dependent Ag is far stronger and involves the secretion of IgG. The T lymphocyte-independent Ag does not induce class switching. Memory induction by T lymphocyte-independent Ag is also decreased.

Typically, uterine infections in livestock are derived from pathogenic bacteria. Bacteria evoke the specific immune responses discussed above. However, depending on the type of

bacteria, the severity of the infection, and the hormonal environment the “dominant” immune responses change.

Uterine Infections

The exact cause of postpartum uterine infections is unknown. The pathogenic organisms involved in uterine infections are usually indigenous to the livestock environments. The term uterine infection indicates that the uterus is contaminated with pathogenic organisms. Nonspecific uterine infections are defined as uterine infections in which neither the initial colonizing bacterium nor the bacteria causing the clinical signs of the disease are known. This is in contrast to infections such as chlamydiosis, which is considered a specific infection. Uterine infections in cattle are associated with several factors including dystocia, retained placenta, twins, stillbirth, and various metabolic disorders. Metritis associated with PHS in swine often affects the highest performing pigs in terms of litter size. This may relate to difficult farrowing. Ninety percent of the sows that receive assistance at farrowing develop metritis. Aberrant immune function before and after calving seems to predispose cows to severe uterine infections. This has not been studied in pigs. Changes in the innate and acquired immune response prepartum and postpartum have been studied in cows with and without uterine infections in an attempt to determine the immune factors that predispose cows to postpartum uterine infections.

For the purpose of this dissertation, uterine infections are classified when possible according to period postpartum and clinical signs. Olson et al. (1986) offered the most detailed description of postpartum period and clinical signs. However, his classification system is based on the postpartum period in cows. Physiologically the events, if not the days, are similar in sheep and pigs. The postpartum period is the interval from parturition to complete uterine involution (Olson et al., 1986) The postpartum period can be divided into three subperiods: puerperal,

intermediate, and postovulatory. The puerperal period is defined as the interval from calving until the pituitary becomes responsive to GnRH (i.e, 7 to 14 d postpartum). The intermediate period is the interval from the time that the pituitary becomes responsive to GnRH to the first postpartum ovulation. The postovulatory period is the interval from the first ovulation to complete uterine involution. Complete uterine involution is defined as the time when the uterine horns and cervix are approximately 40 mm in diameter and the caruncles are epithelialized (Lewis et al., 1984).

Generally, uterine infections are classified according to clinical signs and degree of severity (Youngquist and Little, 1988). Endometritis indicates that the endometrium is inflamed. Metritis indicates that all layers of the uterine wall are inflamed, and pyometra indicates that purulent exudate has accumulated at the site of infection. Following the puerperal period, metritis is the least severe and pyometra is the most severe category of uterine infection. These three classifications are related and may, in fact, develop sequentially (Bartlett et al., 1986; Olson et al., 1986).

During the puerperal period, perhaps 90% of the cows develop a mild, nonpathological endometritis (Griffin et al., 1974; Olson et al., 1986). This percentage is even higher in pigs. Nonpathological endometritis that develops during this time period is a consequence of uterine involution. In particular, uterine involution involves a series of inflammatory responses that void uterine contents and promote restructuring of the uterus. During this period, there is a continuous change in the uterine microflora. During the puerperal period, bacteria invade the uterus, and the composition and concentration of bacteria fluctuates over the next 7 wk. The uterus undergoes a cycle of contamination and clearance of the organisms. The majority of cows resolve the nonpathological endometritis that results. It is not known whether these organisms are necessary

to stimulate uterine involution. It is possible the increase of bacteria in the uterus following parturition signals the initiation of the inflammatory responses that aid in uterine involution. All palpable uterine fluids are voided during the first 2 wk postpartum (Olson et al., 1986). Therefore, the presence of vaginal discharge during this period cannot be viewed as a sign of uterine infection. However, if the discharge becomes fetid, it is a fairly accurate indicator of uterine infection. Puerperal metritis can be a severe problem, causing uterine infections that are life threatening. The likelihood of developing severe infections is higher in pigs than in cattle and sheep.

During the intermediate period, cows reduce or eliminate pathogenic organisms that are typically found in the postpartum uterus (Olson et al., 1986). Uterine infections that persist during this period are classified as endometritis or metritis. Both conditions can become chronic (Lewis, 1997). When cows with chronic endometritis or metritis ovulate, pyometra often develops within a few days (Arthur et al., 1989). Pyometra is detected almost exclusively in livestock with active CL.

When the uterus is unable to clear the organisms associated with uterine involution and the organisms proliferate, an infection is established. If an infection is established, inflammatory cells infiltrate the endometrium, superficial epithelium becomes necrotic, the endometrium becomes hyperemic and congested, and the number of B and T lymphocytes and neutrophils increases in the uterine lumen (Studer and Morow, 1978; Bretzlaff, 1987; and Del Vecchio et al., 1994). The endometrium is often damaged (Studer and Morow, 1978; Bretzlaff, 1987; Del Vecchio et al., 1994). Inflammatory exudates accumulate in the uterus, and the infection spreads to the oviducts in 70% of the cows. The uterus accumulates a considerable amount of purulent exudate, which is typically palpable, because the cervix is constricted enough to prevent drainage

(Arthur et al., 1989). In pigs, the infection spreads to the oviducts, cervix, and vagina (K. Pelzer, personal communication) often presenting clinically as severe vaginitis. This is probably an artifact of differences in cervical structure between pig and cows. However, drainage alone in pigs does not resolve the infection, suggesting that if drainage occurred in cows the infection would not be cleared. This contradicts many of the current treatments for uterine infections that focus on draining of the uterine contents.

The central question associated with postpartum uterine infections revolves around the clearance of the nonpathological organisms associated with "normal" involution. Why do some animals manage bacterial contamination without uterine infections, while others in the same herd and under the same management system develop severe uterine infections?

A number of researchers have reported that neutrophil function is impaired in cows that develop uterine infections (Hussain, 1989; Lewis et al., 1984). In "normal" cows, the number of peripheral neutrophils increases during the last 10 to 15 d of pregnancy and then decreases during the first 7 d after calving (Olson et al., 1986). This may be indicative of neutrophils moving from circulation to the uterus to actively participate in the clearance of bacterial organisms during uterine involution. In correlation with the movement of neutrophils, the phagocytic activity of neutrophils increases prepartum, decreases at calving, and increases during the first 14 d postpartum (Saad et al., 1989). For cows that developed metritis, the prepartum increase and postpartum decrease was less than in cows that did not develop metritis (Olson et al., 1986). Neutrophil function was also reduced throughout this period in cows that developed metritis (Olson et al., 1986; Hussain, 1989). In addition, in cows that developed metritis, the movement of neutrophils, ability to digest pathogens, Ab-dependent, and cell-mediated cytoxicity were reduced (Olson et al., 1986). Many of the factors that predispose cattle to uterine

infections including dystocia, manual removal of retained placenta, and intrauterine infusions reduced phagocytic activity of uterine and blood neutrophils (Paisley et al., 1986; Hussain, 1989). Impairment of neutrophils prepartum and postpartum may predispose certain cows to uterine infections.

Treatment of Uterine Infections

Treatment of uterine infections in livestock varies greatly with species and production system. In dairy cattle worldwide, the most popular treatment of uterine infections is a regimen of systemic or local antibiotics. However in the United States, the most popular treatment is the use of $PGF_{2\alpha}$-analogues. Most management systems for beef cattle and sheep prefer to allow uterine infections to resolve themselves (Wenzel et al., 1993). In which case, the animals clear the infections after a single estrous cycle, or the animal is culled for perpetual reproductive problems. In the United States and abroad, pig producers use massive doses of systemic antibiotics. The incidence of uterine infections is greatest during the postpartum period; therefore, the following description regimens refer to treatment of postpartum uterine infections.

To justify treating postpartum uterine infection in livestock, the treatments must be efficacious and economical. A treatment is deemed efficacious if it resolves the uterine infection and prevents long-term reproductive damage. However, the treatments are only considered economical if the cost of the treatment is less than not treating the animal. Factored into these analyses are culling rates and product yields. Typically, it is cheaper to treat infections than to cull the animals. After establishing an economic necessity for treating, it becomes necessary to analyze the expense versus the return of the various treatments. The easiest criteria to measure are the actual cost of the treatment and the administration labor, the number of days open, and the number of services to conception in treated versus untreated animals.

Cows with postpartum uterine infections treated with various antibiotic protocols have either no change or an increase in days open and services per conception when compared with untreated cows with postpartum uterine infections (Fuquay et al., 1975; Steffan et al., 1984; Thurmond et al., 1993). Treatment of uterine infections with $PGF_{2\alpha}$ analogues reduces the calving to conception interval when compared with appropriate controls (Etherington et al., 1995; Pankowski et al., 1995). Similar studies have not been performed with sheep.

In pigs, there are no formal guidelines for the treatment of postpartum uterine infections; however, there are formal outlines for the treatment of PHS. The syndrome PHS is treated with a combination of successive administration of oxytocin and antibiotics. Oxytocin intiates milk let down. Oxytocin treatments have also been linked to clearance of metritis associated with PHS; however, experiments demonstrating this link were not designed to test it (Sims and Eiler, 1979;Eiler and Sims, 1979). Oxytocin is a $PGF_{2\alpha}$ secretogue and may work through the same mechanism associated with clearance of uterine infections by $PGF_{2\alpha}$ in ruminants. The antibiotic use associated with treatment of PHS and uterine infection in swine has not been properly evaluated. Often antibiotics are given as a preventive to animals that are at "risk" for uterine infections; however, the efficacy of these treatments has not been tested. Administration of $PGF_{2\alpha}$ to induce parturition has been linked to decreased incidence of PHS and uterine infections; however, it is not advocated as a treatment (Einarsson et al., 1975). Intensive management of pigs in intensive production settings has fostered the use of antibiotics and other treatments as a preventative for uterine disorders. Companies including Pharmacia & Upjohn are currently investigating the use of $PGF_{2\alpha}$ as a preventative treatment in pigs (J. Lauderdale, personal communication 1997).

Arcanobacterium pyogenes and *Escherichia coli*: Occurrence and Virulence

Arcanobacterium pyogenes is a species of Gram-positive bacteria common to many livestock infections. *Arcanobacterium pyogenes* is responsible for severe clinical cases of mastitis in cows, goats, sheep, and pigs. Thick, purulent secretions with a noxious odor characterize infections that are caused by strains of *A. pyogenes*. Anaerobic bacteria that are also present at the site of infection often cause the odor associated with these infections. *Arcanobacterium pyogenes* is often the colonizing species of bacteria associated with the initial infection; however, the presence of *A. pyogenes* increases the likelihood of other opportunistic species colonizing. Antibiotic therapy is often ineffective against *A. pyogenes* infections for a variety of reasons including 1) antibiotic resistant strains of *A. pyogenes*, 2) *A. pyogenes* is a Gram-positive bacteria, and 3) *A. pyogenes* infections often involve multiple other species and strains of bacteria. Major sources of *A. pyogenes* include 1) wound infections, 2) teat injuries, 3) udder infections, 4) abscesses, and 5) genital tracts (male and female). The bacteria are spread by contact with contaminated environments and are more common in humid weather.

Gram positive bacteria such as *A. pyogenes* have a thick cell wall (30 to 90 nm) that serves as a barrier against antibiotic induced cytotoxicity and as the initial site of recognition of antibodies against these bacteria. Electron microscopy reveals a uniform appearance. Approximately, 40 to 80% of the wall is composed of complex polymer peptidoglycans. Peptidoglycans consist of linear heteropolysaccharide chains that are cross-linked with short peptides forming three-dimensional net like structures that envelop the protoplast. The sacculus or net like structure consists of multilayered peptidoglycans. During growth, new peptidoglycans are added to the cytoplasmic face of the wall. As growth continues, layers move outward toward the cell surface, the oldest layers eventually being shed as fragments. Teichoic acids are

covalently bound to peptodoglycans. In some bacteria, the cell wall contains lipids, and in others it contains carbohydrates.

Escherichia coli is a common gastrointestinal tract Gram-negative bacterial species indigenous to almost every environment and livestock species. In nonruminant species, *E. coli* represents approximately .1% of the total bacteria in a mature animal's intestines (Yamamoto et al., 1996). Among the symbiotic bacterial strains of *E. coli*, there are several pathogenic strains that cause disease. Pathogenicity of *E. coli* may also change with the environment. *Escherichia coli* that are an essential component of the nonpathogenic "gut" microflora are pathogenic in the urogenital tract and cause severe urinary and reproductive tract infections (Tay et al., 1996). Pathogenic strains of *E. coli* are the most prevalent cause of diarrhea in neonatal and adult livestock. The disease syndromes associated with *E. coli* are referred to as collibacillosis. In livestock, the most common cause of diarrhea is entertoxigenic *E. coli*. Traditionally, entertoxigenic bacteria possess two virulence determinants: 1) adhesins and 2) the ability to produce heat stable and heat labile enterotoxins (Thorns et al., 1989). However, selection pressure from various antibiotics has led to the production of new and more virulent toxins from *E. coli*, including verotoxins and shiga toxins (Wray et al., 1993). The incidence of disease caused by pathogenic *E. coli* is greatly influenced by management of the herd and facilities. A large number of *E. coli* are usually present in the immediate environment whenever it is dirty and wet, the ventilation is poor, and the humidity is high. However, the most common source of *E. coli* based infections is other animals.

Cellular components of Gram-negative bacteria such as *E. coli* increase the antigenicity and susceptibility of these bacteria to antibiotics. However, the rapid cell cycle and prevalence of *E. coli* in the environment increase the rate of mutation, therefore causing rapid development of

antibiotic resistance. Gram negative bacteria have a thinner cell wall (20 to 30 nm). Electron microscopy reveals a layered appearance. The cytoplasmic layer contains peptidoglycan. The outer membrane is a protein containing lipid bilayer resembling the cytoplasmic membrane. The inward facing lipids are phospholipids, and the outward facing lipids are macromolecules called lipopolysaccharides. The core oligosacharide contains glucose and galactose residues and substituted residues of other sugars. The O-specific chains forming the outermost part of the cell wall consist of linear or branched chains of oligosaccharide subunits; the chemical composition of the O-specific chain can vary from strain to strain. About half of the mass of the outer membrane consists of proteins. In *E. coli* and related bacteria, this includes Braun proteins that link the proteins to the peptidoglycan layer. There are also enzymes, proteins involved in specific uptake mechanisms, and porins. Porins are inked via ionic bridges to the peptidoglycans. In *E. coli* and related bacteria, the outermost membrane contains several types of porins. The relative proportion of these porins can vary according to the cell's environment. Components of the outer membrane are held together by ionic and other interactions. Adjacent core oligosaccharides seem to be linked by divalent cations, most often Mg^{2+} and Ca^{2+}. Lipid A is hydrophobically bound to the fatty acid residues of the phospholipids. Some proteins seem to be linked to the core oligosaccharies. The outer membrane is generally permeable to small ions and to small hydrophillic molecules; however, the membrane is much less permeable to hydrophobic or amphipathic molecules.

In the experiments described in this dissertation, we used a combination of *A. pyogenes* and *E. coli* to produce nonspecific uterine infections. The original isolates of these two species and strains were obtained from a dairy cow with a uterine infection from the Virginia Tech Dairy herd (Del Vecchio et al., 1992). Although virulent, we ensured that these isolates were not

antibiotic resistant, decreasing the opportunity to inadvertently contaminate our herds. Before initiating these studies, we knew that these isolates caused uterine infections in sheep; however, we were unsure whether these isolates were pathogenic in pigs. A dose response curve was used to determine the virulence of these strains in swine. In sheep and pigs, intrauterine infusion of these bacteria during the luteal phase resulted in uterine infections characterized by pyometra.

Antibiotics and Antibiotic Resistance

Antibiotics. Originally, antibiotic was defined as any microbial product that inhibited or killed certain microorganisms. The term is now used in a broader sense to incorporate any semisynthetic or synthetic substance that inhibits or kills microorganisms. No antibiotic is effective against all bacteria. Some are active against a narrow range of species, whereas others are active against a broad spectrum of organisms. Antibiotics can be bactericidal or bacteriostatic. Characteristically, antibiotics act at a precise site in the cell. Depending on the antibiotic, the site of action may be the cell wall, the cytoplasmic membrane, protein biosynthesis, or an enzyme involved in nucleic acid synthesis. Structure and mode of action are the main categories of classification of antibiotics used for medical applications. Of the many known antibiotics, relatively few are suitable for treating disease.

The two most commonly prescribed antibiotics to treat uterine infections are derivatives of penicillin and tetracycline (K. Pelzer, personal communication). Penicillin belongs to the β-lactam family of antibiotics that also includes the cephalosporins, clavams, and monobactams. In each case, the molecule includes a four-membered nitrogen containing ring, the β-lactam ring. These antibiotics either disrupt synthesis of the cell envelope in growing cells or induce apoptosis. They inactivate penicillin-binding proteins and inhibit the synthesis of peptidoglycans. These antibiotics can only kill growing cells. For many of these antibiotics, the β-lactam ring is

susceptible to cleavage by enzymes produced by the bacteria they are intended to kill. Cleavage of the ring destroys the antibiotic. Organisms that produce these enzymes are resistant to β-lactam antibiotics. The original penicillins had low activity against Gram-negative bacteria, because they could not penetrate the outer membrane. They also had no effect against Gram-positive bacteria that form β-lactamases. Tetracyclins are broad spectrum antibiotics that inhibit protein synthesis by binding to ribosomes and inhibiting the binding of aminoacyl-tRNA to the A site.

Other families of medicinal antibiotics include aminoglycoside antibiotics, polymyxins, nalidixic acid, novobiocin, and sulphonamides. Aminoglycoside antibiotics are broadspectrum bactericidal antibiotics. They act on Gram-positive and Gram-negative bacteria. Aminoglycoside antibiotics bind to one or both subunits of the ribosome and inhibit translocation in protein synthesis. Polymyxins are peptides that are active against many Gram-negative bacteria; most Gram-positive bacteria are resistant. In Gram-negative bacteria, polymyxins increase the permeability of the cytoplasmic membrane and damage the outer membrane. Nalidixic acid is a synthetic antibiotic that acts against a range of Gram-negative bacteria. This antibiotic prevents DNA replication by inhibiting the actions of gyrase, an enzyme involved in supercoiling. Novobiocin is a antibiotic derived from *Strepotomyces niveus* that has the same activities as nalidixic acid. Sulphonamides are synthetic bacteriostatic compounds that interfere with the synthesis of folic acid, a coenzyme necessary for a variety of metabolic actions.

Bacterial Resistance. Resistance to antibiotics is conferred to bacteria in one of two forms: 1) innate or 2) acquired. In the innate situation, bacteria are “naturally” resistant to specific antibiotics. The bacteria may lack the target structure or may not undergo the process targeted by the antibiotic. Resistance can also be due to the ability of a cell to exclude an

antibiotic from the target site. Some bacteria produce enzymes that inactivate particular antibiotics. Resistance can also be acquired. Chromosomal mutation or acquisition of a resistance gene conveys acquired resistance. A single mutation usually confers resistance to a single antibiotic or to closely related antibiotics with the same target sites. Bacteria have evolved several ways to "share" their resistance traits with one another (Miller, 2000). Resistance genes are commonly carried on plasmids and on the bacterial chromosome. A bacterium can pass resistance traits to another bacterium by transferring a plasmid. Viruses that extract a gene from one bacterial cell and inject it into another can also transfer resistance genes. In addition, after a bacterium dies and releases it contents into the environment, another may take up the liberated gene. In the last two situations, the gene will retain its structure and provide protection from an antibiotic only if integrated correctly into the plasmid or chromosome. Integration occurs frequently, because resistance genes are often embedded in small units of DNA called transposons that readily integrate into other DNA molecules. An R plasmid or a transposon may encode resistance to one or many related or unrelated antibiotics.

Antibiotic Promotion of Resistance. Administration of an antibiotic causes the bacteria that are highly susceptible to the antibiotic to die. But cells that have some resistance from the start or acquire it through mutation or gene exchange may survive, especially if too little antibiotic is given. The remaining resistant bacteria, facing reduced competition from susceptible bacteria, will proliferate. When antibiotics "attack" pathogens they also affect benign endogenous bacteria. Antibiotics eliminate drug-susceptible benign bacteria that would otherwise limit the expansion of pathogens. Simultaneously, antibiotics permit the growth of resistant benign, bacteria and increase the reservoir of resistance traits in the bacterial population.

Antibiotics used to treat disease, as a livestock growth promoter, and an element of crop production has soared since the first commercial applications were introduced. In 1954, 900,000 kg of antibiotics were produced in the United States, by 1998 more than 22,670,000 kg were produced (Levy, 1998). Human treatments account for approximately half of the antibiotics consumed yearly in the United States, but only about half of that use seems necessary (Levy, 1998). The same drugs prescribed for human therapy are widely prescribed in the livestock and crop industries. Approximately 40% of the antibiotics produced in the United States is given to nonhuman animals as disease treatments or growth promoters (Levy, 1998). In particular, antibiotic supplementation increases the utilization of the genetic potential for growth of pigs and poultry, improves feed conversion, and reduces waste product output from intensive livestock production systems.

The magnitude of the impact of livestock antibiotic use on public health and production is unknown. There is very little evidence that directly links use of subtherapeutic doses of antibiotics as growth promoters to the generation of resistance in specific bacterial strains. However, it seems clear that the use of antibiotics, particularly subtherapeutic doses, leads to the selection of resistant bacteria.

Although large numbers of new antibiotics are developed yearly, bacterial resistance to these new compounds continues to emerge, producing a never ending need for the production of more and more powerful antibiotics. Accompanying the need to produce new and more powerful antibiotics is the need to reduce the use of antibiotics and develop solutions to resolve bacterial challenge that does not involve the use of antibiotics.

Antibiotics, Uterine Infections, and Resistance. Internationally, the most common treatment of uterine infections is uterine lavage with antibiotics. This treatment perpetuates the

development of antibiotic resistance. Even though the effectiveness of uterine lavage is questionable, it is believed that uterine lavage stimulates uterine contractions leading to the physical expulsion of uterine infections. Broad-spectrum antibiotics are infused into the uterine lumen during this process to ensure that the bacteria are killed. Unfortunately, most uterine infections are nonspecific, with a large variety of opportunistic bacteria contributing to the signs of infection. There is no guarantee that a broad-spectrum antibiotic is appropriate for the bacterial species involved in the infection or that it will kill all of the bacteria. In fact, it is more likely that the antibiotic will be ineffective against one or more of the species and that, even within the species in which the antibiotic is effective, there will be individuals that are susceptible to the antibiotic and individuals that are resistant.

Uterine lavage with antibiotics perpetuates a cycle of development of antibiotic resistance. In the case of a dairy cow with a postpartum uterine infection, the cow is diagnosed with a uterine infection, systemic and local antibiotics are administered, the susceptible bacteria are killed, and the contents of the uterus slowly drains. Following antibiotic treatment, her milk does not enter the food chain for the appropriate duration. Throughout the entire treatment, the cow is kept in the same pasture or lot with the other cows. As the uterine contents, which contain antibiotics and infectious material, are expelled, the pasture or lot becomes contaminated. The antibiotic on the pasture or lot then affects pathogenic and nonpathogenic indigenous bacteria. The indigenous bacteria are killed or acquire antibiotic resistance genes. The susceptible populations of bacteria in the pasture are killed, and the pasture is repopulated with resistant bacteria. The antibiotic residue on the pasture is consumed by all of the cows in subtherapeutic doses, and it enters the milk supply and continues to confer antibiotic resistance. The next cow that is diagnosed with a uterine infection is treated the same way; however, this time the contents

of the uterus are expelled, because of antibiotic resistance, fewer of the bacteria have been killed. The cycle continues until farms accumulate a large load of antibiotic-resistant organisms, and common diseases, such as scours, become difficult to treat without moving to "new-generation" antibiotics that are "reserved" for life-threatening conditions.

Cyclooxygenase, Lipooxygenase, the Generation of Eicosanoids, and General Reproductive and Immune Function of Eicosanoids

General. Eicosanoids are a family of oxygenated derivatives of dihomo-γ-linolenic acid, arachidonic acid, and eicosapentaenoic acid. Eicosanoids include PG, TX, LT, lipoxins, hydroperoxyeicosatetraenoic acids, and hydroxyeicosatetraenoic acids. In most conditions, the principal precursor for these compounds is arachidonic acid. Eicosanoids produced from arachidonic acid seem to be more potent biologically than those produced from dihomo-γ-linolenic acid and eicosapentaenoic acid. Two oxidative pathways involved in the production of eicosanoids have been identified: cyclooxygenase and lipoxygenase. The primary products of the lipoxygenase pathway are the LT. The cyclooxygenase pathway results in the formation of the PG and TX. The pathways of eicosanoid synthesis begin with either cyclooxygenase or with the 5-, 12-, or 15-lipoxygenases. The amounts and types of eicosanoids synthesized are determined by the availability of arachidonic acid, by the activities of phospholipase A_2, and phospholipase C, and by the activities of cyclooxygenase and lipoxygenase enzymes.

Prostanoids including the PG and TX are derived from C-20 unsaturated fatty acids that enter the cyclooxygenase pathway. Prostaglandins contain a cyclopentane ring and two side chains called α and ω attached to the ring. Thromboxanes contain an oxane ring, rather than a cyclopentane ring. Thromboxane is derived in platelets from the presence of TX synthase, which converts PGH_2 into TXA_2. All other TX are derived from TXA_2. According to the modifications

of the cyclopentane ring in prostanoids, they are classified into Types A through I. Types A, B, and C do not seem to occur in nature. The are produced artificially during various extraction procedures or from spontaneous decay. Prostaglandins G and H share the same ring structure, but differ at C-15, having a hydroperoxy and a hydroxy group, respectively. Prostanoids are further classified into one of three series based on the number double bonds in their side chains. The Series 1 prostanoids contain a 13-trans double bond, the Series 2 prostanoids have 5-cis and 13-trans double bonds, and the Series 3 prostanoids have 5-cis, 13-trans, and 17-cis double bonds. The Series 1, 2, and 3 prostanoids are synthesized from dihomo γ-homolinolenic acid, arachidonic acid, and 5,8,11,14,17-eicosapentaenoic acid, respectively. Under "normal" circumstances, arachidonic acid is the most abundant of these fatty acid precursors. Arachidonic acid serves as the major precursor of the Series 2 prostanoids that are the predominant PG in the body.

Leukotrienes are derived from C-20 unsaturated fatty acids that enter the lipooxygenase pathway. Lipooxygenases catalyze the incorporation of molecular oxygen into arachidonate. Various LT differ in the position of the peroxide that is introduced by the lipoxygenase enzyme. The name LT was applied to this group of eicosanoids because of their origin in leukocytes and their conjugated triene structures.

The first step in the synthesis of eicosanoids is the release of substrate, primarily arachidonic acid from phospholipid storage pools. This occurs via activation of an archidonic acid specific cytoplasmic phospholipase A_2. Agonists that stimulate eicosanoid synthesis bind to cell surface receptors that are members of the seven-transmembrane domain family of receptors. These receptors interact with heterotrimeric GTP-binding proteins, which in turn activate phospholipase C, resulting in the production of diacylglycerol and inositol-1,4,5-trisphosphate.

Inositol-1,4,5-trisphosphate induces release of Ca^{2+} from the endoplasmic reticulum. Diacylglycerol binds to protein kinase C, promoting binding of Ca^{2+} to protein kinase C, its translocation to the membrane, and activation. Active protein kinase C phosphorylates and activates Raf proteins. The Raf proteins are serine/threonine kinases that can phosphorylate MEK, which can phosphorylate MAP kinase. Cytoplasmic phospholipase A_2 is a protein substrate of the MAP kinase cascade. Once phosphorylated, cytoplasmic phospholipase A_2 binds Ca^{2+} and becomes associated with intracellular membranes. Cytoplasmic phospholipase A_2 cleaves arachidonic acid from phosphatidylcholine, making it available to eicosanoid synthesizing enzymes. The second step in the synthesis of eicosanoids is the oxygenation of arachidonic acid. Following liberation from the membrane, these fatty acids are converted to the various eicosanoids by the sequential actions of cyclooxygenase, lipooxygenase, and other synthases. The arachidonic acid cascade is influenced by numerous factors including arachidonic acid stores, enzyme availability, free Ca^{2+}, hormone concentrations, glucocorticoids, and pharmaceutical products. Substrate availability is dictated initially by diet and biosynthesis from essential fatty acids.

The cyclooxygenase pathway involves the conversion of arachidonic acid to endoperoxide intermediates. Cyclooxygenase is the rate limiting enzyme in the production of prostanoids from arachidonic acid. There are two forms of COX. The constitutive isoform, COX-1, plays a critical role in maintaining the background production of arachidonic acid metabolites that is essential for the maintenance of homeostasis. The inducible isoform, COX-2 is selectively expressed in certain cell types such as leukocytes, allowing these cells to produce large quantities of prostanoids at the site of an inflammatory response. The COX-2 isoform is also expressed in reproductive tissues, where the steroid milieu is an important regulator of COX-2 activity.

Cyclooxygenase directs the oxidation of arachidonic acid to the intermediate diperoxide PGG_2. This complex reaction results in the formation of a product with five chiral centers from an achiral substrate. The unstable PGG_2 is subsequently reduced to PGH_2 in a typical peroxidase reaction by the same enzyme. These two reactions are similar for the COX-1 and COX-2 isoforms. Thus, PGH_2 represents the common precursor for the biosynthesis of the PG. Several unique enzymes chemically convert PGH_2 into potent biological molecules. Prostaglandin F synthetase and 9-keto-isomerase are two such enzymes. Prostaglandin F synthetase replaces the peroxide moiety with hydroxyl groups on carbons 9 and 11 to form $PGF_{2\alpha}$. The enzyme 9-keto-isomerase converts PGH_2 into PGE_2. Numerous other specific enzymes are involved in PG and TX synthesis.

The oxidation of arachidonic acid at carbon 5 to form 5-hydroperoxyeicosatetraenoic acid is catalyzed by 5-lipoxygenase. This enzyme can also convert 5-hydroperoxyeicosatetraenoic acid to LTA_4. The synthesis of LTA_4 is facilitated by 5-lipoxygenase activating protein. The FLAP protein is an integral membrane protein that binds free arachidonic acid and presents it to 5-lipoxygenase. Leukotriene A_4 is unstable and undergoes rapid nonenzymatic hydrolysis to 6-trans-LTB_4, which is a biologically inactive metabolite. Alternatively, LTA_4 may be enzymatically converted into other possible products. It may be converted into LTB_4, which is a potent chemotactic agent in the immune system. If glutathione is added to carbon 6, it forms LTC_4. Leukotriene C_4 can be converted to LTD_4 or LTE_4 following the sequential removal of glutamyl and glycine residues from glutathione. Finally, LTA_4 can undergo a second oxidation at carbon 15 to form lipoxins A and B.

Eicosanoids do not easily penetrate the cell membrane. Their transport out of the cells that produce them is regulated by specific transport proteins, and they exert their effects on target

cells by binding to cell surface receptors. Prostanoids are released outside of the cell immediately after synthesis. Prostaglandin G, PGH, PGI, and TXA are chemically unstable and are rapidly degraded into inactive products under physiological conditions. Other PG, although "more" chemically stable, are also metabolized rapidly. The first step in metabolism of these PG involves the oxidation of the 15-hydroxyl group to a keto-moiety. Prostaglandin $F_{2\alpha}$ is metabolized via 15-hydroxy-prostaglandin-dehydrogenase and Δ^{13}-reductase into 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (Lands, 1979). There are further steps for the metabolism of the PG to more water soluble forms that are excreted in urine; however, once the hydroxyl group has been oxidized, the associated PG loses its biological activity. Most are inactivated following a single passage through the lungs. This leads to the belief that most prostanoids are local mediators, acting within a close proximity of production. It is this characteristic that has led to the classification of prostanoids as cytokines.

Prostanoid Receptors. Because of the hydrophobic nature of prostanoids, it was once believed that they were incorporated into the cell membrane and exerted their action by perturbing lipid fluidity. However, prostanoids are not as hydrophobic as was originally believed, they do not incorporate into or permeate the cell membrane, and each prostanoid has a unique activity profile that does not exactly overlap the others, indicating a specific site of action. Biochemical evidence also indicated the presence of prostanoid receptors. The actions of prostanoids were associated with changes in the amount of second messengers. There is a comprehensive classification of prostanoid receptors. Researchers proposed the presence of receptors specific for TX, PGI, PGE, PGF, and PGD, and they named these receptors TP, IP, EP, FP, and DP receptors, respectively. Researchers further classified the EP receptor into four subtypes: EP1, EP2, EP3, and EP4 based on their response to PGE_2.

Eight subtypes of prostanoid receptors are characterized as G protein-coupled rhodopsin-type receptors with seven transmembrane domains. Different genes encode each type. Each of the eight subtypes of prostanoid receptors shows selective ligand-binding specificity that distinguishes it from the others. This specificity has been characterized by the selective responses of tissues to prostanoid and their analogs. Each of the prostanoid receptors was initially identified by its preferential responsiveness to a particular type of prostanoid and was subsequently characterized by its response to various synthetic prostanoid analogs. Different degrees of responsiveness to the same receptor type have been noted between species.

Prostanoid receptors can be grouped into three categories on the basis of their signal transduction and action: 1) the relaxant receptors; 2) the contractile receptors; and 3) the inhibitory receptors. The relaxant receptors mediate increases in cAMP and induce smooth muscle relaxation, and they consist of the IP, DP, EP2, and EP4 receptors. The contractile receptors consist of the TP, FP, and EP1 receptors which, mediate CA^{2+} mobilization and induce smooth muscle contraction. The EP3 receptor is an inhibitory receptor that mediates decreases in cAMP and inhibits smooth muscle relaxation. Sequence homology is greater among the functionally related receptors than between the receptors from the three groups.

Prostanoid receptors of one type or another are expressed in almost every tissue in the body. However, it is difficult to ascertain the exact distribution of each receptor and the identities of cells expressing each receptor, because of the relatively small amounts expressed and the expression of multiple receptor types in a single tissue. The DP receptor type is the least abundant. It is expressed in small amounts in the digestive tract and selective areas of the brain. The prostanoid receptors EP3 and EP4 are widely distributed throughout the body. The mRNA for these receptors has been found in almost all of the mouse tissues tested (Honda et al., 1993;

Sugimoto et al., 1992). In contrast, the distribution of EP1 mRNA seems to be restricted to kidney, lung, and stomach (Watabe et al., 1993). The EP2 receptor is inducible rather than constitutive; therefore, expression requires a stimulus such as LPS or gonadotropins (Katsuyama et al., 1997; Katsuyama et al., 1998). The organ that expresses FP receptor mRNA most abundantly is the CL. The expression of FP mRNA is closely related to the stage of the estrous cycle suggesting a close relationship between FP gene expression and luteolysis (Hasumoto et al., 1997). The IP receptors are expressed in areas that potentate pain and in lymphocytes; however, the function of IP receptors located on the membrane of lymphocytes is unknown. The TP receptor is expressed on immature thymocytes and throughout the vascular system. The TP receptor may play a role in thymoctye differentiation and development.

Phylogenetic trees have been generated to infer the evolutionary relationship among lipid mediators (Boie et al., 1995; Toh et al., 1995). The prostanoid receptors constitute a distinct cluster within the rhodopsin-type receptors. Receptors for platelet activating factor and lipoxin belong to a separate cluster shared by peptide receptors such as bradykinin and endothelin. The prostanoid receptor clusters are further divided into three sub-clusters that relate to the functionality distinctions made earlier. These results indicate that the cyclooxygenase pathway may have initiated as a system composed of PGE and its receptor. The subtypes of the PGE receptor that evolved from the primitive PGE receptor mediated different signal transduction pathways, and the receptors for other PG and the TX evolved from functionally related PGE receptor subtypes by gene duplication. Findings concerning the receptors for LT indicate that the cyclooxygenase and lipooxygenase pathways evolved separately and later integrated forming the arachidonic acid cascade.

General Reproductive Actions of Eicosanoids. The PG, particularly $PGF_{2\alpha}$ and PGE_2 are directly involved in many reproductive functions. This includes mediating local and systemic reproductive mechanisms. The role of eicosanoids in ovulation is similar to their role during an inflammatory response. This includes eicosanoid action at the hypothalamic and ovarian level. Luteinizing hormone releasing hormone is secreted by the hypothalamus in response to PGE_2 (Ojeda and Campbell, 1982). In the ovary, the PG content of a follicle increases as the follicle matures. Indomethacin abolishes LH induced ovulation, and this effect can be reversed by treatment with PGE_2 or $PGF_{2\alpha}$ (Murdoch et al., 1993). The increase in PG synthesis is associated with an increase in the COX-2 mRNA induced by gonadotropins. Prostaglandin E_2 stimulates adenylate cyclase activity, particularly in granulosa cells, and this may increase the steroidogenic action of LH within the follicle. The increase in PGE_2 also contributes to the increase in follicular blood flow that occurs during the early phase of the ovulatory process. During the later phase of the ovulatory process, $PGF_{2\alpha}$ stimulates phospholipase C causing a reduction in follicular blood flow. Prostaglandins may also activate proteolytic enzymes that contribute to the breakdown of the follicular wall. Leukotrienes also accumulate in follicular tissue during the ovulatory process. Following ovulation, PG have a role in luteal growth, maintenance, and regression. Both PGE_2 and PGI_2 stimulate progesterone secretion from luteal cells in vitro by activating adenylate cyclase and inducing accumulation of cAMP. Depending on species, $PGF_{2\alpha}$ is considered the causative agent of luteolysis during the estrous cycle. Various PG affect uterine motility. Changes in the motility of the reproductive tract affect processes leading to the evacuation of the uterine contents such as cervical dilation, parturition, and uterine involution. Aspirin-like drugs delay parturition in many species. Prostaglandin $F_{2\alpha}$ is found in large quantities in uterine tissues during late pregnancy, and it is thought to be a strong uterotonic

substance. Prostaglandin $F_{2\alpha}$ plays important roles in initiating and maintaining parturition by causing and sustaining uterine contractility. Studies on COX-2-deficient mice showed multiple reproductive failures, including failure of ovulation, fertilization, implantation, and deciduization.

Immune Involvement I. The Generation of Fever. Fever is a representative component of the acute-phase response to immunological challenge. Fever is elicited by cellular components of infectious organisms (i.e., LPS) and by noninfectious inflammatory insults. Exogenous pyrogens and noninfectious insults stimulate the production of cytokines that work as endogenous pyrogens. These cytokines include IL-1, IL-6, TNF-α, INF-α, and INF-γ. These cytokines exert their effects on the preoptic area stimulating the neuronal pathways that raise body temperature (Saper et al., 1994). Nonsteroidal anti-inflammatory drugs including aspirin and indomethacin can suppress fever. Because these drugs inhibit PG biosynthesis, it is assumed that PG are involved in fever generation (Vane, 1971). The identity of the PG that mediates fever is still unknown. Milton and Wendlandt (1970) suggested PGE_2 as a central mediator of fever. Prostaglandins of the E type induce fevers when injected into the brain. Administration of indomethacin decreases LPS-induced fever and increases the concentrations of PGE_2 in the preoptic area in response to LPS challenge (Stitt and Webb, 1986; Sirko et al., 1989; Sehic et al., 1996). In rabbits, microinjections of $PGF_{2\alpha}$ into the brain also induce fever (Morimoto et al., 1988). Fever induced by the intracerebroventricular injection of $PGF_{2\alpha}$ differs from the fever produced by PGE_2 (Morimoto et al., 1988; Rothwell, 1990; Coelho et al., 1993). These differences suggest that $PGF_{2\alpha}$ may stimulate thermogenesis via different mechanisms than PGE_2. The intracerebral administration of PGI_2 can also produce a limited febrile response, whereas PGD_2 and TXA_2 do not evoke fever production. Nitric oxide has been postulated to

have a role in the production of fever. Nitric oxide produced by pyrogens induces the production of various cytokines including PGE_2 and $PGF_{2\alpha}$ that induce fever (Lin and Lin, 1996).

Immune Involvement II. Inflammation, Vascular Permeability, and Immunity. Local reddening, heat generation, swelling, and pain are the four classical signs associated with acute inflammation. The first three are caused by increased blood flow and vascular permeability with resultant edema. Previous studies suggested that PG are primarily involved in vasodilation in the inflammatory response and synergize with other mediators such as histamine and bradykinin to cause an increase in vascular permeability and edema. In particular, PGE_2 and PGI_2 are the most potent mediators of these events (Narumiya et al., 1999). Although PGI_2 and IP receptor have been shown to have a primary role in mediating the inflammatory response in certain experimental models, it is more likely that there is a context-dependent interaction between this system and the PGE_2 and EP receptor system (Narumiya et al., 1999).

In addition to acute inflammation, PG are likely to play physiological roles in the regulation of immunity and allergy. Thromboxane and IP receptors are expressed by immature and mature thymocytes (Oida et al., 1995). A TXA_2 mimetic induces apoptosis of immature thymocytes in vitro, leading to the suggestion that the TXA_2 and TP system may have an antigen-dependent immunomodulatory role. The E type-PG have potent immunosuppressive effects. Prostaglandin E_2 induces apoptosis in thymocytes and inhibits several T lymphocyte functions such as the production of IL-2 (McConkey et al, 1990). In particular, PGE_2 probably acts by redirecting the actions of T lymphocytes; PGE_2 activates the Th-2 subset of T cells while suppressing the Th-1 (Phipps et al., 1991). Prostaglandin E_2 acts on the EP2 and (or) EP4 receptor of B cells and synergizes with LPS or IL-4 to facilitate IgE production (Fedyk and Phipps, 1996). These findings suggest that PGE_2 may work as a switch for Th2 mediated allergic

responses. A role for PGD_2 in the immune response has also been postulated, because it is produced in abundance by mast cells (Lewis et al., 1982).

Regulation of the Estrous Cycle

General. Four organs are principally involved in the control of reproduction in females: the hypothalamus, pituitary, ovaries, and uterus. The estrous cycle is defined as the period from one estrus to the next. Based on morphological structures on the ovary, the estrous cycle can be divided into two major phases: follicular and luteal. During the follicular phase depending on species, a dominant follicle is present on the ovary, and during the luteal phase a CL is present on the ovary. Multiple ovulators do not have a single dominant follicle, instead several tertiary follicles are present on the ovary. The duration of these phases varies with species. During estrus, estrogen from ovarian follicles is the dominant hormone influencing the reproductive tract. During the luteal phase, progesterone secreted from the CL is the dominant hormone influencing the reproductive tract. During the follicular phase, the concentration of estrogen increases as follicles grow. This increase in ovarian estradiol is necessary to induce the preovulatory surges of LH and FSH. Estradiol increases the responsiveness of the pituitary to GnRH (Draincourt, 1991; Caraty et al., 1995). Ovulation occurs after the LH surge (Draincourt, 1991). After ovulation, corpus hemorrhagicum forms. A corpus hemorrhagicum is an intermediate ovarian structure. The luteal phase is dominated by the presence of CL in the ovary. The CL replaces the corpus hemoragicum at the site of ovulation. The CL secretes progesterone throughout the luteal phase (Mann et al., 1992). Luteolysis is signaled by the uterine secretion of $PGF_{2\alpha}$. Luteolysis is divided into two phases: structural and biochemical. Biochemical regression results in a decrease in progesterone concentration. As progesterone decreases, estrogen increases, and the frequency of LH impulses increases, initiating a new cycle.

Ovine. Sheep are seasonally polyestrous animals. Depending on breed, sheep have estrous cycles during seasons with short days and long nights. In southwesten Virginia, most breeds of sheep cycle from mid-August through mid-February, although sheep with Dorset or Rambouillet breeding may have estrous cycles through April. In sheep, estrous cycles vary in duration between 14 to 17 d. Progesterone concentrations in ewes range from approximately .4 ng/mL during the first 4 d of the cycle, to as great as 5 ng/mL during d 8 to 13 (McNatty et al., 1973). In ewes, luteolysis is initiated between d 11 and 13.

Porcine. Sexually mature female pigs are polyestrous throughout the year. In pigs, the estrous cycle is approximately 21 d, with an average range of 19 to 23 d. The estrous cycle in pigs commences at puberty and continues throughout the female's life, interrupted only by pregnancy, lactation, or endocrine dysfunction. Estrus lasts for 24 to 72 h, and ovulation occurs 36 to 42 h after the onset of estrus. The duration of the ovulatory process is approximately 3.8 h, with a range in the ovulation rate of 10 to 25 ova. Progesterone secretion increases from d 7 to maximum production between d 12 and 14 of the estrous cycle. Luteolysis begins, and progesterone concentrations decrease to approximately 1 ng/mL or less by d 17 to 18.

Interactions Between Estrogen, Progesterone, Oxytocin, and $PGF_{2\alpha}$

Estrogen and Progesterone Receptors. Oscillating ovarian production of estrogen and progesterone coordinates uterine functions throughout the estrous cycle and early pregnancy. In sheep, changes in the ovarian production of estrogen and progesterone are accompanied by changes in the endometrial steroid hormone receptor populations (Findlay et al., 1982; Cherny et al., 1991; Ott et al., 1993; Wathes and Hamon, 1993). Progesterone and estrogen receptors are greatest at the time of estrus in response to the increased concentrations of estrogen (Wathes and Hamon, 1993). Progesterone and estrogen receptor concentrations are least during the later

portion of the luteal phase in response to prolonged periods of progesterone. Estrogen up regulates estrogen and progesterone receptor expression (Miller et al., 1977; Zelinski et al., 1982; Spencer et al., 1995), whereas progesterone inhibits estrogen receptor expression (Spencer et al., 1995). Specifically, estrogen regulates expression of the estrogen and progesterone receptor genes by modulating transcriptional and post translational regulation (Shapiro et al., 1989).

As progesterone in circulation increases, endometrial estrogen receptor mRNA and protein decrease (between d 1 to 11) (Spencer and Bazer, 1995). Estrogen receptor expression increases between d 13 and 15 and is maximal on d 1 when plasma concentrations of estrogens are increasing and progesterone concentration are basal (Baird and Scaramuzzi, 1976). This suggests that progesterone blocks the effects of estrogen during the luteal phase and early pregnancy.

The mechanism by which estrogen enhances progesterone receptor transcription is well documented (Kastner et al., 1990; Kraus et al., 1994). However, estrogen enhancement of estrogen receptor transcription or translation is not well understood. According to Ing et al (1996), estrogen regulates estrogen receptor expression at the mRNA and protein level, not at the level of the gene. The short half lives (2 to 3 h) of the mRNA and protein products of the estrogen receptor gene make estrogen receptor levels susceptible to transcriptional and(or) post transcriptional regulation (Nardulli and Katzellenhogen, 1986; Borras et al., 1994). This accounts for the rapid turnover of estradiol receptors following a low dose of exogenous estradiol-17β. Following the administration of the exogenous estradiol-17β, the mRNA products are modified, and the protein product is produced and remains for approximately 2 to 3 h unless there is continued stimulation with estrogens (Ing et al., 1996).

Oxytocin. In ewes, the uterus does not seem to be responsive to oxytocin during portions of the luteal phase (Roberts et al., 1976). The decrease of oxytocin receptors during the luteal phase prevents oxytocin from causing an effect. The uterus is least responsive to oxytocin during periods of progesterone dominance. The amount of uterine activity seems to correspond to uterine sensitivity to oxytocin. In sheep, the uterus is most active at estrus or just before estrus (Croker and Shelton, 1973; Naaktgeboren et al., 1973).

Estrogen, Progesterone, and Oxytocin Receptors. Estrogen increases oxytocin receptor expression (Soloff, 1975; Hixon and Flint, 1987; Beard and Lamming; 1994), and progesterone decreases oxytocin receptor expression (Burgess et al., 1990; Beard et al., 1994). During the luteal phase, the endometrium is under the influence of progesterone. Progesterone seems to block the action of estrogens, probably at the level of the estrogen response element on the oxytocin receptor gene (E. E. Custer, personal communication). In ewes, increased progesterone concentrations on d 12 down regulate the progesterone receptor. The decrease in response to progesterone correlates with an increase in the response to estrogen. Increased estrogen up regulates the production of oxytocin receptors. In rats and cows, there is clear evidence that estrogen binds to an estrogen response element on the oxytocin receptor gene (Adan et al., 1991; Adan et al., 1993; Zingg et al., 1995; Bale and Dorsa, 1997). It is reasonable to conclude that similar events occur in other animals. After estrogen binds to the response element on the oxytocin receptor gene, synthesis of oxytocin receptors begins. Progesterone may block the effects of estrogen; however, the block is limited. Initially, in ovariectomized ewes, progesterone suppresses the effects of estrogen on oxytocin induced secretion of $PGF_{2\alpha}$, but, after 10 d of progesterone treatments, it enhances the effects of estrogen (McCracken et al., 1981). Homanics and Silvia (1988) reported similar findings after 15 d of progesterone treatment. These findings

indicate a short-term suppressive effect of progesterone on oxytocin-induced secretion of $PGF_{2\alpha}$ that disappears after longer periods of treatment. After longer periods of treatment, it also suggests an interaction between estrogen and progesterone that results in an increased responsiveness of the uterus to oxytocin. Endometrial concentrations of oxytocin receptors are greater after 12 d than after 5 d of progesterone treatment (Zhang et al., 1992). The interaction between progesterone and estradiol that enhances $PGF_{2\alpha}$ secretion may not be because of changes in oxytocin receptor concentrations.

Because $PGF_{2\alpha}$ is secreted by the endometrium in response to oxytocin (Sharma and Fitzpatrick, 1974; Mitchell et al., 1975; Roberts et al., 1976), a role has been postulated for oxytocin in luteolysis (Flint et al., 1990). However, reports of the effects of exogenous oxytocin on CL have been equivocal. Hatjiminaoglo et al. (1979) is the only report that exogenous oxytocin given between d 1 and 7 of the estrous cycle causes premature luteolysis in some ewes. However, the statistical validity of this experiment is questionable. Premature luteal regression was observed in a significant number of the ewes treated with oxytocin; however, the significance was inflated by the small number of ewes on the study. Milvae et al. (1991) administered exogenous oxytocin over a series of 4-d periods throughout the estrous cycle, without observing premature luteolysis or a decrease in cycle length.

Progesterone, Oxytocin, and $PGF_{2\alpha}$ Receptors. To date there is no explanation for changes in sensitivity of the CL to $PGF_{2\alpha}$. The sensitivity of the cow and sheep CL to $PGF_{2\alpha}$ increases progressively throughout the luteal phase (Skarzynski et al., 1997). Initially, change in sensitivity was attributed to changes in receptor concentration and affinity. Currently, the change in differential sensitivity is attributed to an increase in oxytocin (Lasma et al., 1989; Skarzynski

et al., 1997) indicating the presence of intracellular mechanisms that regulate different reactions of CL to $PGF_{2\alpha}$ over time (Skarzynski et al., 1999).

If $PGF_{2\alpha}$-induced luteolysis is a receptor mediated event, it would stand to reason that the changes in the sensitivity of the CL to $PGF_{2\alpha}$ would depend on the density and affinity of FP receptors in the CL. However, Sakamoto et al., (1994) demonstrated that expression of FP receptor mRNA and the number and affinity of the $PGF_{2\alpha}$-binding sites were only slightly greater in the late luteal phase than in early luteal phase. High affinity $PGF_{2\alpha}$-binding sites have also been detected on the luteal membrane in early cow CL (Sakamoto et al., 1995). The FP receptor concentration and affinity are the same in early and active CL (Wiltbank et al., 1995). Therefore, it is difficult to attribute an increase in sensitivity to $PGF_{2\alpha}$ to changes in FP receptor concentration or affinity.

Recently, it has been suggested that the sensitivity of the cow and sheep CL to $PGF_{2\alpha}$ is mediated via intraluteal factors including progesterone and oxytocin (Skarzynski and Okuda, 1999). The CL is a site of progesterone, PG, and oxytocin production. High affinity binding sites for oxytocin and progesterone have been demonstrated in the CL (Okuda et al., 1992). These luteal factors may act on the CL independently or in concert to modify the actions of one another.

Luteolysis

General. The CL is a transient endocrine organ formed from cells of the follicle following ovulation. The primary function of the CL is to secrete progesterone. The CL consists of two distinct steroidogenic cell types: large luteal cells and small luteal cells. These two cell types are morphologically distinct. In most species, the function of the CL is regulated by the anterior pituitary, the uterus, and in the case of pregnancy the conceptus. Luteolysis is the

regression of the CL. An initial decline in progesterone secretion characterizes functional luteolysis. Functional luteolysis is distinct from structural luteolysis. Structural luteolysis describes the subsequent change in cellular structure of the CL. However, it is difficult to make the distinction between functional and structural luteolysis. In many species, luteolysis represents the "end" of a reproductive cycle and the initiation of the next cycle.

In livestock "normal" luteolysis is dependent on the presence of the uterus. Hysterectomy of cows, ewes, and pigs delays luteolysis and extends the luteal period. Prostaglandin $F_{2\alpha}$ from the uterus is the luteolysin in these species (Hansel et al., 1973; McCracken et al., 1970). On the basis of partial hysterectomy and vascular anastamosis studies, initiation of luteolysis by $PGF_{2\alpha}$ appears to be a local effect between the uterus and the ovaries. It has been postulated that $PGF_{2\alpha}$ enters the ovarian artery from the uteroovarian vien via a countercurrent exchange mechanism, allowing $PGF_{2\alpha}$ to travel to the ovarian artery without entering the pulmonary circulation, where it would be enzymatically inactivated in the lungs.

One of the intriguing questions about luteolysis is: What is the signal that initiates the luteolytic release of $PGF_{2\alpha}$? In sheep, the pulsatile release of luteolytic amounts of $PGF_{2\alpha}$ is coordinated with pulsatile releases of oxytocin from the CL and the posterior pituitary (Fairclough et al., 1980). These two events provide a coupled feedback loop (Fairclough et al., 1980; Tsai and Wiltbank, 1997). Hypophysial and ovarian oxytocin stimulate release of small quantities of uterine $PGF_{2\alpha}$ (Fairclough et al., 1980). Prostaglandin $F_{2\alpha}$ than initiates a positive feedback loop involving release of additional luteal oxytocin and luteal and uterine $PGF_{2\alpha}$ (Lewis and Wade, 1996; Tsai and Wiltbank, 1997).

The actual role of oxytocin in luteolysis in sheep is controversial. Injections of oxytocin alone will not cause regression of CL (Milvae et al., 1991; Wulster-Radcliffe et al., 1999), and

regression of the CL occurs in the absence of oxytocin. Platelet activating factor-1 is an ether phospholipid that is involved in a number of physiological pathways. Platelet activating factor-1 is produced by the uterus in sheep and mimics the actions of oxytocin, inducing $PGF_{2\alpha}$ release. It is has been proposed that platelet activating factor-1 is the uterine pulse generator for pulsatile release of $PGF_{2\alpha}$ in sheep (Chami et al., 1999). Concentrations of oxytocin secreted from porcine CL are extremely low, suggesting that pigs are dependent on pituitary oxytocin for the initiation of luteolytic pulses of $PGF_{2\alpha}$ (McCracken et al., 1999).

In ewes, $PGF_{2\alpha}$ causes a rapid decrease in luteal blood flow. This decrease in blood flow is correlated with decreases in progesterone concentration. Because endothelial cells express receptors for $PGF_{2\alpha}$, it has been postulated that $PGF_{2\alpha}$ acts directly on the luteal endothelial cell population (Mamluk et al., 1998). Prostaglandin $F_{2\alpha}$ causes degeneration of luteal endothelial cells (O'shea et al., 1977) resulting in a reduction in capillary bed density, decreasing blood flow to the luteal tissue (Braden et al., 1988).

Prostaglandin $F_{2\alpha}$ elicits a number of morphological changes in luteal cells. Twenty four hours after injecting ewes with $PGF_{2\alpha}$ during the luteal phase, there is a decrease in the proportion of steroidogenic luteal cells occupying the CL (Braden et al., 1988). Morphological changes in these cells do not become evident until 24 to 36 h after exposure to $PGF_{2\alpha}$, although the steroidogenic activity has already declined (Sawyer et al., 1990). Even though it is easy to measure decreases of progesterone in relation to increases in $PGF_{2\alpha}$, it is has been difficult to isolate the details of this mechanism. Prostaglandin $F_{2\alpha}$ could decrease progesterone synthesis through a number of intracellular mechanisms including: 1) downregulation of receptors for lutetropic hormones, 2) decreased cellular uptake of cholesterol, 3) decreased transport of

cholesterol through the cell or across the mitochondrial membrane, and 4) decreased activity of the steroidogenic enzymes needed for biosynthesis of progesterone.

Prostaglandin $F_{2\alpha}$ binds to specific receptors localized on large luteal cells. The $PGF_{2\alpha}$ receptor was described in more detail earlier in this dissertation. Upon binding, $PGF_{2\alpha}$ induces activation of membrane bound PLC (Berridge and Irvine, 1984) via a stimulatory G protein (Miwa et al., 1990). Phospholipase C catalyzes the hydrolysis of phospatidylinositol 4,5-bisphosphate to IP_3 and DAG. Increased cytosolic concentrations of IP3 result in release of free Ca^{2+} from the smooth endoplasmic reticulum. Increased free Ca^{2+} and DAG stimulate the catalytic activity of Ca^{2+}-dependent PKC. Protein kinase C mediates many of the antisteroidogenic actions of $PGF_{2\alpha}$ in large luteal cells, and activation of PKC induces expression and activation of proteins involved in apoptosis. Apoptosis in CL can be broken down into a simplistic series of steps: 1) $PGF_{2\alpha}$ binds to its recptor on the large luteal cell, 2) PKC is activated or inhibited leading to protection or an increased rate of apoptosis, 3) increase in intracellular calcium, 4) altered gene expression, 5) increase in the expression of proteins that mediate apoptosis, and 6) fragmentation of DNA, 7) cellular changes, and 8) cell death.

Role of Immune Cells and Cytokines in Luteolysis. The immune system has a critical role in luteolysis. Leukocytes are present in the CL or infiltrate during luteolysis. These cells are actively involved in the biochemical and structural regression of the CL. Splenectomy in rats results in elevated concentrations of progesterone in the serum and delayed luteal regression (Matsuyama et al., 1987). Injection of splenocytes reverses this effect (Matsuyama et al., 1987). The presence of immune cells and the necessity of a functioning immune system to maintain cyclicity suggest a role for immune cells and their products in luteolysis. The effects of the

immune system are mediated indirectly by changes in hormone profiles in the hypothalamo-pituitary-gonadal axis and directly by interactions with ovarian cells.

Earlier it was proposed that $PGF_{2\alpha}$ causes the initial fall in progesterone by acting directly on luteal cells via capillary endothelial cells and regulation of blood flow. However, another likely role for luteal $PGF_{2\alpha}$ is to alter the local immune response. Prostaglandin $F_{2\alpha}$ can modulate activation of immune cells and cytokine secretion. It is possible that immune cells within the CL must be exposed to $PGF_{2\alpha}$ to become activated and to facilitate luteolysis. Luteal $PGF_{2\alpha}$ may act to enhance the function of immune cells to promote luteal regression or modify the local immune response so that it does not extend to the surrounding ovarian tissues.

Macrophages and T lymphocytes are present in the CL during the luteal phase of the estrous cycle (Brannstrom et al., 1994). In rabbit CL, the number of lymphocytes remains constant, but the number of macrophages increases before the onset of luteolysis. Fewer immune cells infiltrate the CL during early pregnancy than in a nonfertile cycle.

Cytokines implicated in ovarian function include IL-1, IL-2, TNF-α, IFN-γ, IFN-α, granulocyte-macrophage colony stimulating factor, and platelet activating factor. The role of platelet activating factor in luteolysis was discussed in the last section and will not be addressed in this section. Interleukin-1β does not significantly affect basal progesterone by luteal cells (Nothnick and Pate, 1990); however, IL-1β does inhibit LH-stimulated steroidogenesis (Pitzel et al., 1993). In particular, IL-1β decreases basal and LH stimulated progesterone in pig luteal cells (Pitzel et al., 1993). In bovine luteal cell cultures, IL-1β stimulates PG synthesis. Treatment with IL-1β resulted in dose dependent increases in $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ a stable metabolite of PGI_2. This stimulatory effect was blocked by indomethacin (Nothnick and Pate, 1990), cyclohexamide, and antinomycin (Townson and Pate, 1994). The acute stimulation of

luteal PG production by IL-1β seems to be via the activation of PLA_2 and to a lesser extent PLC. Thus, a primary action of IL-1β is to increase availability of arachidonic acid for conversion to PG. After chronic exposure to IL-1β, luteal cells have an enhanced ability to convert exogenous arachidonic acid to $PGF_{2\alpha}$, suggesting an up-regulation of COX (Townson and Pate, 1994).

The actions of TNF-α on bovine luteal cells are similar to the actions of IL-1β. Gonadotropin stimulated steroidogenesis is suppressed, and PG sysnthesis is stimulated (Benyo and Pate, 1992). Tumor necrosis factor-α also activates PLA_2, but not PLC. When combined, TNF-α and IL-1β exert synergistic effects on luteal PG production (Benyo and Pate, 1992). Tumor necrosis factor-α inhibits basal and gonadotropin-stimulated progesterone secretion and enhances the inhibitory effects of $PGF_{2\alpha}$ and oxytocin in pig luteal cells (Pitzel et al., 1993).

Interferon-γ is also involved in luteolysis. It exerts a biphasic effect on cultured bovine luteal cells. During the first 24 h of culture, IFN-γ has no effect on progesterone synthesis, but it inhibits PG production. After 72 h, no difference is observed in either progesterone or PG secretion as compared with control cells. However, after 72 h, IFN-γ completely inhibits LH-stimulated progesterone and markedly increases PG synthesis (Fairchild and Pate, 1991).

Certain cytokines also have direct cytotoxic effects on cells. Marked cytotoxic effects are observed when cells are treated with TNF-α and IFN-γ (Benyo and Pate, 1992). These cytokines enhance expression of receptors for the other cytokines, and this could explain why both are necessary to achieve a cytotoxic effect on luteal cells (Pate, 1995).

Activation of Immune Cells During Luteolysis. Recognition of MHC-associated antigenic peptide and exposure to sufficient concentrations of appropriate cytokines activates T lymphocytes. Nearly all nucleated cells express MHC class I glycoproteins, which are recognized by T cytotoxic lymphocytes. The MHC class II molecules are recognized by T helper

lymphocytes, which secrete cytokines that activate cytotoxic T lymphocytes. The MHC class II molecules are normally expressed only on antigen-presenting cells, such as macrophages and B lymphocytes. However, aberrant expression of MHC class II molecules can occur in pathogenesis and may be a cause of certain autoimmune endocrine disorders. It has been postulated that alteration in expression of MHC glycoproteins on the luteal cell surface may stimulate immune involvement in luteolysis. Cultured bovine luteal cells express MHC class I molecules and very low concentrations of MHC class II molecules. Treatment with TNF-α or IFN-γ for 72 h results in an increase in MHC class I glycoproteins (Fairchild and Pate, 1991; Benyo and Pate, 1992). Increased class I expression could promote cytotoxic T cells against luteal cells. Of greater interest is the profound induction of MHC class II molecules on luteal cells by IFN-γ (Fairchild and Pate, 1989; Petroff et al., 1997). It is presumed that expression of MHC class II glycoproteins on the surface of luteal cells confers the function of antigen presentation to these cells (Pate, 1995). Luteal cells may present autoantigen to T lymphocytes, and this could result in an immune response against the CL.

Sensitivity to Exogenous $PGF_{2\alpha}$ in Sheep

Exogenous $PGF_{2\alpha}$ is luteolytic in midluteal phase sheep. In fact, administration of exogenous $PGF_{2\alpha}$ and $PGF_{2\alpha}$ anologues has been used to help determine the role of uterine $PGF_{2\alpha}$ in luteolysis (Wade and Lewis, 1996). Unlike in pigs, sheep CL are sensitive to administration of $PGF_{2\alpha}$ as soon as the CL becomes active (Inskeep et al., 1975). Single and multiple doses of $PGF_{2\alpha}$ induce luteolysis, although multiple doses are more effective. Intrauterine, interarterial, and uterine vein infusions of $PGF_{2\alpha}$ are more efficient than i.m. administration (Douglas and Ginther, 1973; McCracken et al., 1970). Exogenous $PGF_{2\alpha}$-induced luteolysis in sheep is dose dependent. Corpora lutea from ewes given 6 mg of $PGF_{2\alpha}$ i.m. on d 8

of the estrous cycle weighed significantly less than those from ewes treated with 2 mg of $PGF_{2\alpha}$ (Douglas and Ginther, 1973).

Sensitivity to Exogenous $PGF_{2\alpha}$ in Pigs

Unlike in sheep, before d 12 of the estrous cycle, pig CL are resistant to $PGF_{2\alpha}$. The mechanism that prevents premature luteolysis in response to exogenous $PGF_{2\alpha}$ in pigs is unknown. Resarchers have attempted to induce premature luteolysis in pigs by administration of single or multiple doses of $PGF_{2\alpha}$ or $PGF_{2\alpha}$–analogues at various times during the estrous cycle (Diehl and Day, 1974; Guthrie and Polge, 1976; Halford et al., 1975; Lindloff et al., 1976; Estill et al., 1993). Depending on the protocol, these researchers met with little or no success. Administration of $PGF_{2\alpha}$ or a $PGF_{2\alpha}$-analogue before d 12 does not consistently disrupt luteal function. Disruptions in luteal function are measured by changes in progesterone concentrations, changes in the interval between the initiation of the next estrous cycle, and premature luteolysis. Although administration of exogenous $PGF_{2\alpha}$ does not induce luteolysis, there is evidence that exogenous $PGF_{2\alpha}$ can transiently affect luteal function, causing reductions in progesterone concentrations (Guthrie and Polge, 1976).

Neither a dose nor an administration method have been calibrated that consistently cause premature luteolysis in pigs before d 12 of the estrous cycle. The dose and frequency of administration may vary between sows and gilts and the initiation time of the treatment. Neither a single intrauterine inoculation on d 10 nor an i.m. dose of $PGF_{2\alpha}$ (2 or 5 mg) on d 12 disrupted luteal function or caused luteolysis (Diehl and Day, 1974). Gilts early in the estrous cycle (i.e., beginning on d 4) may be treated as often as four times (20 mg $PGF_{2\alpha}$ i.m.) at 12 h intervals without causing a change in luteal function (Hallford et al., 1975). However, gilts receiving the same dose of $PGF_{2\alpha}$ later in the cycle (i.e., beginning on d 9 or 10) had decreased progesterone

concentrations 8 h after the injections are administered. The change in progesterone concentration is transient; 96 h after injections are administered, serum progesterone concentrations return to normal and follow a pattern similar to the controls throughout the rest of the estrous cycle. Multiple injections of a $PGF_{2\alpha}$-analogue are more likely to be luteolytic than a single dose (Guthrie and Polge, 1976). Four injections of a synthetic $PGF_{2\alpha}$-analogue administered every 12 h beginning on d 10 are more likely to induce luteolysis than a single injection on d 10 (Guthrie and Polge, 1976). Following infusion of $PGF_{2\alpha}$ into the anterior uterine vein on d 6, 8, 10, 12, 14, and 15 of the estrous cycle, there is a reduction in serum progesterone concentration. At each stage of the cycle, $PGF_{2\alpha}$ suppressed luteal function, although the decrease in progesterone secretion was greater when the infusion was performed on d 12, 14, and 15 of the estrous cycle than on d 6, 8, and 10. In gilts, premature luteolysis and concomitant shortening of the estrous cycle can be achieved by administering multiple injections of $PGF_{2\alpha}$ before d 12 (Estill et al., 1993). Because of the relative refractoriness of pig CL to the luteolytic effects of exogenous $PGF_{2\alpha}$ during the first 12 d of the cycle, multiple injections are required to consistently causes luteolysis.

Small numbers of specific high-affinity $PGF_{2\alpha}$ receptors have been detected on large luteal cells before d 12 (Gadsby et al., 1990). The number of high affinity binding sites increased dramatically on d 13 (Gadsby et al., 1990), coinciding with the apparent onset of enhanced sensitivity to the luteolytic effects of $PGF_{2\alpha}$ between d 12 and 13.

The possibility exists that premature luteolysis in response to repeated exogenous $PGF_{2\alpha}$ injections administered during the early luteal phase is mediated through specific $PGF_{2\alpha}$ receptors (Gadsby et al., 1990; Balapure et al., 1989) and subsequent activation of postreceptor mediators. There are three possible explanations. Multiple doses of $PGF_{2\alpha}$ stimulate an increase

in $PGF_{2\alpha}$ receptors. Prostaglandin $F_{2\alpha}$ causes an accumulation of cellular mediators such as protein kinase C or calcium within the luteal cells that sensitize the corpus CL to $PGF_{2\alpha}$ (Wiltbank et al., 1990). Repeated administration of $PGF_{2\alpha}$ results in an increase of the ratio of luteolytic $PGF_{2\alpha}$ to the luteotropic PGE_2 decreasing luteal steroidogenesis and shortening the cycle. Administration of exogenous $PGF_{2\alpha}$ could further alter this ratio by increasing the endogenous $PGF_{2\alpha}$ release.

Summary

Impaired or down-regulated immune function before and(or) after parturition increases the susceptibility of livestock to uterine infections. When systemic or local immune function is suppressed, benign bacteria such as the nonpathogenic organisms associated with “normal” uterine involution become established in the uterus, proliferate, and cause uterine infections. Changes in the immune function are mediated by changes in leukocyte function and population. Impaired or down-regulated leukocyte function increases the susceptibility to uterine infections. Prostaglandins and other eicosanoids regulate many leukocyte functions, in particular lymphocyte and neutrophil functions.

The experiments described throughout this dissertation were conducted with sheep and pigs as models to study the interactions between exogenous and endogenous prostaglandins on the uterine immune response to bacterial infection. Because the ruminant CL is responsive to Lutalyse it is difficult to use a ruminant model to distinguish between the effects of luteolysis and changes in prostaglandin concentrations on uterine infections. Therefore, pigs allow us to further separate out the general and independent actions of steroids and prostaglandins on the clearance of uterine infections. This dissertation and the associated experiments were designed to

study the in vivo and in vitro effects of $PGF_{2\alpha}$ on the uterine immune system and, consequently, the clearance of uterine infections in sheep and pigs.

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Chapter IV

PROGESTERONE DOMINATED UTERINE RESPONSES TO INFECTIOUS BACTERIA IN GILTS

ABSTRACT: A progestogenated uterus is susceptible to infections, but this is not well characterized in gilts. Therefore, the effects of day of the estrous cycle and progesterone were evaluated. Gilts (n = 5/group) were assigned to treatments in 2 x 2 factorial arrays. In Exp. 1, day of estrous cycle and bacterial challenge were main effects. On d 0 or 8, uteri were inoculated with either 70 x 10^7 cfu of *E. coli* and 150 x 10^7 cfu of *A. pyogenes* in PBS (10 mL) or PBS. In Exp. 2, OVEX and progesterone supplementation were main effects. On d 0, gilts were OVEX, or a sham procedure was performed. After surgery, gilts received i.m. injections of progesterone (10 mg/5 mL) or 5 mL of safflower oil diluent twice daily. On d 8, gilts were inoculated with the same doses of bacteria as in Exp. 1. In Exp. 1 and 2, vena caval blood was collected for 4 d for progesterone, estradiol-17β, $PGF_{2\alpha}$, and PGE_2, gilts were killed, and uteri were collected. Sediment and ability to culture *E. coli* and *A. pyogenes* from uterine flushings were used to diagnose infections. Differential white blood cell counts and lymphocyte blastogenic response to mitogens (i.e., Con A and LPS) were used to evaluate immune function. In Exp. 1, d-8 gilts receiving bacteria developed infections (PCV > 5%), but d-0 gilts receiving bacteria did not (PCV < 1%). Daily percentages of neutrophils and lymphocytes changed ($P < .01$) in response to day of estrous cycle and bacterial challenge. Unstimulated and Con A-stimulated blastogenesis were greater ($P < .01$) for d-0 than for d-8 gilts. In Exp. 2, gilts exposed to progesterone (i.e., ovarian or injected) had infections (PCV > 5%). Daily percentages of neutrophils and lymphocytes changed ($P < .01$) in response to OVEX, and neutrophils changed ($P < .05$) in response to progesterone. The Con A- and LPS-stimulated blastogenesis increased in response to OVEX ($P < .05$) and decreased in response to progesterone ($P < .01$). Prostaglandin $F_{2\alpha}$

concentrations increased ($P < .01$) in response to bacterial challenge independently of the stage of the cycle or progesterone status. Prostaglandin E_2 concentrations decreased ($P < .01$) in response to bacterial challenge in gilts. Day of the estrous cycle and progesterone supplementation changed the uterine immune response to infectious bacteria in gilts.

Introduction

The major determinant of lifetime productivity in female livestock is reproductive performance (Lewis, 1997). Uterine infections decrease reproductive performance resulting in increased culling and herd health costs. The incidence of uterine infections in pigs is unknown. However, severe metritis is recognized as a sign of the lactation disorder now known as PHS and formerly known as MMA. The number of sows or gilts affected during a farrowing is highly dependent on the environment and the management system. From 10 to 100% of gilts or sows are affected during a farrowing (Martineau et al., 1999). Impaired or down-regulated neutrophil and lymphocyte function increases the susceptibility of cows and sheep to uterine infections, and bacteria that are found commonly in the environment can then become established in the uterus (Lewis, 1997; Ramadan et al., 1997). This has not been evaluated in pigs.

In cows, sheep, and rabbits, the resistance to uterine infections is greatest at estrus (Black et al., 1953a,b; Rowson et al., 1953; Ramadan et al., 1997). It has been speculated that in pigs some bacteria are able to survive this phase and multiply, leading to infections during the luteal phase that seem to be associated with increased embryonic death (Scofield et al., 1974).

This study was conducted with gilts to evaluate 1) the immune response to bacterial challenge during the follicular and luteal phase of the estrous cycle, 2) the ability of $PGF_{2\alpha}$ to modulate in vivo primed lymphocytes under in vitro conditions, and 3) the value of pigs for detailed studies of uterine infections and prostaglandin interactions.

Materials and Methods

General. Gilts that were 9 to 12 mo of age, produced at the Virginia Tech Swine Center, and had had at least two consecutive estrous cycles of 19 to 22 d in duration were used for this study. All of the gilts were healthy and had no history of uterine infections. Gilts were penned twice daily with a boar. Gilts standing firmly to be mounted were considered to be in estrus.

Estrus Synchronization. Fourteen days after gilts were detected in estrus, they began receiving 15 mg of altrenogest once daily for 10 d. Altrenogest was mixed into the feed. Gilts were housed in stalls and were individually fed. After removal of altrenogest from the diets, gilts were penned twice daily with boars to check for estrus after synchronization treatment. Gilts that were not detected in estrus within 6 d after removal of altrenogest were not assigned to the experiment.

Vena Caval Catheterization. The vena cava was catheterized 12 h before each gilt received intrauterine inoculations. For a full review of the procedure refer to Benoit and Daily (1991). Gilts were anesthetized using a combination of xylazine (2mg/mL of BW) and Telazol (3mg/kg). The right rear leg was anesthetized dorsal and lateral to the hock with 6 to 10 mL of 2% lidocaine hydrochloride. The skin was incised approximately 3 cm dorsal to the hock and 2 cm lateral to the Achilles tendon, the subcutaneous fat was separated, and the saphenous vein was exposed (Benoit and Daily, 1991). A 180-cm-long catheter (inner diameter, 0.42 mm; outer diameter, 0.74 mm; ICO-Rally, Palo Alto, CA) was inserted into the vein through a small incision and then passed up through the saphenous vein into the caudal vena cava. The catheters were marked 50, 55, 60, and 65 cm from the internal end. The external end of the catheter was positioned within the subcutaneous fat of the hind leg, the rump, and the back. A trocar and cannula were used to “tunnel” under the skin. The external end of the catheter was ultimately

placed in a Velcro pocket glued to the center of the back to ensure that the gilts would not pull out the catheter. One 4-mL blood sample was taken at each marked position on the catheter as it was passed up the vein, and then the catheter was positioned temporarily. The final catheter position was determined according to progesterone or estradiol-17β concentrations depending on which day of the estrous cycle that the surgery was performed; the greatest progesterone or estradiol-17β concentration indicated the region where the uteroovarian blood entered the vena cava. The catheters were placed at that position.

Intrauterine Inoculations. For a full review of the laparoscopic procedure refer to Ramadan et al. (1997) or Wulster-Radcliffe et al. (1999). Gilts were anesthetized with the same combination of drugs as was used for inserting vena caval catheters. Briefly, gilts were placed at a 45° angle in the dorsal recumbent position. A 2-cm incision was made in between the first and second most dorsal mammary glands on each side of the gilt. The subcutaneous fat was blunt dissected away from the body wall. After visualizing the body wall, a trocar and cannula were stabbed through the body wall in each of the incisions. The abdominal cavity was insulflated with CO_2. The trocars were removed and replaced with a laparoscope and a probe. The uterus was visualized with a laparoscope and positioned with both uterine horns in view. The probe used for positioning the uterus was removed and replaced with a 17 g aspiration needle. The needle was plunged into the uterine horn. A 20 mL syringe containing either PBS or bacterial treatments was attached to the end of the aspiration needle. A second person slowly depressed the plunger of the syringe until the syringe was empty. The needle and syringe were removed.

Inoculations. The strains of *A. pyogenes* and *E. coli* used for this study were isolated from a cow with endometritis at the Virginia Tech Dairy Center. Both strains of bacteria were purified and stored in skim-milk broth medium at -20° until they were used to prepare intrauterine

inoculations. Inoculations prepared with bacteria produced endometritis in cows (Del Vecchio et al., 1992) and sheep (Ramadan et al.,1997).

The bacteria were cultured in brain-heart infusion broth (Difco, Detroit, MI) to obtain enough colony-forming units to prepare inoculations. The optical density of the cultures was measured periodically at 560 nm to determine bacterial growth. The optical density was used in a regression equation, which was derived from optical densities of cultures and direct counts of the number of colony forming units on blood agar plates after a sample of the cultures had been incubated for 24 h to estimate the number of colony forming units per 10 μL of medium. To prepare inoculations, culture medium was transferred into sterile culture tubes and centrifuged at 10 x g for 20 min at 4°C. The supernatant, which can act as a chemoattractant, was removed, and the pellet was resuspended in 10 mL of sterile isotonic saline. The inoculations were held on ice until they were used. Five mL of the suspension was injected into each uterine horn. On the basis of a dose response curve, each inoculation contained 150 x 10^7 cfu of *A. pyogenes* and 70 x 10^7 cfu of *E. coli.*

Lymphocyte Separation and Blastogenic Assay. Twelve mL of heparinized blood was centrifuged at 2,800 x g for 20 min at 4°C. The buffy coat was mixed with 4 mL of HBSS at pH 7.4 (Gibco BRL, Grand Island, NY). The mixture was layered on top of 4 mL of Ficoll-Paque (Sigma Chemical, St. Louis, MO) and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte-containing portion was transferred to sterile culture tubes. The suspension was centrifuged 1,000 x g for 10 in at room temperature. The supernatant was removed, and the lymphocytes were washed twice with HBSS. The cells were then suspended in complete RPMI-1640 medium (Gibco) that contained fetal bovine serum (.1 mL/mL; Gibco), penicillin (100 IU/mL; Sigma), and streptomycin (100 mg/mL; Sigma). The number of live lymphocytes was

determined using a hemocytometer and a trypan blue dye exclusion procedure. The final concentration of live cells was adjusted to 1 x 10^6/mL of RPMI-1640.

The blastogenic assay was similar to the one described in Burrells and Wells (1977). Lymphocytes (1 x 10^5 live cells) from each vena caval sample were cultured in 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ). Mitogenesis was stimulated with Con A (stimulates T-lymphocytes; 1 μg/well; Sigma) or LPS (stimulates B lymphocytes; .5 μg/well; Sigma), or other cells were left unstimulated (100 μL o RPMI-1640/well) so that unstimulated mitogenesis could be estimated. Incubation treatments were in triplicate.

The plates were held at 37° for 48 h in a humidified chamber with an atmosphere of 5% CO_2 in air. [^{3}H]Thymidine (1 μCi; specific radioactivity 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in RPMI-1640 was added to each well, and the plates were maintained under the same conditions for another 16 h. At the end of culture, lymphocyte viability was determined with a trypan blue dye-exclusion procedure, and cells were transferred to fiberglass filters (Whatman, Madistone, England). Filter discs corresponding to each well were transferred to separate scintillation vials, and lymphocytes were solubilized to release [^{3}H]thymidine. Disintegrations per minute were determined with a liquid scintillation counter and used to calculate the pmol of [^{3}H]thymidine incorporated into newly synthesized DNA.

Immunoassays. Radioimmunoassays were used to measure progesterone, estradiol-17β, and PGE_2. For catheter positioning and measurement of sample, a [^{125}I]progesterone and [^{125}I]estradiol-17β RIA kits (Diagnostic Products, Los Angeles, CA) were used. All samples for progesterone and estradiol 17-β collected after final catheter positioning were evaluated for both studies in a single progesterone and single estradiol-17β assay with a CV of 8.6 and 9.7%, respectively. The concentration of PGE_2 was determined using tritated tracer (Lewis et al.,

1978). The PGE_2 intra- and interassay CV for Exp. 1 were 11.2 and 23.1% respectively. The PGE_2 intra- and interassay CV for Exp. 2 were 9.7 and 21.4% respectively.

The concentrations of $PGF_{2\alpha}$ were determined with an EIA (Del Vecchio et al., 1992; Fortin et al., 1994). The intraassay and interassay CV for $PGF_{2\alpha}$ for Exp. 1 were 7.8 and 24.2%, respectively. The intraassay and interassay CV for $PGF_{2\alpha}$ for Exp. 2 were 5.9 and 26.2%, respectively.

Slaughter. All gilts were killed at the end of the experiment. Gilts were anesthetized with sodium pentobarbital (65 mg/mL) and then electrocuted using a 220 V electrocution set. Immediately after slaughter, the reproductive tracts were collected and examined for signs of infection. Clear uterine flushings with small amounts of sediment (PCV), no signs of endometrial inflammation, and the inability to culture *A. pyogenes* and *E.coli* collectively indicated that the uterus was not infected. Cloudy or colored uterine flushings with large amounts of sediment (PCV), inflamed endometrium, and the ability to culture *A. pyogenes* and *E.coli* collectively indicated that the uterus was infected. Ovaries were also observed to confirm stage of the estrous cycle.

*Experiment 1**(Chapter 2; Question 5)**: Does the uterine immune response to infectious bacteria differ during the follicular and luteal phases of the estrous cycle in gilts?*

Experimental Design. In Exp. 1, the effects of stage of the estrous cycle on the immune response to intrauterine inoculation with *A. pyogenes* and *E. coli* were evaluated. Day of estrous cycle and bacterial challenge were main effects. Gilts (n=5/treatment) were assigned to one of four randomized treatments: 1) estrus-PBS, 2) estrus-bacteria, 3) luteal-PBS, and 4) luteal-bacteria.

Estrus was synchronized. Vena caval catheters were inserted either within 2 h after finding the gilts in heat or on d 7. Twelve hours after inserting vena caval catheters, the uteri were infused with either saline or bacteria. More specifically, on d 0 or 8, uteri were inoculated with either 70 x 10^7 cfu of E. coli and 150 x 10^7 cfu of A. pyogenes in PBS (10 mL) or PBS. Four d after inoculations gilts were killed, the ovaries were observed, and the uterus was harvested and flushed with 20 mL of saline to determine PCV of the total flushing and bacterial regrowth.

Blood Sampling. Vena caval blood was collected twice daily for 4 d. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12-mL sample was taken from the catheter placed cranial to the site where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple gilts, blood samples were kept in an ice-water bath until centrifuged for the collection of plasma and the buffy coat. Before centrifugation, a small portion of the heparinized sample was used for differential WBC counts. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for blastogenic assay. The lymphocyte blastogenic response to mitogens was measured. Plasma samples were assayed for steroid hormones (progesterone and estradiol-17β) and for prostaglandins ($PGF_{2\alpha}$ and PGE_2). Blood samples were collected twice daily approximately 12 h apart. A sample was taken before treatments were applied. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin et al., 1994).

Statistical Analysis. The General Linear Models procedures of the Statistical Analysis System (SAS, 1985) were used to analyze the data. The GLM model included the independent variables: stage of estrous cycle, bacteria, stage of estrous cycle x bacteria, gilt nested within

stage of estrous cycle and bacteria, time, stage of estrous cycle x time, bacteria x time, and stage of estrous cycle x bacteria x time. Gilt nested within stage of estrous cycle and bacteria was the main plot error term, and the residual was the subplot error term. The variance associated with the main plot error term was used to calculate overall standard errors (SEM) associated with main plot variables. When appropriate, the PDIFF (i.e., a method for comparing all possible least squares means) option in SAS was used to compare individual means.

The shapes of the response curves for unstimulated lymphocyte blastogenic activity were determined for gilts in each treatment group, and then unstimulated activity was used as a covariant for Con A- and LPS-stimulated lymphocytes. The incorporation data were reported after the adjustment for unstimulated activity rather than reporting a stimulation index. The reason for this is because a stimulation index represents a percentage change from unstimulated activity, and the index is based on the assumption that the relationship between unstimulated activity and stimulated incorporation is linear, which was not always true for the data from this experiment. The prediction option in SAS was used to generate a new set of data that contained the adjusted values for Con A and LPS. The adjusted values were analyzed with the original GLM model.

Experiment 2 ***(Chapter 2; Question 7)****: Does progesterone mediate the changes in uterine response to infectious bacteria during the estrous cycle in gilts?*

Experimental Design. In Exp. 2, the effects of exogenous and endogenous progesterone on immune response to intrauterine inoculation with *A. pyogenes* and *E. coli* were evaluated. Ovariectomy and progesterone supplementation were the main effects, all gilts received bacterial inoculations. Gilts (n=5/treatment) were assigned to one of four randomized treatments: 1) sham-oil, 2) sham-progesterone supplementation, 3) OVEX-oil, and 4) OVEX-progesterone supplementation.

Estrus was synchronized. On d 0, gilts were OVEX or a sham procedure was performed. After surgery, gilts received i.m. injections of progesterone (10 mg/mL) or 5 mL of safflower oil diluent twice daily. On d 7, vena caval catheters were inserted. On d 8, gilts were inoculated with either 70 x 10^7 cfu of E. coli and 150 x 10^7 cfu of A. pyogenes in PBS (10 mL) or PBS. Four d after inoculations gilts were killed, the ovaries were observed, and the uterus was harvested and flushed to determine PCV and bacterial regrowth.

Blood Sampling. Vena caval blood was collected twice daily for 4 d. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12-mL sample was taken from the catheter placed caudal to the site where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple gilts, blood samples were kept in an ice-water bath until they were centrifuged for the collection of plasma and the buffy coat. Before centrifugation, a small portion of the heparinized sample was used for differential WBC counts. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for blastogenic assay. The lymphocyte blastogenic response to mitogens was measured. Plasma samples assayed for steroid hormones (progesterone and estradiol-17β) and for prostaglandins ($PGF_{2\alpha}$ and PGE_2). Blood samples were collected twice daily approximately 12 h apart. A sinle sample was taken before administration of treatments. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin et al., 1994).

Ovariectomy. On d 0, gilts were either OVEX or a sham procedure was performed. Gilts were anesthetized using a combination of xylazine (2mg/mL of BW) and Telazol (3mg/kg). Gilts were placed on surgery tables in a dorsal recumbent position. A midventral laparotomy was performed. The sham procedure included several minutes of massage of the reproductive tract to

stimulate the effects of surgery. For OVEX, the ovaries were removed, but the uterus was left intact.

Statistical Analysis. The General Linear Models procedures of the Statistical Analysis System (SAS, 1985) were used to analyze the data. The GLM model included the dependent variables: OVEX, progesterone, OVEX x progesterone, gilt nested within OVEX and progesterone, time, OVEX x time, progesterone x time, and OVEX x progesterone x time. Gilt nested within OVEX and progesterone was the main plot error term, and the residual was the subplot error term. The variance associated with the main plot error term was used to calculate overall standard errors (SEM) associated with main plot variables. When appropriate, the PDIFF (i.e., a method for comparing all possible least squares means) option in SAS was used to compare individual means.

The shapes of the response curves for unstimulated lymphocyte blastogenic activity were determined for gilts in each treatment group, and then unstimulated activity was used as a covariant for Con A and LPS stimulated lymphocytes. The incorporation data were reported after the adjustment for unstimulated activity rather than reporting a stimulation index. The prediction option in SAS was used to generate a new set of data that contained the adjusted values for Con A and LPS. The adjusted values were analyzed with the original GLM model.

Statistical Analysis and Adjustments: An Explanation. Blastogenic data is analyzed using the method described above throughout this dissertation. In each experiment, the shapes of the response curves for unstimulated lymphocyte blastogenic activity were determined in each treatment group, and then the unstimulated activity was used as a covariant for Con A- and LPS-stimulated lymphocytes. The incorporation data were reported after this adjustment. These adjustments were made for both statistical and physiological reasons. Statistically, data of this

sort is reported as a stimulation index; however, this would be incorrect in these types of data sets. A stimulation index infers a linear relationship and is usually used when a sample is being taken at a single time. It is inaccurate to assume that a linear relationship is maintained over time. Particularly in a physiological system that is influenced by the hormonal environment which changes over time. Before adjustments were made the unstimulated data was analyzed to determine if it was significant covariant and if it changed over time. By analyzing the shape of the response curve we were more accurately able to determine the influence of basal incorporation on any given day in response to a particular hormonal environment. In the final models, the dependent variable = the independent variable*adjustement + the error term. Although not exactly the same concept, this is similar to the use of initial body weight in the analysis of growth in many nutrition experiments. This description can be applied throughout this dissertation. Therefore, it will not be repeated in every chapter.

Results

Experiment 1.

Uterine Infections. All of the gilts inoculated with *A. pyogenes* and *E.coli* on d 8 of the estrous cycle developed uterine infections, but none of the gilts inoculated with bacteria on d 0 or with saline developed infections (Figure 1). Uterine infections were declared if uterine flushings contained greater than 5 % sediment at slaughter and if we were able to culture *A. pyogenes* and *E. coli* from the uterine flushings (Figure 1).

Lymphocyte Blastogenic Activity. Unstimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was greater ($P < .01$) for gilts inoculated with bacteria or saline on d 0 rather than on d 7 (Figure 2, Panel A). The stage of the estrous cycle x bacteria, the bacteria x time, and the stage of the estrous cycle x time interactions were significant ($P < .01$; Figure 2,

Panel A). The Con A-stimulated incorporation of [^{3}H]thymidine was approximately 2.4 pmol throughout the sampling period for gilts inoculated with either bacteria or saline on d 7 (Figure 2, Panel B). The time following inoculation, the bacteria x time, and the stage of the estrous cycle x time interaction were significant ($P < .01$; Figure 2, Panel B). The stage of the estrous cycle, and bacterial inoculation were significant for [^{3}H]thymidine incorporation into LPS-stimulated lymphocytes. Incorporation was greater for gilts inoculated on d 0 ($P < .01$; Figure 2, Panel B)

Differential White Blood Cell Counts. The main effects of stage of the cycle and bacteria were significant for neutrophils and lymphocytes ($P < .01$, Figure 3). The numbers of lymphocytes per 100 WBC in gilts inoculated on d 0 increased, ($P < .01$, Figure 3) and the numbers of neutrophils decreased ($P < .01$, Figure 3). For neutrophils and lymphocytes, the time after inoculation $P < .01$, Figure 3), stage of the estrous cycle x time, and bacteria x time interactions were significant ($P < .01$, Figure 3).

The numbers of eosinophils changed ($P < .01$) with time after inoculation, but changes were not striking and the numbers were close to their means (Figure 3). The numbers of monocytes did not change ($P > .1$, Figure 3).

Steroids and Prostaglandins. Stage of estrous cycle affected vena caval progesterone; concentrations were less ($P < .001$) in gilts inoculated on d 0 than in gilts inoculated on d 7 (6.3 vs 52 ng/mL, SEM = 3.4; Figure 4). The time following inoculation and the stage x time interaction were also significant for changes in progesterone concentration ($P < .01$; Figure 4). Stage of estrous also affected vena caval estradiol-17β; concentrations were greater ($P <.01$) in gilts inoculated on d 0 than in gilts inoculated on d 7 (39.8 vs 11.4 ng/mL, SEM = 3.6; Figure 5). The time following inoculation and the stage of estrous cycle x time following inoculation were also significant for changes in estradiol-17β concentration ($P < .01$; Figure 5).

Vena caval $PGF_{2\alpha}$ was greater ($P < .01$) in gilts inoculated with bacteria than in gilts inoculated with saline (.35 vs .16 ng/mL, SEM = .05), and $PGF_{2\alpha}$ concentrations changed with time after inoculation (Figure 7).

Vena caval PGE_2 concentrations differed ($P < .01$) between gilts inoculated with saline and those inoculated with versus bacteria (Figure 7). Concentrations of PGE_2 also changed with the stage of cycle ($P < .05$) and time with time after inoculation ($P < .01$; Figure 7).

Experiment 2.

Uterine Infections. All of the gilts inoculated with *A. pyogenes* and *E.coli* that were exposed to either endogenous or exogenous progesterone or a combination of the two developed uterine infections (Figure 8). Gilts that were not exposed to endogenous or exogenous progesterone did not develop infections (Figure 8). Uterine infections were diagnosed in the same manner as for Exp. 1.

Lymphocyte Blastogenic Activity. Unstimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes decreased ($P < .01$) in response to progesterone (.09 pmol vs .05 pmol; Figure 9, Panel A) and increased in response to OVEX (.06 vs .09 pmol; Figure 9, Panel A). The progesterone x time after inoculation and the OVEX x time after inoculation interactions were significant for unstimulated incorporation of [^{3}H]thymidine ($P < .01$; Figure 9, Panel A). The main effects of OVEX and progesterone were significant for Con A-stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes ($P < .01$; Figure 9, Panel B). The progesterone x OVEX interaction was also significant for Con A-stimulated lymphocytes ($P < .01$; Figure 9, Panel B). The main effects of OVEX and P4 were also significant for [^{3}H]thymidine incorporation into LPS-stimulated lymphocytes ($P < .01$; Figure 9, Panel C). For

LPS-stimulated [^{3}H]thymidine incorporation, the OVEX x time after inoculation and the progesterone x time after inoculation was also significant ($P < .01$; Figure 9, Panel C).

Differential White Blood Cell Counts. The main effects of OVEX and progesterone were significant for neutrophils. The numbers of neutrophils per 100 WBC were greater ($P < .01$) in gilts that were not OVEX than gilts that were (72 vs 58; Figure 10). Progesterone supplementation resulted in a greater ($P < .05$) number of neutrophils per 100 WBC than did oil treatment (63 vs 68; Figure 10). For neutrophils, the time after inoculation, OVEX x time after inoculation, and progesterone x time after inoculation were significant ($P < .01$; Figure 10). The main effect OVEX was significant for lymphocytes ($P < .01$; Figure 10). Gilts that were OVEX had greater ($P < .01$) numbers than gilts that were not (19.8 vs. 31.1; Figure 10). Time after inoculation and the time after inoculation x OVEX interactions were significant ($P < .01$) for lymphocytes. Monocytes and eosinophils changed with time after inoculation ($P < .01$; Figure 10).

Steroids and Prostaglandins. Ovariectomy and progesterone supplementation affected vena caval progesterone; concentrations were decreased in gilts that were OVEX and increased in gilts that received progesterone supplemetation ($P < .001$; Figure 11). Concentrations of estradiol-17β were decreased ($P <.01$; Figure 12) in gilts that were OVEX ($P < .01$; Figure 12). Vena caval $PGF_{2\alpha}$ concentrations changed with time after inoculation (Figure 13).

Discussion

The general relationship among immune function, hormone environment, and uterine infections has been described for cattle, but these relationships have not been adequately described for other species. In particular, there is practically no data available describing these relationships in pigs. In addition, the biochemical mechanisms of action have not been fully

elucidated in any livestock species, primarily because of the expense and difficulty of developing repeatable experimental models (Black et al., 1953; Rowson et al., 1953; Lander Chacin et al., 1990). To address this void in our knowledge of uterine infections, we evaluated the effects of stage of the estrous cycle and progesterone dominance on the uterine immune response in pigs.

The results of this study indicate that vena caval lymphocytes from gilts inoculated with either bacteria or saline on d 0 of the estrous cycle had greater capacities for unstimulated and for Con A- and LPS-stimulated incorporation of [^{3}H]thymidine than did lymphocytes from gilts inoculated on d 7. Also, vena caval blood from gilts inoculated on d 0 contained more lymphocytes, fewer neutrophils, greater concentrations of estrogens, and lesser concentrations of progesterone than did vena caval blood from gilts inoculated on d 7. The differences in immune function and hormonal environment between follicular and luteal phase gilts probably explains how the uterus in all gilts inoculated with bacteria on d 0 was able to prevent the development of infections.

In cows, sheep, and rabbits, the resistance to uterine infections is greatest at estrus (Black et al., 1953a,b; Rowson et al., 1953; Ramadan et al., 1997) than during the luteal phase of the estrous cycle. This also seems to be true in pigs. However, it has been speculated that in pigs some bacteria are able to survive the follicular phase and multiply, leading to infections during the luteal phase associated with increased embryonal death (Scofield et al., 1974). Neither Exp. 1 or 2 were designed to test this hypothesis; however, our inability to regrow bacteria at the end of the follicular phase suggests that the strains of bacteria used in this set of experiments does not remain viable in the uterus through the luteal phase.

In the present study, immune function seemed to be up-regulated during the follicular phase or in the absence of progesterone. Several previous studies indicated that uterine immune

function is enhanced during the follicular phase of gilts, cattle, and sheep and estrogen treatment enhanced uterine immune function in ovariectomized gilts, mares, and cows (Hawk et al., 1961; Brinsfield et al., 1963; Washburn et al., 1982; Roth and Kaelberle, 1981; Lander Chacin et al., 1990; Ramadan et al., 1997). Thus, the results from the present study are consistent and begin to characterize these changes in pigs.

Immune function was up-regulated when estrogens were increased and down-regulated when progesterone was decreased. This was demonstrated better in Exp. 2 than in Exp. 1. In Exp. 2, if gilts were not exposed to progesterone they were resistant to infections, whereas gilts that were exposed to exogenous or endogenous progesterone were susceptible. Because OVEX reduced the influence of other steroids and other ovarian factors, it is possible to relate the increased susceptibility to infection directly to the presence of progesterone.

The distribution of WBC in vena caval blood changed with stage of the estrous cycle and the presence or absence of progesterone. During periods that gilts were more susceptible to infections, there were greater numbers of neutrophils in circulation, and this may indicate a decrease in chemotaxis or the rate of migration. The decreased numbers of neutrophils in blood when estrogen was increased and progesterone was decreased is probably due to migration of neutrophils into the uterine lumen. The numbers of lymphocytes were decreased during progesterone dominance.

The numbers of vena caval lymphocytes increased in response to bacteria, and this was more dramatic during the estrous phase than the luteal phase. The increase in numbers of vena caval lymphocytes in response to bacterial challenge may reflect the movement of immunocompetant secondary lymphoid tissues into the blood.

Intrauterine inoculation with bacteria increased vena caval $PGF_{2\alpha}$ concentrations, regardless of the stage of the cycle or progesterone status. This increase in $PGF_{2\alpha}$ is consistent with increases reported in cows and sheep with uterine infections (Del Vecchio et al., 1992; Ramadan et al., 1997). We assume that the increase in $PGF_{2\alpha}$ is a result of the inflammation that is normally associated with infections (Tizard, 1996).

Intrauterine inoculations with bacteria decreased vena caval PGE_2. We have not previously detected this in sheep or cattle. This decrease may be associated with the severity of infections. All gilts that had infections had pyometra.

Based on the results of this study, changes in ovarian progesterone regulate uterine immune functions. In conjunction with those changes, uterine eicosanoids seem to be an important component of the uterine defense mechanism against bacteria. Thus, we believe that gilts are well suited for studying uterine infections in livestock.

Implications

Uterine infections are a major problem in cattle. However, the extent of the problem is unknown in other livestock species. It is assumed that, because of management conditions and syndromes with uterine infections as a component, uterine infections are a major problem in the swine industry. Swine provide an excellent model for studying the uterine immune response to infection. Changes in estrogens and progesterone help regulate immune cell function, and changes in immune cell function are related to the susceptibility to uterine infections. Infection-induced increases in uterine prostaglandin production may promote the movement of immune cells into the uterus. Treatments with $PGF_{2\alpha}$ may be useful for modulating uterine immune function and reducing the incidence of uterine infections in pigs.

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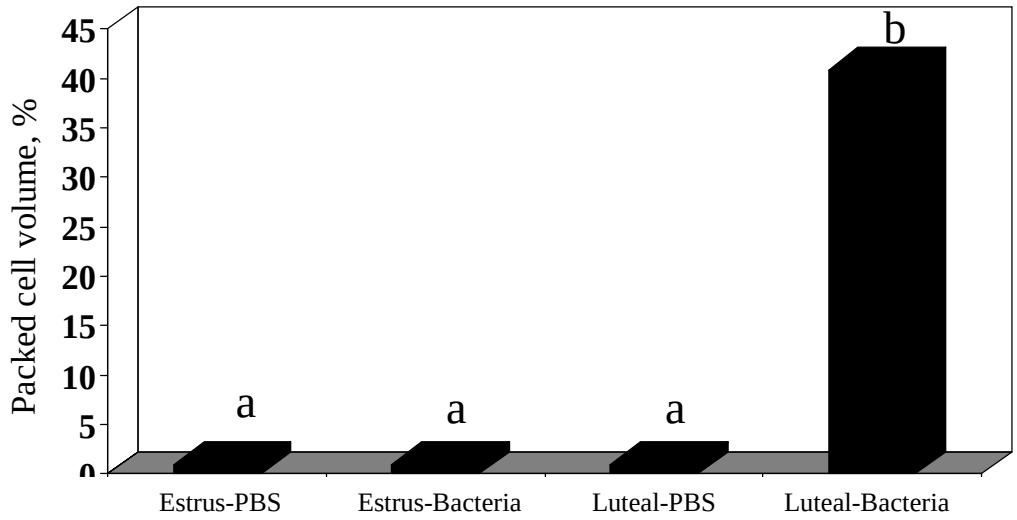

Figure 1. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 1. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts either received an intrauterine inoculation (PBS or Bacteria) on d 0 or 8 of the estrous cycle. [a,b]Different superscripts indicate that means differ ($P < .01$). There were five gilts/treatment and the SEM = 2.7.

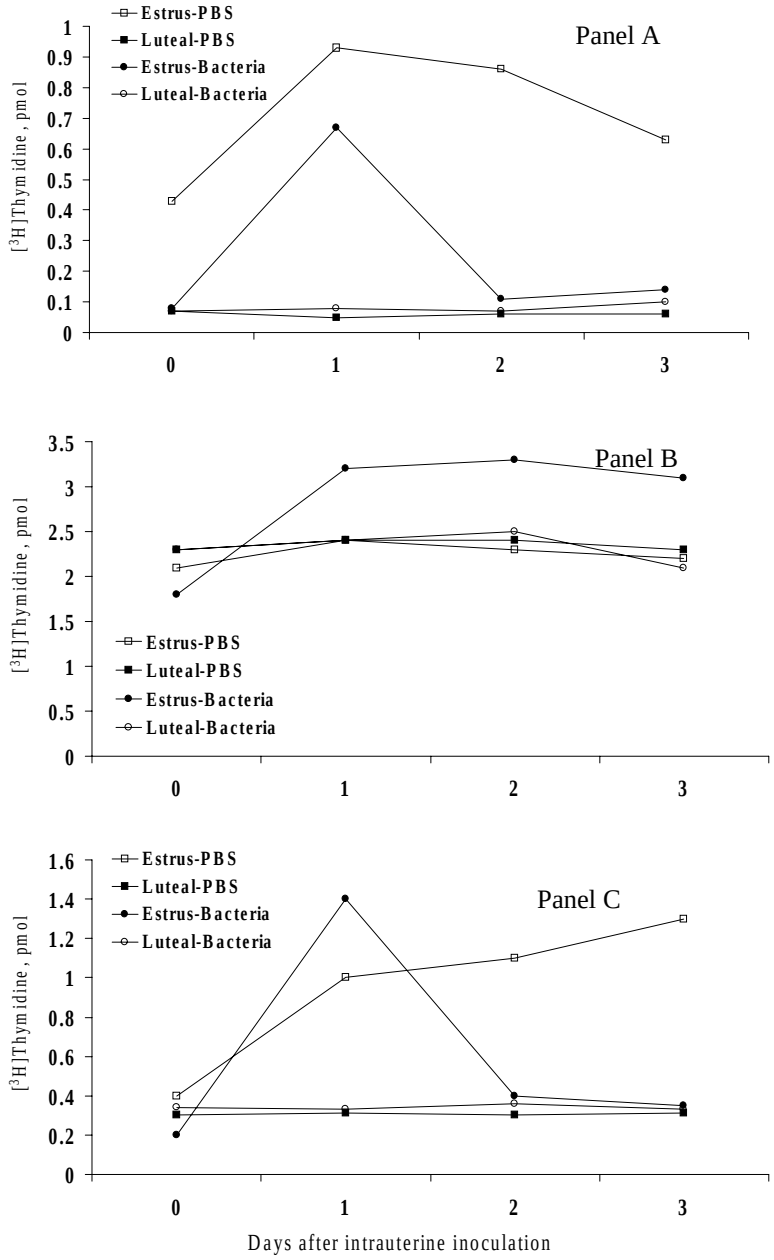

Figure 2. The effect of treatment on incorporation of [^{3}H]thymidine into newly formed lymphocytes in Exp. 1. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts received intrauterine inoculations (PBS or Bacteria) on d 0 or 8 of the estrous cycle. There were effects of bacteria and stage of estrous cycle ($P < .01$; SEM =. 04) on unstimulated incorporation of [^{3}H]thymidine (Panel A). There was a significant effect of time after inoculation, bacteria x time interaction, and stage of estrous cycle x time interaction ($P < .01$; SEM = .1) on Con A-stimulated incorporation of [^{3}H]thymidine (Panel B). There was a significant effect of bacteria, stage of estrous cycle, time and all the interactions ($P < .01$; SEM = .05) on LPS-stimulated incorporation of [^{3}H]thymidine (Panel C).

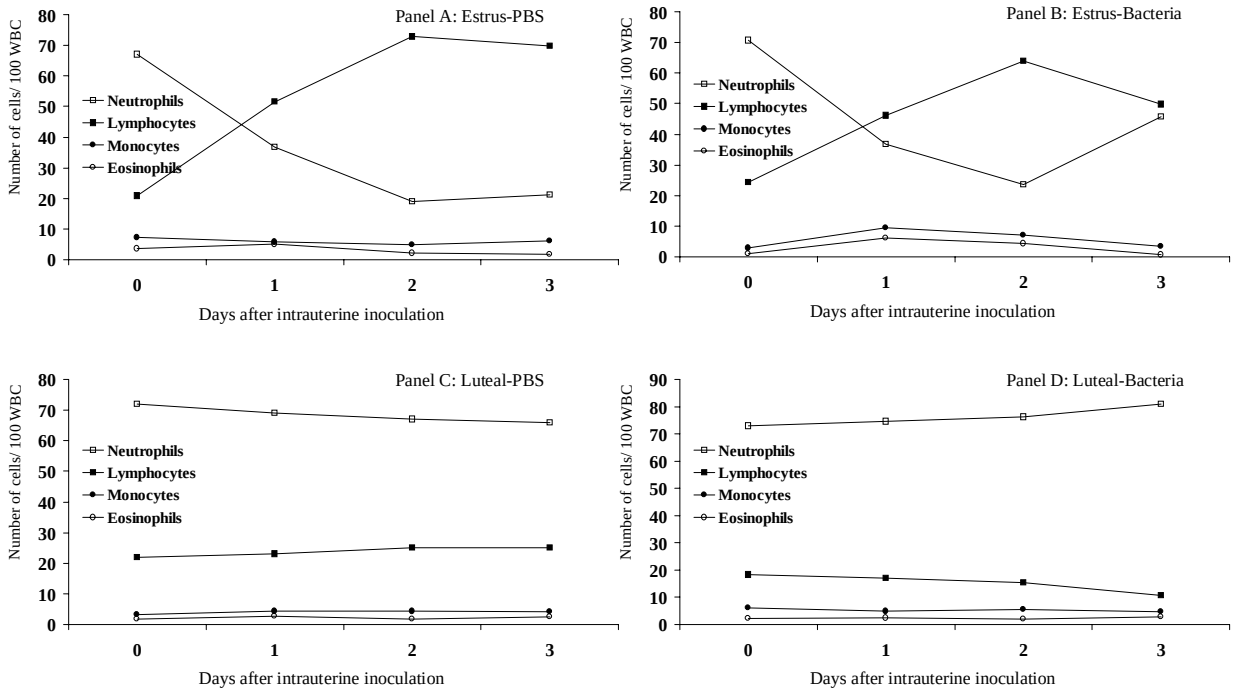

Figure 3. The effect of treatments on the average number of different types of WBC in Exp.1. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts received intrauterine inoculations (PBS or Bacteria) on d 0 or 8 of the estrous cycle. Different types of WBC (lymphocytes, neutrophils, eosinophils, and monocytes) were counted out of 100 WBC from a thin blood smear stained with Wright's stain. Treatments are defined in each panel. The stage of the cycle and bacterial inoculation affected neutrophil ($P < .01$) and lymphocyte ($P < .01$) numbers, and time after inoculation ($P < .01$) affected neutrophils and lymphocytes. Neutrophils (SEM = 3.5); Lymphocytes (SEM = 3.3); Monocytes (SEM = 1.1); Eosinophils (SEM = .95)

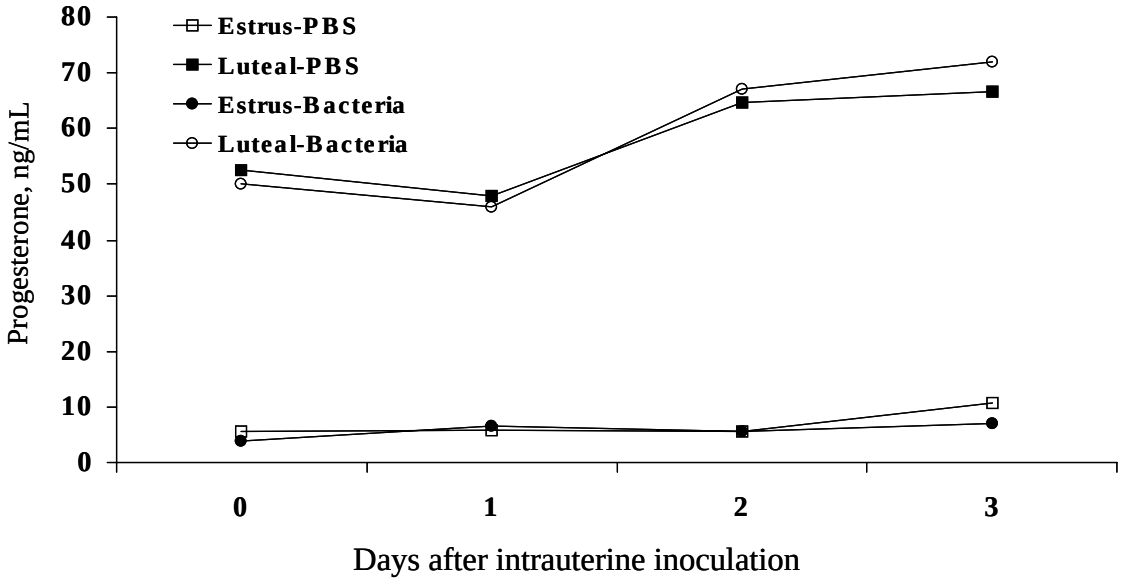

Figure 4. Concentrations of progesterone in venal caval blood collected from gilts given intrauterine inoculations of PBS or bacteria on d 0 or 8 of the estrous cycle. Concentrations of progesterone were greater ($P < .01$) in gilts during the luteal phase than at estrus, and progesterone concentration changed ($P < .01$; SEM =3.4) with time after inoculation.

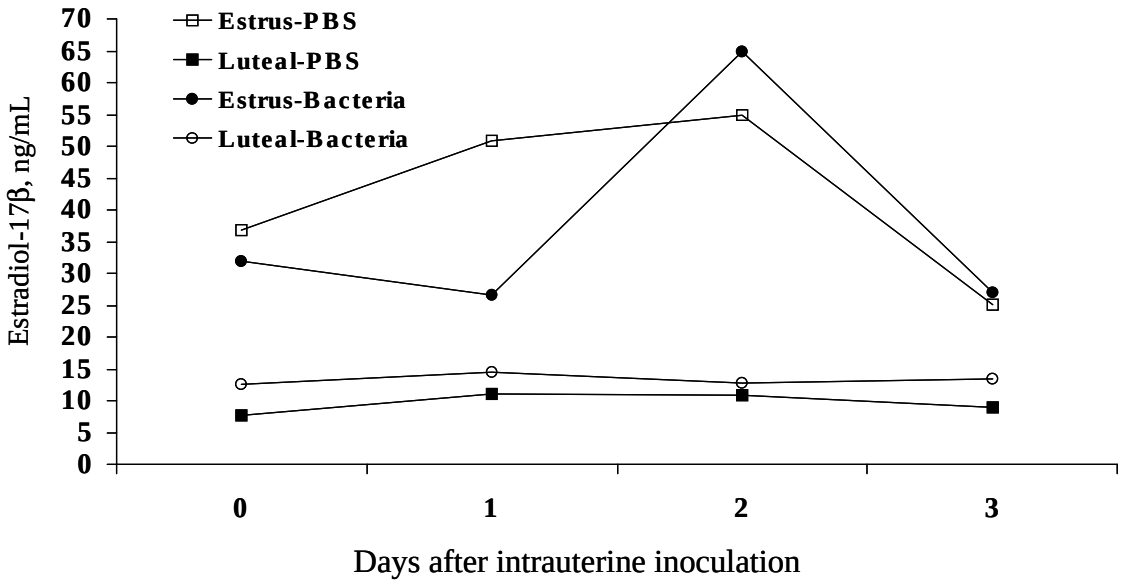

Figure 5. Concentrations of estradiol-17β in venal caval blood collected from gilts given intrauterine inoculations of PBS or bacteria on d 0 or 8 of the estrous cycle. Concentrations of estradiol-17β were greater ($P < .01$) in gilts during the estrous phase than during the luteal phase, and estradiol-17β concentration changed ($P < .01$; SEM =3.6) with time after inoculation.

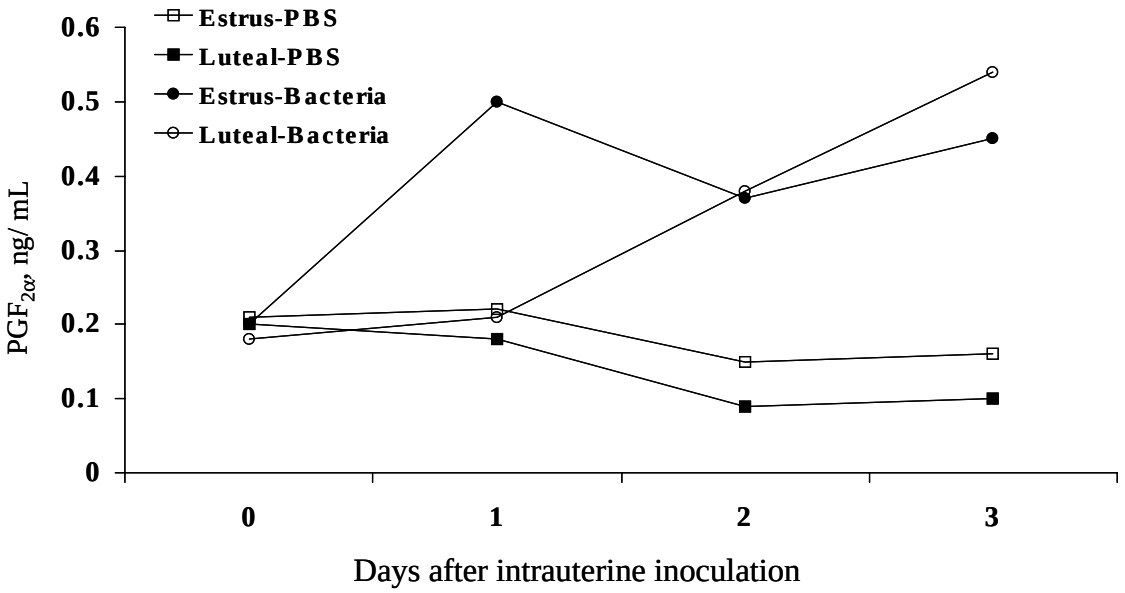

Figure 6. Concentrations of $PGF_{2\alpha}$ in venal caval blood collected from gilts given intrauterine inoculations of PBS or bacteria on d 0 or 8 of the estrous cycle. Concentrations of $PGF_{2\alpha}$ were greater ($P < .01$) in gilts inoculated with bacteria than in gilts inoculated with PBS, and $PGF_{2\alpha}$ concentrations changed ($P < .01$; SEM =.03) with time after inoculation.

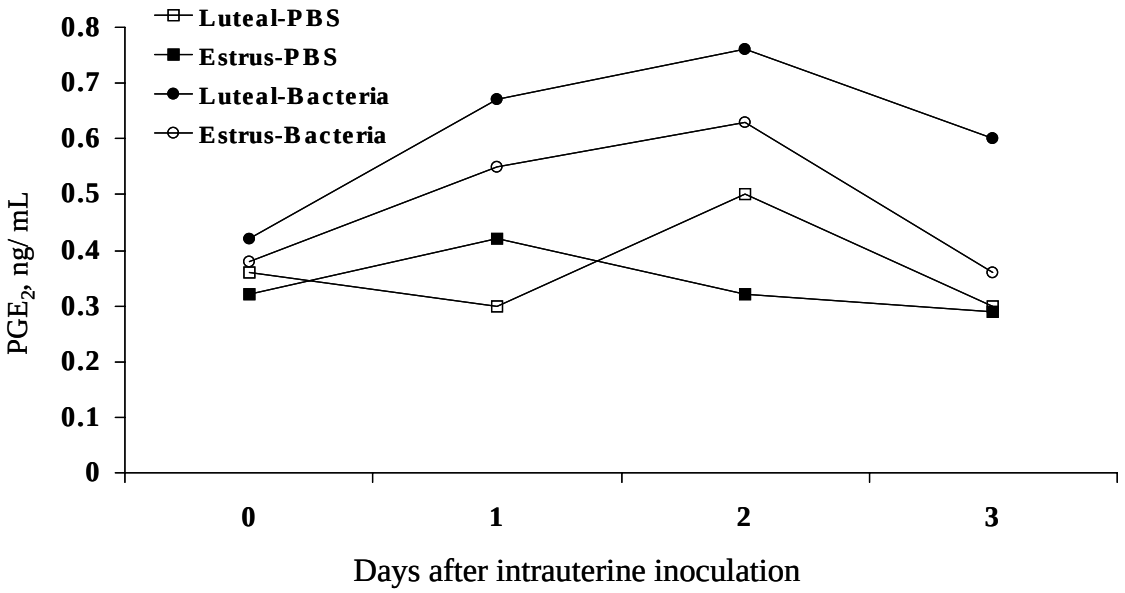

Figure 7. Concentrations of PGE_2 in venal caval blood collected from gilts given intrauterine inoculations of PBS or bacteria on d 0 or 8 of the estrous cycle. Concentrations of PGE_2 were higher ($P < .05$) in gilts inoculated with bacteria than in gilts inoculated with PBS, and PGE_2 concentrations were differed with stage of estrous cycle ($P < .01$) Concentrations of PGE_2 changed ($P < .01$; SEM =.05) with time after inoculation.

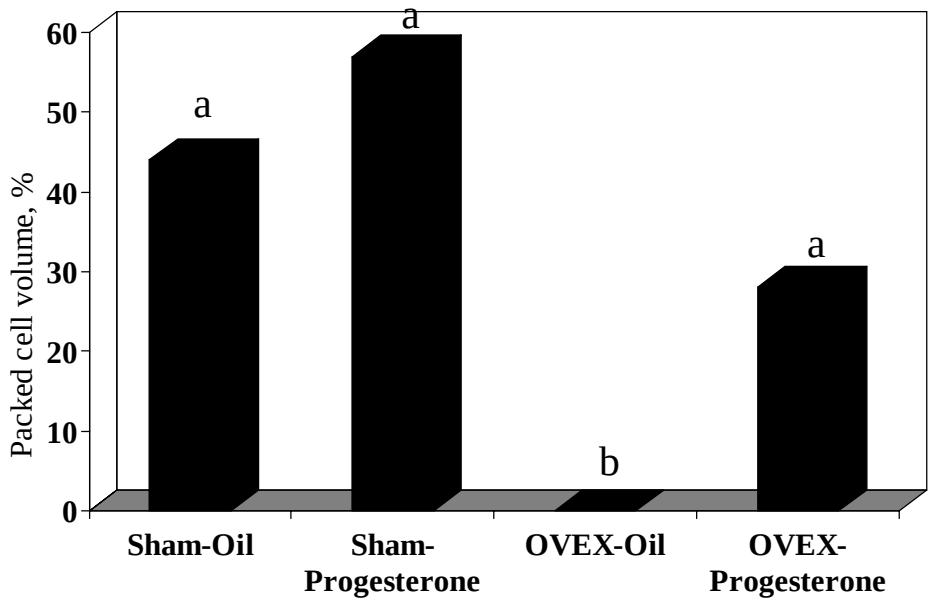

Figure 8. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 2. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts either received OVEX or a sham procedure was performed on d 0. Gilts either received progesterone supplementation or oil injections. All gilts were inoculated with bacteria on d 8 of the estrous cycle. [a,b]Different superscripts indicate that means differ ($P < .01$). There were five gilts/treatment and the SEM = 3.1.

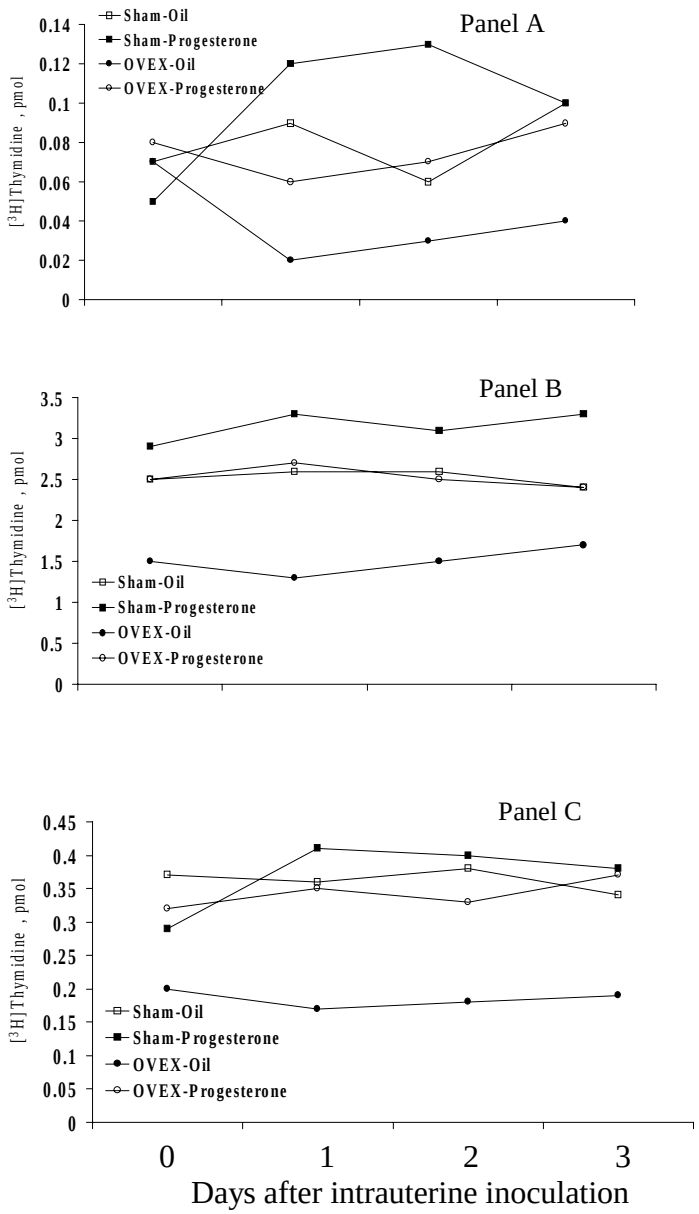

Figure 9. The effect of treatments on incorporation of [^{3}H]thymidine into newly formed lymphocytes in Exp. 2. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts were OVEX or a sham procedure was performed on d 0. Gilts either received progesterone or oil supplementation. All gilts were inoculated with bacteria on d 8 of the estrous cycle. There was a significant effect of OVEX and progesterone ($P < .01$; SEM =. 01) on unstimulated incorporation of [^{3}H]thymidine (Panel A). There was a significant effect of OVEX and progesterone ($P < .01$; SEM = .1) on Con A-stimulated incorporation of [^{3}H]thymidine (Panel B). There was a significant effect of OVEX and progesterone ($P < .01$; SEM = .01) on LPS-stimulated incorporation of [^{3}H]thymidine (Panel C).

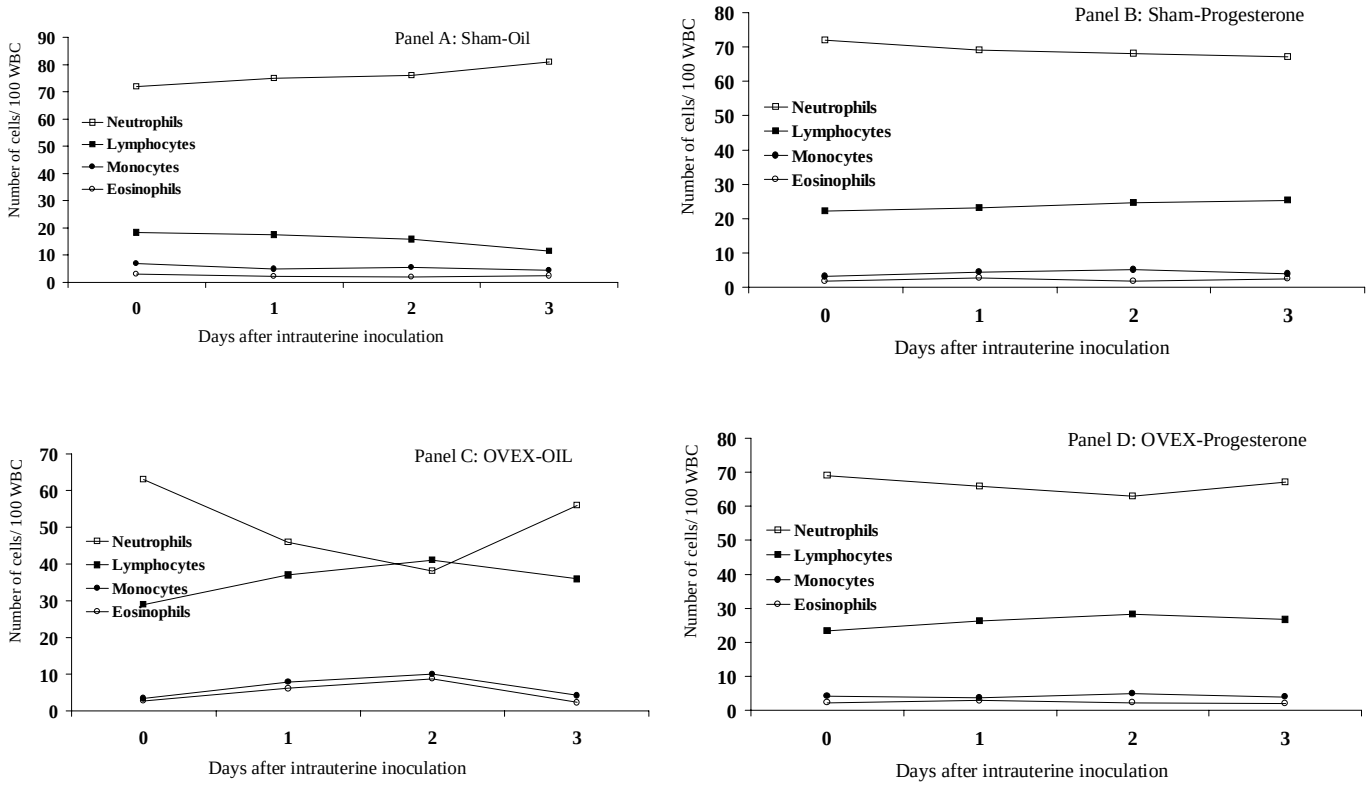

Figure 10. The effect of treatments on the average number of different types of WBC in Exp.2. Gilts were assigned to randomized treatments, estrus was synchronized, and gilts were OVEX or a sham procedure was performed on d 0. Gilts either received progesterone or oil supplementation. All gilts were inoculated with bacteria on d 8 of the estrous cycle. All gilts were inoculated with bacteria on d 8 of the estrous cycle. Different types of WBC (lymphocytes, neutrophils, eosinophils, and monocytes were counted out of 100 WBC from a thin blood smear stained with Wright's stain. Treatments are defined in each panel. The OVEX and progesterone affected neutrophil ($P < .05$). The OVEX affected lymphocyte ($P < .01$) numbers, and time after inoculation ($P < .01$) affected neutrophils and lymphocytes. Neutrophils (SEM = 1.9); Lymphocytes (SEM = 1.4); Monocytes (SEM = .8); Eosinophils (SEM = .8)

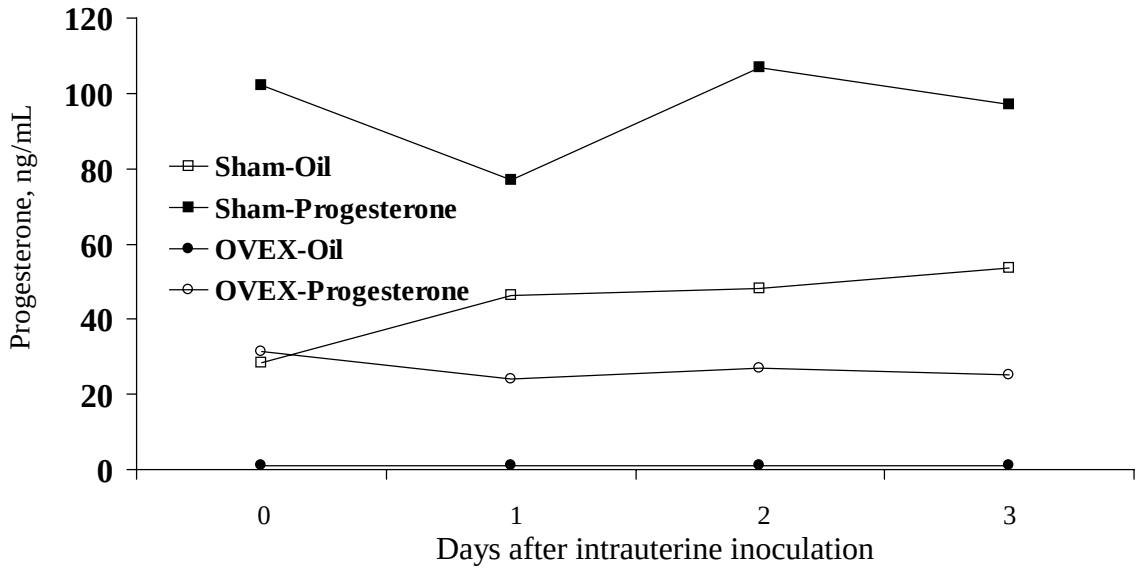

Figure 11. Concentrations of progesterone in venal caval blood collected from gilts that were OVEX or had a sham procedure on d 0 and received progesterone or oil supplementation in Exp. 2. All gilts were inoculated with bacteria on d 8. Concentrations of progesterone were affected by OVEX and progesterone ($P < .01$; SEM = 6.5).

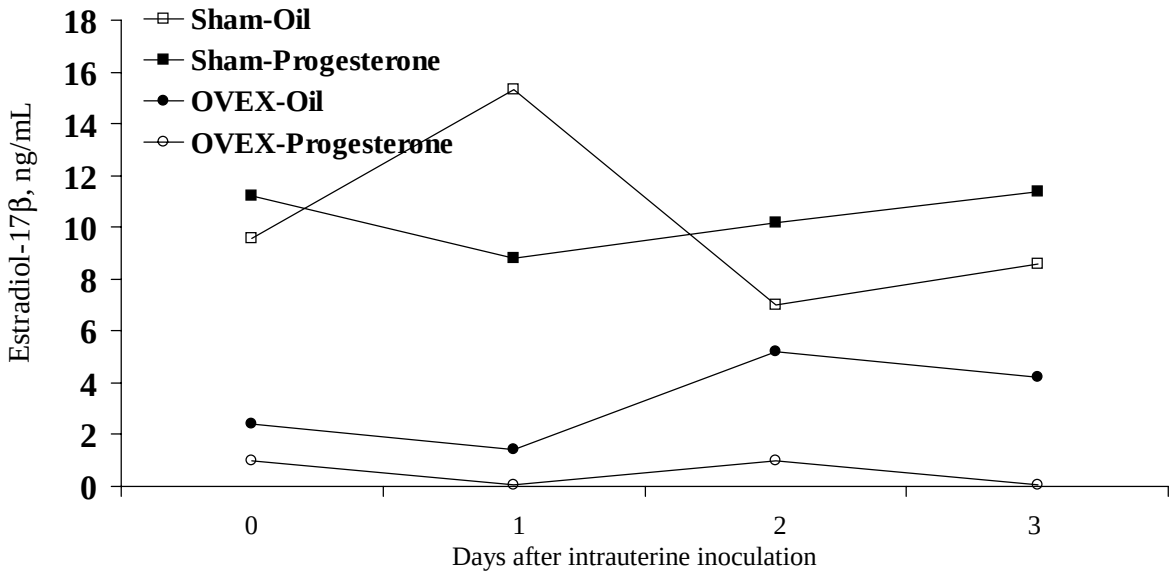

Figure12. Concentrations of estradiol-17β in venal caval blood collected from gilts that were OVEX or had a sham procedure on d 0 and received progesterone or oil supplementation in Exp. 2. All gilts were inoculated with bacteria on d 8. Concentrations of estradiol-17β were affected by OVEX ($P < .01$; SEM = 2.3).

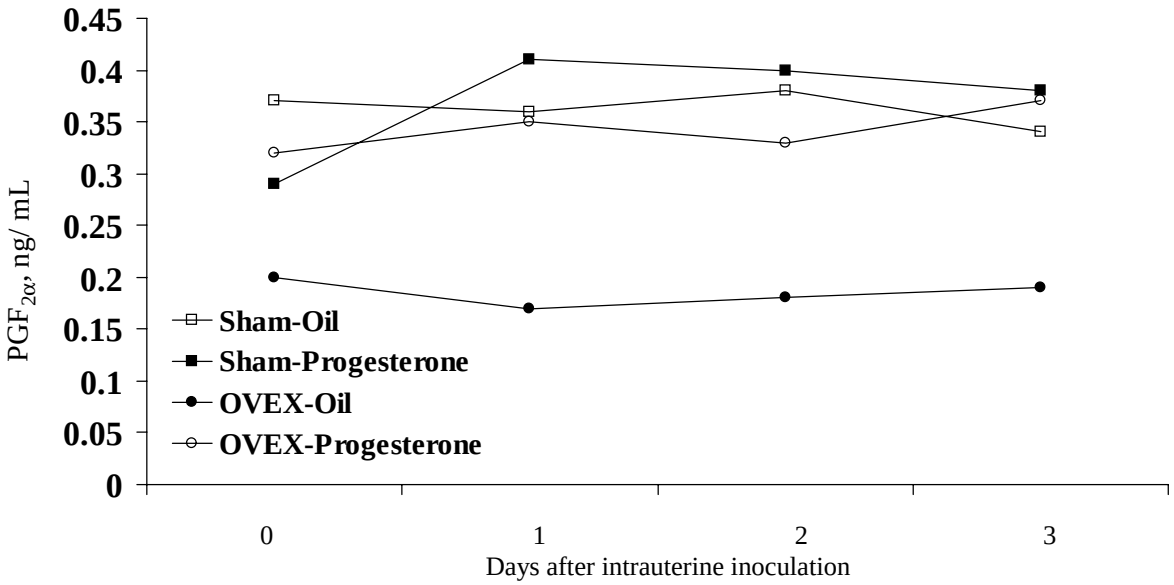

Figure 13. Concentrations of $PGF_{2\alpha}$ in venal caval blood collected from gilts that were OVEX or had a sham procedure on d 0 and received progesterone or oil supplementation in Exp. 2. All gilts were inoculated with bacteria on d 8. Concentrations of $PGF_{2\alpha}$ were affected by time after inoculation ($P < .01$; SEM = .06).

Chapter V

UTERINE RESPONSE TO MULTIPLE INOCULATIONS WITH *E. COLI* AND *A. PYOGENES* IN NULLIPAROUS EWES

ABSTRACT: Uterine infections seem to be more severe in nulliparous ewes that have not been exposed to bacterial challenge. Thus, we attempted to determine whether multiple exposures of a combination of *E. coli* and *A. pyogenes*, known to cause nonspecific uterine infections, altered the uterine immune response. Nulliparous ewes (n = 9/group) were assigned to one of four randomized treatments. Treatments were administered over three synchronized treatment cycles. On d 6 of the first synchronized treatment cycle, each ewe received an intrauterine inoculation of either 35 X 10^7 cfu of *E. coli* and 75 X 10^7 cfu of *A. pyogenes* in PBS or PBS. Vena caval blood was collected on d 6 through 12 of the treatment cycle. On d 12 of the first treatment cycle, all ewes were administered a 15 mg i.m. injection of Lutalyse to ensure clearance of infections. Ewes were rested for an estrous cycle. Estrous was again synchronized. Ewes either received bacteria or PBS on d 6 of the third treatment cycle. Vena caval blood was collected on d 6 through 12 of the second treatment cycle. The lymphocyte blastogenic response to mitogens was determined to evaluate the immune status of the ewes, white blood cells were counted, and progesterone, $PGF_{2\alpha}$, and PGE_2 were quantified. Uteri were collected at slaughter on d 12 following the second inoculation. The PCV in uterine flushings and the ability to culture *E. coli* and *A. pyogenes* were used to diagnosis uterine infections. Treatments affected ($P < .01$) daily percentages of neutrophils and eosinophils. Subsequent bacterial challenges increased ($P < .05$) the percentage of neutrophils. Day of the treatment cycle ($P < .01$) and the d x treatment interaction ($P < .01$) were significant for neutrophils. The day of treatment cycle and the treatment x d interaction were significant ($P < .01$) for lymphocytes. Infections (PCV > 1%) were observed in all ewes receiving bacterial inoculations during the second inoculation period at the

time of slaughter; however, ewes receiving the second bacterial challenge had less severe infections ($P > .05$; single bacterial challenge vs second bacterial challenge; PCV 30 vs 10%). None of the ewes that received PBS as a second challenge developed infections (PCV < 1%). Multiple exposures to bacterial challenge enhanced ($P < .01$) the lymphocyte blastogenic response to the mitogens Con A and LPS. Neither treatment nor day of treatment cycle affected vena caval progesterone ($P > .1$). Vena caval $PGF_{2\alpha}$ was increased in ewes inoculated with bacteria ($P < .01$). The decreased severity in uterine infections in ewes exposed twice to the bacteria indicates that multiple exposures enhance the immune response to bactera in sheep.

Introduction

Nonspecific uterine infections reduce the reproductive efficiency of livestock; however, the physiological characteristics that increase the susceptibility of some livestock to uterine infections are unknown (Arthur et al.1989; Lewis, 1997). Induced nonspecific uterine infections seem to be more severe in nulliparous ewes and in gilts that have not been exposed to bacterial challenge than in multiparous animals (unpublished data). Multiparous ewes probably have been exposed to the bacteria before, during breeding and during parturition, and have acquired immunity to the organisms, decreasing the severity of infection and increasing the rate of clearance.

A recent study provided evidence that intrauterine inoculation of heifers with *A. pyogenes* might produce active immunity (Watson et al., 1990). Other studies indicate that multiparous cows may be more resistant to *A. pyogenes* infections than are primiparus cows (Hussain, 1989). These studies indicate that older cows can recognize *A. pyogenes* and mount a humoral and cell-mediated defense against the organism resolving the infection faster or preventing the infection from occurring. However, parity and bacterial exposure are confounded in these studies, making

it difficult to separate out the effects of parity from previous bacterial exposure. Similar research has not been done with sheep. This study was conducted with nulliparous, yearling ewes that had not been exposed to rams to evaluate the immune response to multiple bacterial challenges with the same bacteria.

Materials and Methods

Experiment 3 ***(Chapter 2; Question 8)****: Does the uterine immune response change after multiple exposures to infectious bacteria in nulliparous ewes?*

General. Ewes that were 12 to 15 mo of age, produced at the Virginia Tech Sheep Center, and had two consecutive estrous cycles of 14 to 17 d in duration were used for this study. All of the ewes were healthy and had no history of uterine infections. In order to ensure ewes had not been exposed to the strains of bacteria used for intrauterine inoculation, estrus was checked using rams with aprons. An apron cover diverted a ram's penis, preventing intromission and vaginal exposure to bacteria. Ewes had the same number of estrous cycles to ensure similar reproductive status. Based on age and seasonality, ewes were placed on treatments after their first two estrous cycles of the season.

Experimental Design. The uterine immune response to multiple inoculations of infectious bacteria in nulliparous ewes was evaluated. Nulliparous ewes (n = 9/treatment) were assigned to one of four randomized treatments: 1) first inoculation (PBS) – second inoculation (PBS); designated PBS-PBS, 2) first inoculation (PBS) – second inoculation (bacteria); designated PBS-bacteria, 3) first inoculation (bacteria) – second inoculation (PBS); designated bacteria-PBS, and 4) first inoculation (bacteria) – second inoculation (bacteria); designated bacteria-bacteria.

The third treatment cycle of the season was synchronized. During the first treatment cycle following synchrony, vena caval catheters were inserted (Bennoit and Daily, 1991; Wade and Lewis, 1996) on d 5. On d 6, ewes received intrauterine inoculations of isotonic PBS (5mL) or

bacteria (75 x 10^7 cfu of A. pyogenes and 35 x 10^7 cfu of E. coli resuspended in 5 mL of PBS). Lutalyse (15 mg) was administered i.m. on d 12 of the first treatment cycle. During the second estrous cycle ewes were "rested." Procedures and treatments from the first treatment cycle were repeated during the third treatment cycle. However, on d 12 of the third treatment cycle, ewes were exsanguinated, CL were counted, and the uterus in each ewe was harvested and flushed to determine PCV and bacterial regrowth.

Estrus Synchronization. Ten to 12 d after nulliparous ewes were detected in estrus for the second time, they received one half of a Syncro-Mate-B (Sanofi, Overland Park, KS) implant. Implants were removed 10 d after they were inserted. Ewes were penned twice daily with rams with aprons to check for estrus after synchronization treatment. Ewes not detected in estrus within 60 h after implant removal were not assigned to the experiment. On d 12 of the first synchronized treatment cycle, ewes received a 15 mg i.m. injection of Lutalyse (Pharmacia & Upjohn, Kalamazoo, MI). Again ewes were penned twice daily with rams with aprons 24, 36, and 48 after the injection of Lutalyse to check estrus. Fourteen days after ewes were detected in estrus, they were penned with aproned rams every 12 h until estrus was detected. The onset of estrus was considered d 0. Ewes received treatments during the third treatment cycle relative to detection of estrus.

Vena Caval Catheterization. The vena cava was catheterized 24 h before each ewe received intrauterine inoculations. For a full review of the procedure refer to Benoit and Daily (1991). Briefly, the saphenous vein, just dorsal to the right hock during the first treatment cycle and the left hock during the second treatment cycle, was exposed. A 90-cm-long catheter (inner diameter, 0.42 mm; outer diameter, 0.74 mm; ICO-Rally, Palo Alto, CA) was inserted into the vein through a small incision and then passed up through the saphenous vein into the vena cava.

The catheters were marked 45, 50, 55, and 60 cm from the external end. One 4-mL blood sample was taken at each marked position on the catheter as it was passed up the vein, and then the catheter was positioned temporarily. Final catheter position was determined according to progesterone concentrations; the greatest progesterone concentration indicated the region where the uteroovarian blood entered the vena cava. The catheters were placed at that position.

Intrauterine Inoculations. For a full review of the laparoscopic procedure, refer to Appendix B. Briefly, the uterus was visualized with a laparoscope and positioned with both uterine horns in view. The probe used for positioning the uterus was removed and replaced with a 17 g aspiration needle placed in the 5 mm cannula. The needle was plunged into the uterine horn. A 12 mL syringe containing either PBS or bacterial treatments was attached to the end of the aspiration needle. A second person slowly depressed the plunger of the syringe until the syringe was empty. The needle and syringe were removed.

Blood Sampling. On d 6 to 12 of the first and third treatment cycle blood samples were collected. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12 mL sample was taken from the catheter placed where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple ewes, blood samples were kept in an ice-water bath until centrifuged for the collection of plasma and the buffy coat. Before centrifugation on d 6, 8, 10, and 12 of the first and third treatment cycle, a small portion of the heparinized sample was used for differential WBC. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for blastogenic assay. The lymphocyte blastogenic response to mitogens was measured on d 6 to 12 during the first and third treatment cycles. Samples collected on d 6, 8, 10, and 12 of the first and third treatment cycles were

assayed for progesterone. Samples collected on d 6 through12 of the first and third treatment cycle were assayed for $PGF_{2\alpha}$ and PGE_2. Blood samples were collected twice daily approximately 12 h apart. The first blood sample was taken before administration of any treatments. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin, 1994). On d 6 of the first treatment cycle, d 5 of the second treatment cycle, and d 12 of the third treatment cycle, jugular blood was collected into Vacutainers that did not contain an anticoagulant. Samples were maintained at room temperature for 4 h then centrifuged, and serum was collected. Serum was stored at -20° until Ab was measured.

Inoculations. The strains of *A. pyogenes* and *E. coli* used for this study were isolated from a cow with endometritis at the Virginia Tech Dairy Center (Del Vecchio et al., 1992). Both strains of bacteria were purified and stored in skim-milk broth medium at -20° until they were used to prepare intrauterine inoculations. Inoculations prepared with bacteria produced endometritis in cows (Del Vecchio et al.,1992) and sheep (Ramadan et al.,1997).

The bacteria were cultured in brain-heart infusion broth (Difco, Detroit, MI) to obtain enough colony-forming units to prepare inoculations. The optical density of the cultures was measured periodically at 560 nm to determine bacterial growth. The optical density was used in a regression equation. The regression equation was derived from optical densities of cultures and direct counts of the number of colony forming units on blood agar plates after a sample of the cultures had been incubated for 24 h to estimate the number of colony forming units per 10 μL of medium (Ramadan et al., 1997). To prepare inoculations, an appropriate volume of culture medium was transferred into sterile culture tubes and centrifuged at 10 x g for 20 min at 4°C. The supernatant, which can act as a chemoattractant was removed, and the pellet was resuspended in 5 mL of sterile isotonic PBS. The inoculations were held on ice until they were

used. A 2.5 mL volume of the suspension was injected into each uterine horn. On the basis of a dose response curve, each inoculation contained 75 x 10^7 cfu of *A. pyogenes* and 35 x 10^7 cfu of *E. coli.*

Lymphocyte Separation and Blastogenic Assay. Twelve mL of heparinized blood was centrifuged at 2,800 x g for 20 min at 4°C. The buffy coat was mixed with 4 mL of HBSS at pH 7.4 (Gibco BRL, Grand Island, NY). The mixture was layered on top of 4 mL of Ficoll-Paque (Sigma Chemical, St. Louis, MO) and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte-containing portion was transferred to sterile culture tubes. The suspension was cenrifuged 1,000 x g for 10 min at room temperature. The supernatant was removed, and the lymphocytes were washed twice with HBSS. Then cells were suspended in complete RPMI-1640 medium (Gibco) that contained fetal bovine serum (.1 mL/mL; Gibco), pencillin (100 IU/mL; Sigma), and streptomycin (100 mg/mL; Sigma). The number of live lymphocytes were determined using a hemocytometer and a trypan blue dye exclusion procedure. The final concentration of live cells was adjusted to 1 x 10^6/mL of RPMI-1640.

The blastogenic assay was similar to the one described in Burrells and Wells (1977). Lymphocytes (1 x 10^5 live cells) from each vena caval sample were cultured in 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ). Mitogenesis was stimulated with Con A (stimulates T-lymphocytes; 1 μg/well; Sigma) or LPS (stimulates B lymphocytes; .5 μg/well; Sigma); other cells were left unstimulated (100 μL of RPMI-1640/well) so that unstimulated mitogenesis could be estimated. Incubation treatments were in triplicate.

The plates were held at 37° for 48 h in a humidified chamber with an atmosphere of 5% CO_2 in air. A tracer of 1 μCi of [^{3}H]thymidine (specific radioactivity 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in RPMI-1640 was added to each well, and the plates were held

under the same conditions for another 16 h. At the end of culture, lymphocyte viability was determined with a trypan blue dye-exclusion procedure, and cells were transferred to fiberglass filters (Whatman, Madistone, England). Filter discs corresponding to each well were transferred to separate scintillation vials, and lymphocytes were solubilized to release [^{3}H]thymidine. Disintegrations per minute were determined with a liquid scintillation counter and used to calculate the picomoles of [^{3}H]thymidine incorporated into newly synthesized DNA.

Immunoasays. Radioimmunoassays were used to measure progesterone and PGE_2. For catheter positioning and measurement of sample, an [^{125}I]progesterone kit (Diagnostic Products, Los Angeles, CA) was used to quantify progesterone. All samples collected after final catheter positioning were evaluated in a single assay with a CV of 7.9%. The concentration of PGE_2 was determined using tritiated tracer (Lewis et al., 1978). The PGE_2 intra- and interassay CV were 7.9 and 18.2% respectively.

The concentrations of $PGF_{2\alpha}$ were determined with an EIA (Del Vecchio et al., 1992; Fortin, 1994). The intraassay and interassay CV for $PGF_{2\alpha}$ were 6.2 and 29.3%, respectively.

Antibody Measurements. The presence or absence of Ab for *A. pyogenes* and *E. coli* was measured by titration into microtiter plates using an agglutination procedure (Louks et al., 1985). Antibody was measured separately for *A. pyogenes* and *E. coli*. Live *A. pyogenes* and *E. coli* were used as the Ag. Approximately 2 x 10^5 cfu of A. pyogenes and 1 x 10^5 cfu of E. coli was used as Ag. The bacteria that were used as Ag were obtained from the stock of bacteria that was originally used to inoculate the ewes. Bacteria were prepared in the same manner as prepared for intrauterine inoculation. Serum from all ewes was serially diluted with lamb serum that did not contain Ab to these strains of *A. pyogenes* and *E. coli*. Serum was diluted to a final volume of 200 μL. Serum dilutions of 0, 1, 1:2, 1:4,1:8, and 1:16 were used to measure the presence of *E.*

coli. Serum dilutions of 0, 1, 1:2, 1:5, 1:10, and 1:20 were used to measure the presence of *A. pyogenes*. After addition of the Ag, microtiter plates were left at room temperature for 1 h. At the end of the 1 h incubation period, microtiter plates were observed for the presence of a percipitate. During the incubation period, it is presumed that bacteria continued dividing; therefore, it was not possible to determine the exact dose of bacteria that caused precipitation. A precipitate indicated the presence of Ab. Although serial dilutions were used, it was believed that this assay technique was too primitive to accurately measure Ab titer; therefore, only the presence or absence of a precipitate within these dilutions was recorded.

Slaughter. All ewes were slaughtered on d 12 of the third treatment cycle, and the reproductive tracts were collected and examined for signs of infection. Clear uterine flushings with small amounts of sediment, no signs of endometrial inflammation, and the inability to culture *A. pyogenes* and *E. coli* collectively indicated that the uterus was not infected. Cloudy or colored uterine flushings with large amounts of sediment (PCV), inflamed endometrium, and the ability to culture *A. pyogenes* and *E. coli* collectively indicated that the uterus was infected.

Statistical Analysis. The General Linear Models procedures of the Statistical Analysis System (SAS, 1985) were used to analyze the data. The GLM model included the following variables: treatment, ewe nested within treatment, day of treatment cycle, and the treatment x day interaction. Ewe nested within treatment was the main plot error term, and the residual was the subplot error term. The variance associated with the main plot error term was used to calculate overall standard errors (SEM) associated with main plot variables. When appropriate, the PDIFF (i.e., a method for comparing all possible least squares means) option in SAS was used to compare individual means. Inoculations were administered on d 6 of the treatment cycle. To

represent the physiology associated with changes in the treatment cycle, time will be referred to as day of the treatment cycle.

The FREQ procedure of SAS was used to determine the effect of treatment on the presence of Ab in the serum. Chi-square was used to determine whether the presence of Ab in the serum was affected by treatment.

The shapes of the response curves for unstimulated lymphocyte blastogenic activity were determined for ewes in each treatment group, and then unstimulated activity was used as a covariant for Con A and LPS stimulated lymphocytes. The incorporation data were reported after the adjustment for unstimulated activity rather than reporting a stimulation index. The reason for this is because a stimulation index represents a percentage change from unstimulated activity, and the index is based on the assumption that the relationship between unstimulated activity and stimulated incorporation is linear, which was not always true for the data from this experiment. The prediction option in SAS was used to generate a new set of data that contained the adjusted values for Con A and LPS. The adjusted values were analyzed with the original GLM model.

Data were analyzed for differences between the four treatment groups after the second inoculations during the third treatment cycle. Data accumulated following the first inoculation during the first treatment cycle was analyzed for differences between two treatment groups (PBS vs bacteria).

Results

Experiment 3.

Uterine Infections. A ewe was diagnosed with a uterine infection if the PCV of the uterine flushing obtained at slaughter was greater than 1% and if *A. pyogenes* and *E. coli* were cultured from the uterine flush (Figure 1). All of the ewes inoculated with *A. pyogenes* and *E.*

coli on d 6 of the third treatment cycle developed uterine infections, but none of the ewes inoculated with PBS on d 6 of the third treatment cycle developed an infection (Figure 1). Because ewes were not slaughtered until the end of the third treatment cycle, an accurate measure of the development of uterine infections after the first inoculation with bacteria or PBS was not obtainable. Ewes that received two inoculations of bacteria, the first on d 6 the first treatment cycle and the second on d 6 of the third treatment cycle, had less severe infections than ewes that received their first bacterial inoculation on d 6 of the third treatment cycle (Figure 1; $P < .01$).

Antibody Measurements. Following exposure to *A. pyogenes* and *E. coli,* Ab in serum increased ($P < .01$; Figure 2 and 3). Before the experiment, only 20% of the ewes had a measurable precipitate following exposure of serum to *A. pyogenes* and only 10% following exposure of serum to *E. coli*. The rates remained constant if ewes received a PBS inoculation during the first treatment cycle. The presence of precipitate increased after ewes were exposed twice to bacteria; 100% had a measurable precipitate following exposure of serum to *A. pyogenes* and *E. coli* (Figures 2 and 3)

Lymphocyte Blastogenic Activity: Treatment cycle 1. Unstimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was less ($P < .01$) for ewes inoculated with bacteria on d 6 of the first treatment cycle than for ewes inoculated with PBS (.87 vs 1.68 pmol, SEM .2). The day of the treatment cycle and the treatment x day interaction was significant ($P < .01$) for [^{3}H]thymidine incorporation into newly formed lymphocytes for ewes inoculated with bacteria versus PBS on d 6 of the first treatment cycle (Figure 4, Panel A).

The day of the treatment cycle and the treatment x day interaction were significant for [^{3}H]thymidine incorporation into Con A-stimulated lymphocytes. Incorporation was greater for

ewes inoculated with PBS than for those inoculated with bacteria during the first treatment cycle (Figure 4, Panel B). [^{3}H]Thymidine incorporated for ewes inoculated with bacteria on d 6 of the first treatment cycle increased from 8 pmol before inoculations to 14 pmol 24 h after inoculation. Between d 7 and 9, [^{3}H]thymidine incorporation decreased to 8 pmol. It increased again to 10 pmol by d 11 of the treatment cycle (Figure 5, Panel B). For ewes inoculated with PBS, incorporation was approximately 11 pmol throughout the sampling period.

The LPS-stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was less in ewes that received bacteria on d 6 of the first treatment cycle than in ewes that received PBS (4.1 vs 5.2 pmol; SEM .2; Figure 4, Panel C). The day of the treatment cycle and the treatment x day interaction were significant ($P < .01$; Figure 4, Panel C) for [^{3}H]thymidine incorporation into newly formed lymphocytes (Figure 4, Panel C).

Lymphocyte Blastogenic Activity: Treatment cycle 2. Unstimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was greater ($P < .01$) for ewes inoculated with bacteria on d 6 of the third estrus cycle than for ewes inoculated with PBS (.84 vs .5 pmol; SEM = .04; Figure 5, Panel A). Unstimulated incorporation was increased in ewes receiving their second bacterial inoculaton versus ewes receiving their first bacterial inoculation during the third treatment cycle (.88 vs .8 pmol; Figure 5, Panel A). The day of the treatment cycle and the treatment x day interaction were significant ($P < .01$) for [^{3}H]thymidine incorporation into newly formed lymphocytes (Figure 5, Panel A).

Treatment, day of treatment cycle, and treatment x day interaction were significant for [^{3}H]thymidine incorporation into Con A-stimulated lymphocytes (Figure 5, Panel B). Ewes receiving their second bacterial inoculation during the third treatment cycle had significantly increased [^{3}H]thymidine incorporation compared with ewes receiving their first bacterial

inoculation or PBS inoculations (15.4 vs 9.6 pmol; SEM = .25). In ewes receiving the second bacterial inoculation, [^{3}H]thymidine incorporation increased from 12 to 15.8 pmol during the first 24 h following inoculation. Incorporation continued to increase until d 10 of the treatment cycle and decreased to 14.7 pmol by d 12 of the treatment cycle (Figure 5, Panel B).

Treatment, day of treatment cycle, and treatment x day interaction were significant for [^{3}H]thymidine incorporation of LPS-stimulated lymphocytes (Figure 5, Panel C). Ewes receiving their second bacterial inoculation during the third treatment cycle had significantly increased [^{3}H]thymidine incorporation compared to ewes receiving their first bacterial inoculation or PBS inoculations (5.8 vs 4.0 pmol; SEM = .2). In ewes receiving the second bacterial inoculation, [^{3}H]thymidine incorporation increased from 4.6 to 6.1 pmol during the first 24 h following inoculation. Incorporation continued to increase until d 10 of the treatment cycle and decreased to 5.2 pmol by d 12 of the treatment cycle (Figure 5, Panel C).

Differential White Blood Cell Counts. Treatment cycle 2. Day of the estrous and the treatment x day interaction were significant ($P < .01$) for lymphocytes. The numbers of lymphocytes per 100 WBC in ewes inoculated on d 6 with bacteria of the third treatment cycle increased throughout the remainder of the treatment cycle (Figure 6, Panels B and D). In ewes that received their second inoculation of bacteria on d 6 of the third treatment cycle, lymphocyte percentage decreased from 29 to 22 in the first 48 h after inoculation (Figure 6, Panel D). They then increased to 32 % by d 12 (Figure 6).

For neutrophils, treatment, day, and the treatment x day interaction were significant ($P < .01$). The number of neutrophils per 100 WBC increased from 51 to 62 by 48 h after the second bacterial inoculation (Figure 6, Panel D). Neutrophil numbers remained elevated until d 12 when

they decreased to 51 (Figure 6, Panel D). Neutrophils numbers in ewes receiving PBS or their first bacterial inoculation remained constant (Figure 6, Panels A,B, and C).

The numbers of eosinophils changed ($P < .05$) with treatment, but changes were not striking, and numbers were close to the means (Figure 6). Monocytes were not significantly affected by treatments, day, or the interaction (Figure 6). The data for basophils were not evaluated because the number in the majority of WBC counts was typically zero.

Steroids and Prostaglandins: Treatment cycle 1. Neither treatment nor day of treatment cycle affected vena caval progesterone ($P > .1$). The mean progesterone concentration in ewes between d 6 and 11 was 12.1 ng/mL (SEM=1.8).

Vena caval $PGF_{2\alpha}$ increased in ewes inoculated with bacteria in comparison to ewes inoculated with PBS (.34 vs .15 ng/mL; SEM=.05; Figure 8), and $PGF_{2\alpha}$ concentrations ($P < .01$) changed with time after inoculation (Figure 7). Vena caval PGE_2 concentrations changed ($P < .01$) with day of the treatment cycle (Figure 8).

Steroids and Prostaglandins: Treatment cycle 2. Neither treatment nor day of treatment cycle affected vena caval progesterone. The mean progesterone concentration of ewes between d 6 and 12 was 10.5 ng/mL (SEM=2.1).

Vena caval $PGF_{2\alpha}$ increased in ewes inoculated with bacteria during the third treatment cycle compared with ewes inoculated with PBS (.36 vs .16 ng/mL; SEM=.02), and $PGF_{2\alpha}$ concentrations ($P < .01$) changed with time after inoculation (Figure 9).Vena caval $PGF_{2\alpha}$ was also significantly increased in ewes receiving their second inoculation of $PGF_{2\alpha}$ compared with ewes receiving their first inoculation (.38 vs .34 ng/mL).Vena caval PGE_2 concentrations changed ($P < .01$) with day of the treatment cycle (Figure 10).

Discussion

Ewes that received multiple inoculations of bacteria developed less severe infections after the second bacterial inoculation than did ewes that only received a single bacterial inoculation (Fig. 1). Although ewes appeared to have less severe infections, determination of severity was based on PCV and the ability to regrow bacteria. Therefore, it is not possible to determine whether ewes developed less severe infections or if the uterine immune system was able to clear the infections more quickly.

The results of this experiment indicate that vena caval lymphocytes from ewes inoculated with multiple doses of bacteria on d 6 of two treatment cycles had greater capacities for unstimulated and Con A- and LPS-stimulated incorporation of [^{3}H]thymidine than did lymphocytes from ewes inoculated with a single dose of bacteria or saline.

The increased capacity for unstimulated and Con A- and LPS-stimulated incorporation of [^{3}H]thymidine suggests an increase lymphocyte activity, which is confirmed by the increase of lymphocytes in the vena caval blood. However, the increase in neutrophils in the vena caval blood is puzzling. If there is a rapid innate immune response, neutrophils leave circulation, enter the tissue, and are transported to the site of infection (Tizard, 1996). However, neutrophil numbers were increased following bacterial inoculation in this study. This may be an artifact of sampling. Samples for differential WBC counts were taken before and then every 48 h after bacterial inoculations. If the immune response is rapid, it is possible that neutrophils had already traveled to the site of infection, and the neutrophils present in circulation were new. This would suggest an enhanced innate uterine immune response to bacteria after previous exposure. This may be further supported by the increased Con A-stimulated incorporation of [^{3}H]thymidine in ewes that had received multiple inoculations of bacteria, suggesting faster cell-mediated response and possibly, although we did not test it, the development of T lymphocyte memory systems.

The increase in Con A-stimulated incorporation of [^{3}H]thymidine may be an artifact of our resting period. Concanvalin A- and LPS-stimulated incorporation of [^{3}H]thymidine was greater in ewes that had previously received bacterial inoculations, before the second inoculation was administered, suggesting that the "resting" period was not long enough for lymphocyte activity to return to "normal." Increases in LPS-stimulated incorporation of [^{3}H]thymidine were expected and were indicative of an increase in B lymphocyte activation. An increase in B lymphocyte involvement was confirmed by the increase in presence of Ab to the specific bacteria following bacterial inoculation.

Exogenous $PGF_{2\alpha}$ is administered as a treatment for postpartum uterine infections in cows and sheep (Olson et al., 1986; Youngquist and Little, 1988). However, the mechanism of the action of by which exogenous $PGF_{2\alpha}$ clears uterine infections has not been clearly defined. It is typically believed that $PGF_{2\alpha}$ causes regression of the corpus luteum and a concomitant decrease in progesterone. Progesterone down-regulates the uterine immune system, and, therefore, the decrease in progesterone leads to an increase in immune cell function and clearance of uterine infection. If luteolysis is induced, this may be the primary mechanism of clearance of uterine infections; however, if the CL does not regress and progesterone does not decrease, there must be another mechanism involved. In the presence of bacteria, either a single inoculation or multiple inoculations, vena caval concentrations of $PGF_{2\alpha}$ increase. In ewes that received multiple bacterial inoculations, concentrations of $PGF_{2\alpha}$ were greater than in ewes that only received a single inoculation of bacteria. However, mean concentrations of progesterone did not change (data not shown).

In the present study, immune function seemed to be up-regulated following multiple exposures to the same bacteria. Previous studies (Watson et al., 1990; Hussain, 1989) indicated

that uterine immune function was enhanced in heifers that received intrauterine inoculations with *A. pyogenes*. It was believed that intrauterine inoculation with *A. pyogenes* in those heifers lead to the development of active immunity (Watson et al., 1990). The differences in immune function between ewes previously exposed to intrauterine bacteria and ewes that have never been exposed probably explains differences in the severity and clearance rate of infections.

Implications

Multiple exposures to bacteria decreased the severity of uterine infections in sheep possibly accounting for the decreased incidence and severity of uterine infections in multiparous ewes. The uterine immune system seems to have a "stronger" response to bacteria after multiple exposures, suggesting that the uterine immune system recognizes the bacteria and mounts a "faster" and "stronger" humoral and cell-mediated response against the bacteria, preventing or clearing the infection. If this is the case, it may be possible to inoculate nulliparous ewes with low doses of the bacteria to reduce the incidence of uterine infections. Therefore, preventing, rather than treating, uterine infections may be a possibility. A similar strategy may be possible in other livestock.

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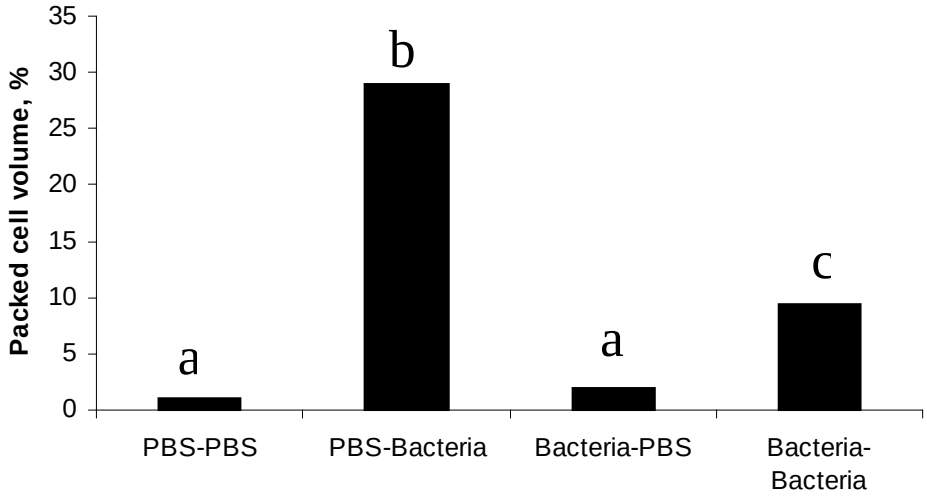

Figure 1. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculation (PBS or Bacteria) on d 6 of the first and third treatment cycles. Ewes were slaughtered on d 12 of the third treatment cycle. Uteri were collected and flushed. Packed cell volume of the uterine flushings was measured. [a,b,c]Different superscripts indicate that means differed between treatment groups ($P < .01$). The SEM = 1.3, and there were nine ewes per treatment.

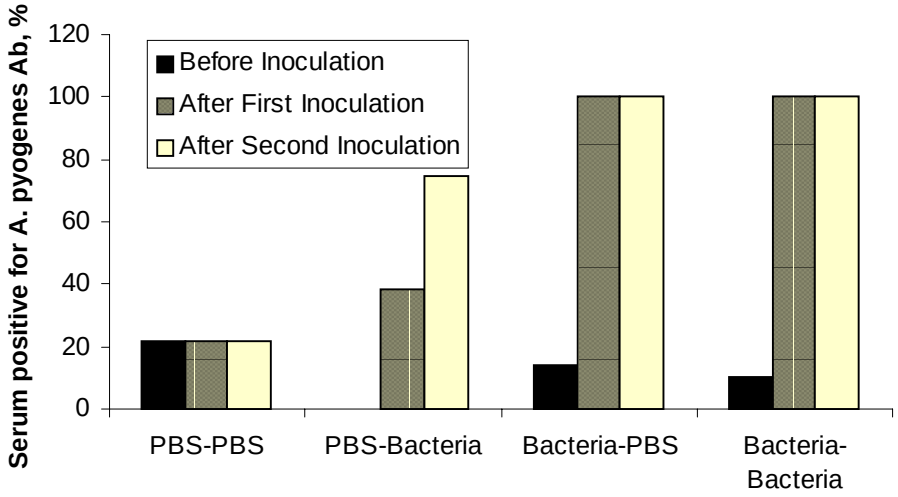

Figure 2. The effect of treatment on the presence of *A. pyogenes* Ab in the serum in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations of (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken before the ewes had received any type of inoculation to quantify the extent of prior exposure to *A. pyogenes* (d 6 first treatment cycle), after the first inoculation (d 5 of the second treatment cycle), and after the second inoculation (d 12 of the third treatment cycle). The presence or absence of Ab was measured via an agglutination test. Chi-square analysis was used to analyze the data. Following exposure to *A. pyogenes*, the presence of *A. pyogenes* Ab in serum increased ($P < .01$).

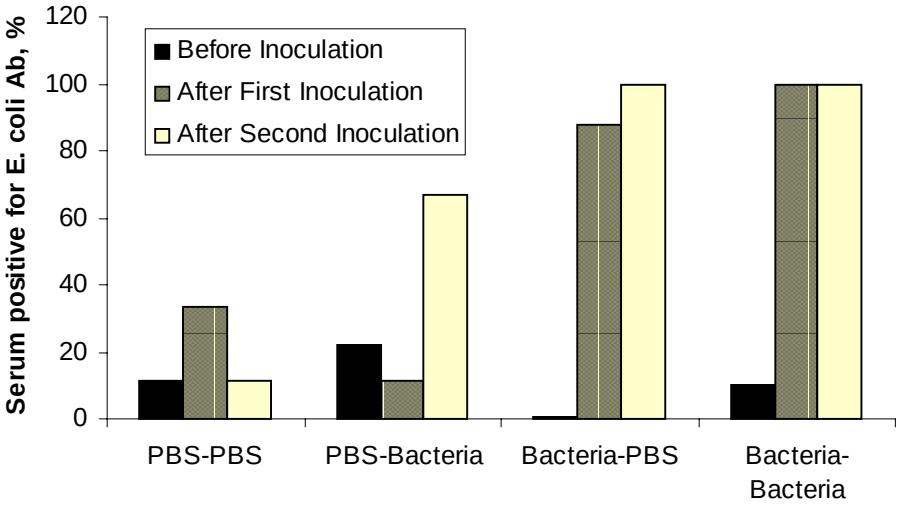

Figure 3. The effect of treatment on the presence of *E. coli* Ab in the serum in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculation (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken before the ewes had received any type of inoculation to quantify the extent of prior exposure to *E. coli* (d 6 first treatment cycle), after the first inoculation (d 5 of the second treatment cycle), and after the second inoculation (d 12 of the third treatment cycle). The presence or absence of Ab was measured via an agglutination test. Chi-square analysis was used to analyze the data. Following exposure to *E. coli*, the presence of *E. coli* Ab in serum increased ($P < .01$).

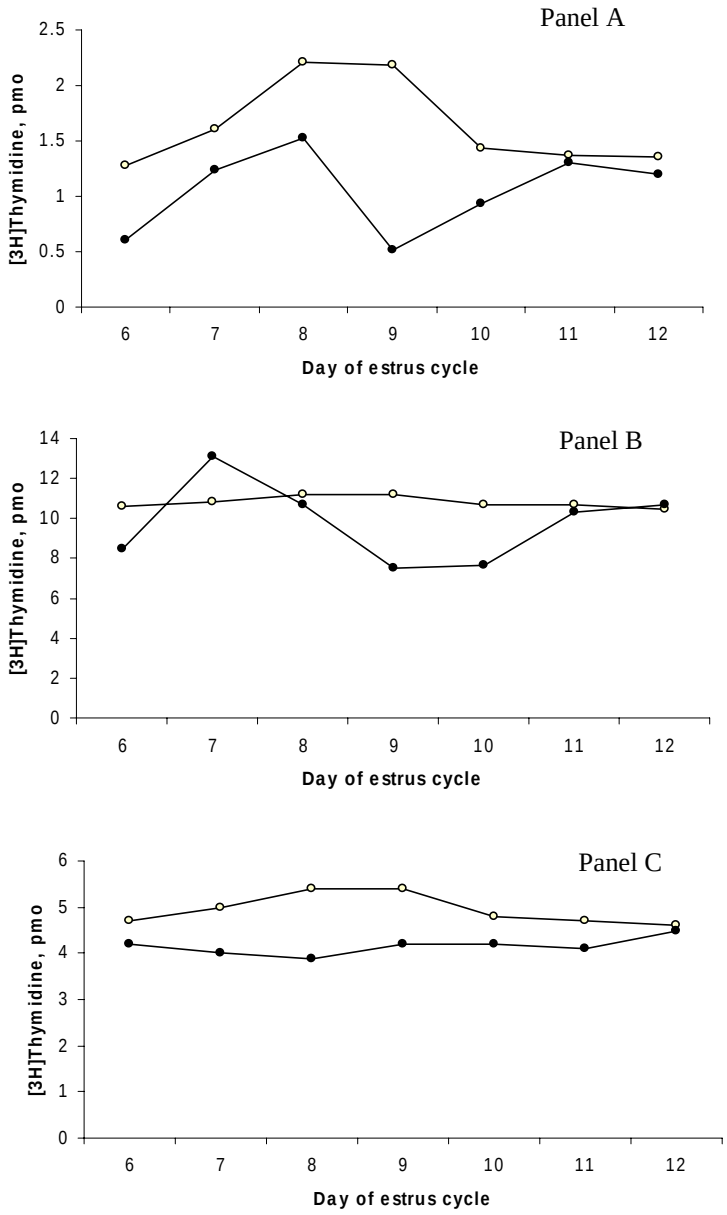

Figure 4. The effect of treatment on incorporation of [^{3}H]thymidine into newly formed lymphocytes during the first treatment cycle in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or bacteria) on d 6 of the first treatment cycle. Samples were taken on d 6 to 12 of the first treatment cycle. There was a significant effect of treatment, day of treatment cycle, and treatment x day interaction ($P < .01$) on unstimulated incorporation of [^{3}H]thymidine (Panel A). There were 18 ewes per treatment, and the SEM = .2. There was a significant effect of day of treatment cycle and treatment x day interaction ($P < .01$) on Con A-timulated incorporation of [^{3}H]thymidine (Panel B). There were 18 ewes per treatment, and the SEM = .3. There was a significant effect of treatment, day of treatment cycle, and treatment x day interaction ($P < .01$) on LPS-stimulated incorporation of [^{3}H]thymidine (Panel C). There were 18 ewes per treatment, and the SEM = .2.● = PBS; o = Bacteria.

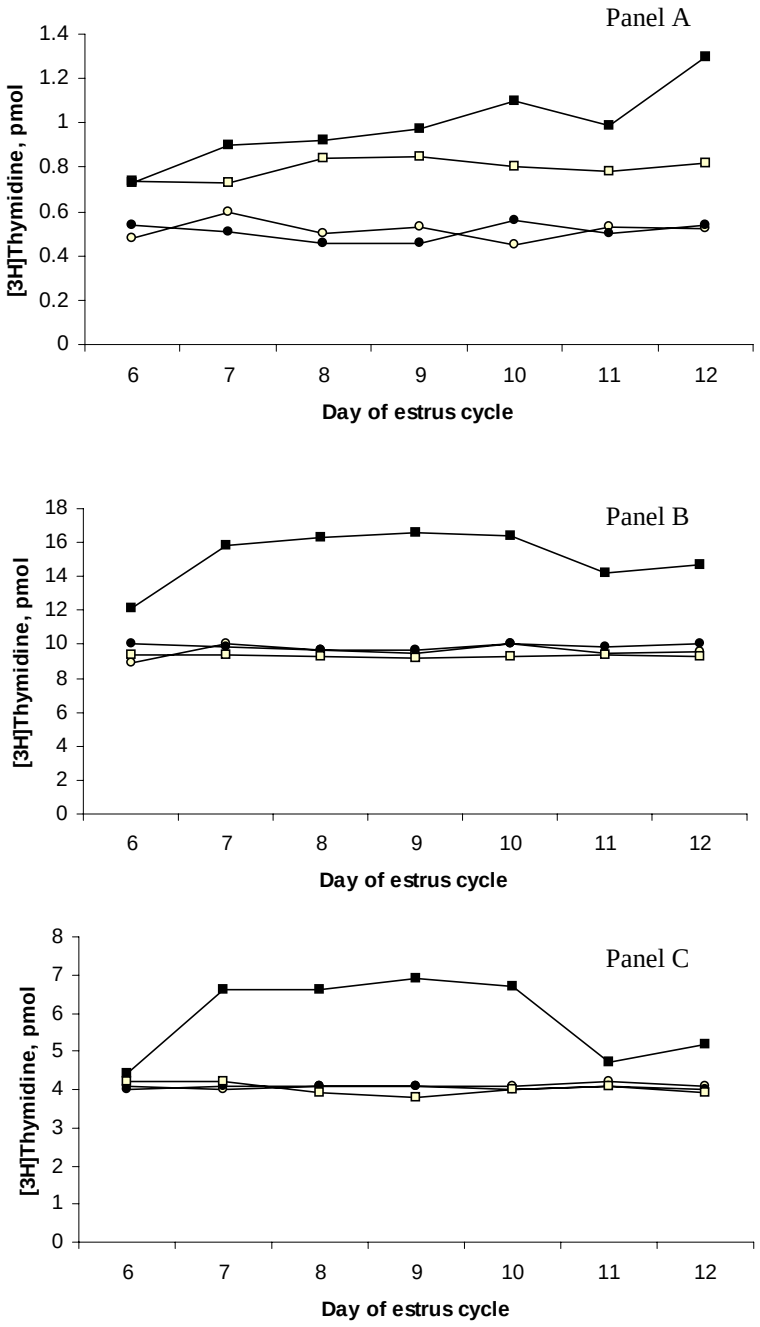

Figure 5. The effect of treatment on incorporation of [^{3}H]thymidine into newly formed lymphocytes during the third treatment cycle in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken on d 6 to 12 of the third treatment cycle. There was a significant effect of treatment, day of treatment cycle, and treatment x day interaction ($P < .01$) on unstimulated, and Con A-, and LPS-stimulated incorporation of [^{3}H]thymidine. There were nine ewes per treatment, and the SEM for Panel A = .04; for Panel B SEM= .25; and for Panel C SEM = .2.

= Bacteria-Bacteria; = Bacteria-PBS; • = PBS-Bacteria; o = PBS-PBS

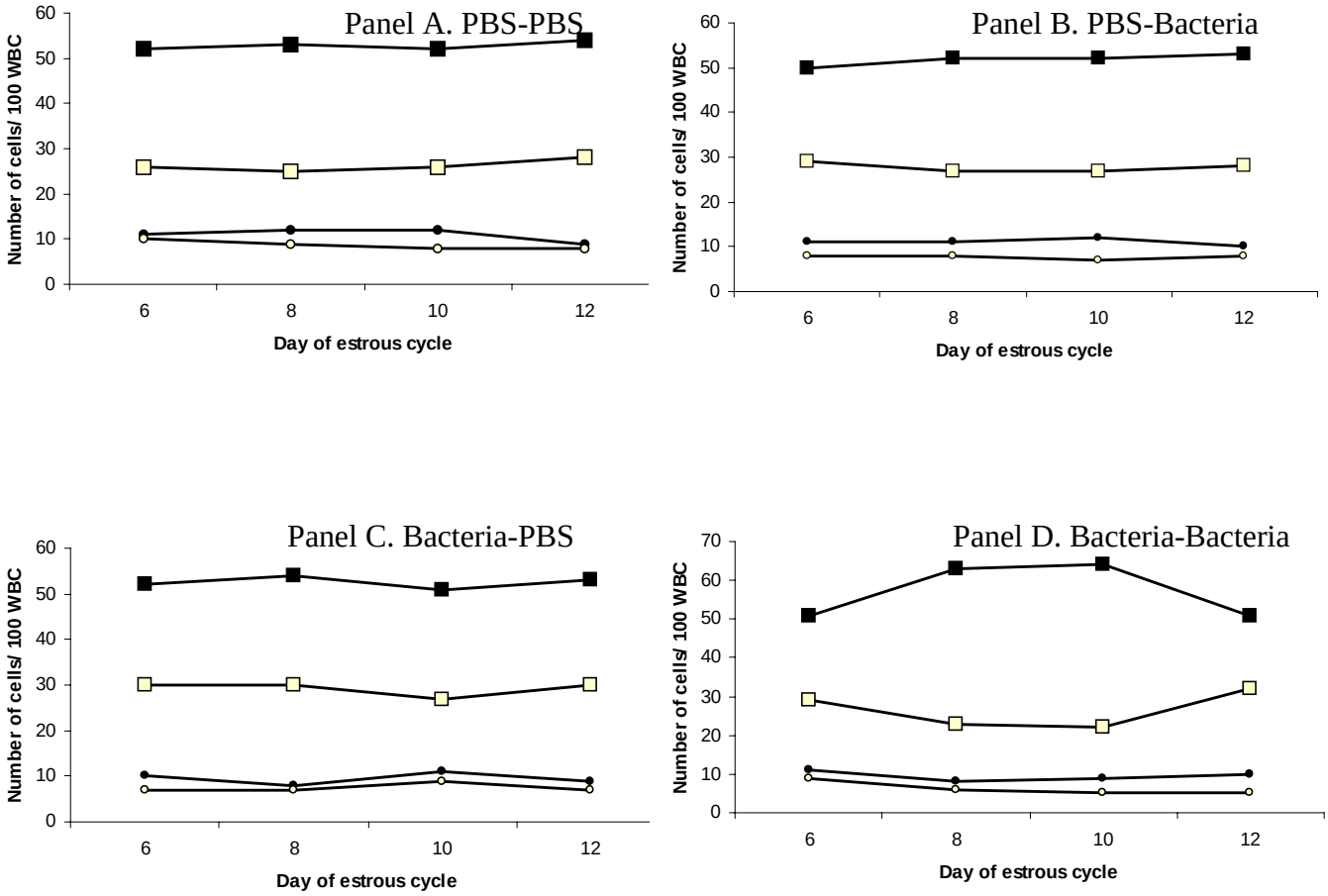

Figure 6.The effect of treatment on the average number of different types of WBC in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculation (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken on d 6, 8, 10, and 12 of the third treatment cycle. Different types of WBC (lymphocytes, neutrophils, eosinophils, and monocytes) were counted out of 100 WBC from a thin blood smear stained with Wright's stain. Treatments are defined in each panel. Treatment did not affect lymphocytes or monocytes, but there was an effect of d and a treatment x d interaction on lymphocytes ($P < .01$). There was a significant effect of treatment on neutrophils and eosinophils ($P < .05$). There was also a significant effect of d and treatment x d interaction on neutrophils ($P < .01$).
 = Neutrophils; = Lymphocytes; • = Monocytes; o = Eosinophils

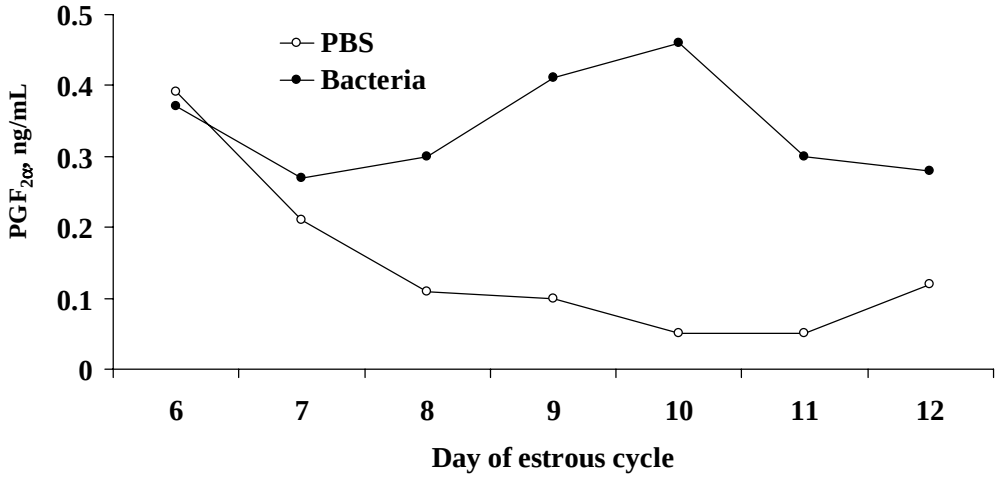

Figure 7. The effect of treatment on $PGF_{2\alpha}$ in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 of the first treatment cycle. Samples were taken on d 6-12 of the first treatment cycle. There was a significant effect of treatment, d of treatment cycle, and treatment x d interaction ($P < .01$). There were 18 ewes per treatment, and the SEM = .05.

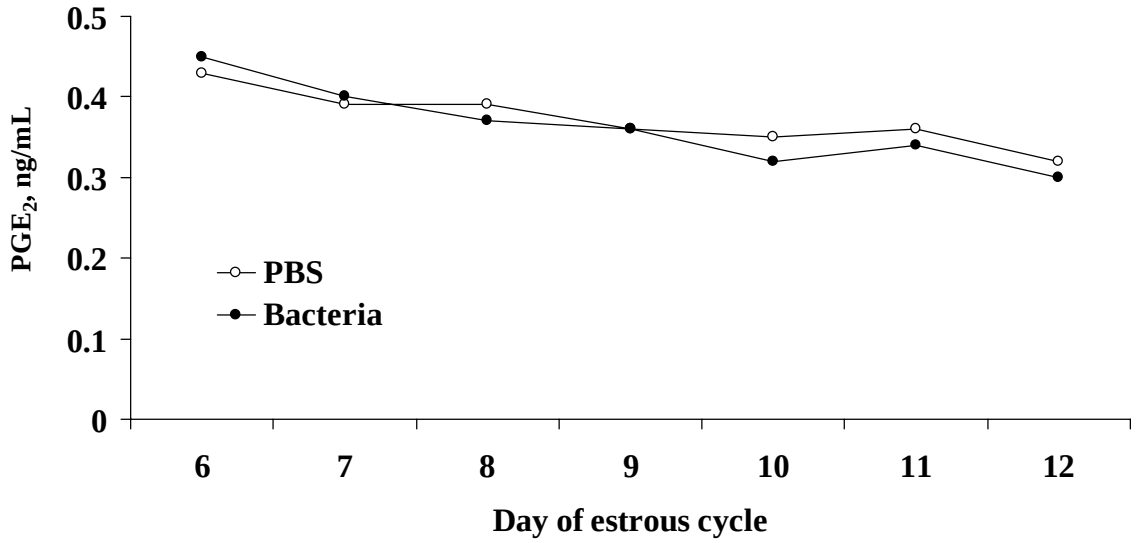

Figure 8. The effect of treatment on PGE_2 in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 of the first treatment cycle. Samples were taken on d 6-12 of the first treatment cycle. There was a significant effect of d of treatment cycle ($P < .01$). There were 18 ewes per treatment, and the SEM = .4.

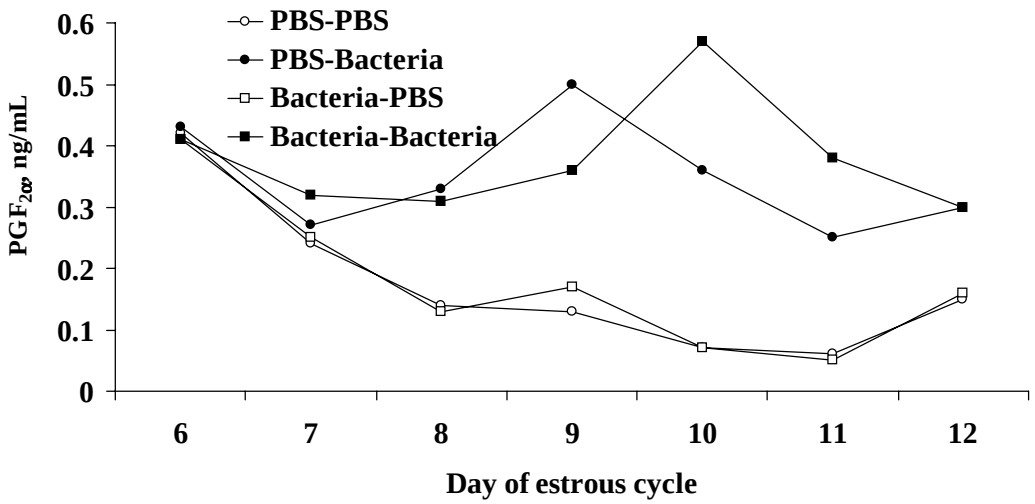

Figure 9. The effect of treatment on $PGF_{2\alpha}$ during the third treatment cycle in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken on d 6-12 of the third treatment cycle. There was a significant effect of treatment, d of treatment cycle, and treatment x d interaction ($P < .01$). There were nine ewes per treatment, and the SEM = .02.

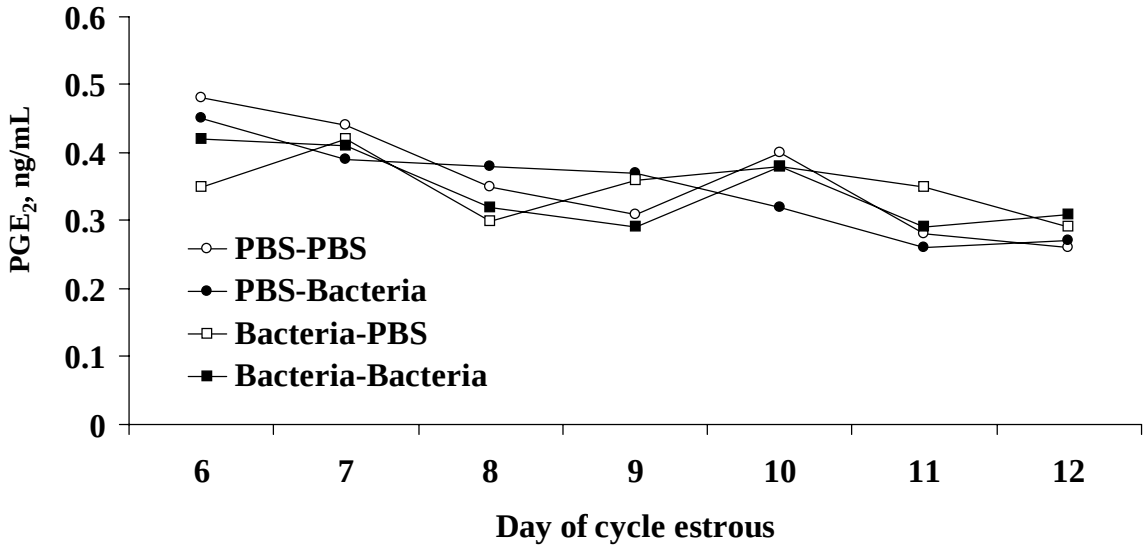

Figure 10. The effect of treatment on PGE_2 during the third treatment cycle in Exp. 3. Ewes were assigned to randomized treatments, treatment cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 of the first and third treatment cycles. Samples were taken on d 6-12 of the third treatment cycle. There was a significant effect day of treatment cycle ($P < .01$). There were nine ewes per treatment, and the SEM = .2.

Chapter VI

PROSTAGLANDIN $F_{2\alpha}$ AND $PGF_{2\alpha}$ SECRETOGUES CHANGE THE IMMUNE RESPONSE IN EWES AFTER EXPOSURE TO INFECTIOUS BACTERIA

ABSTRACT: The uterus is susceptible to infection during the luteal phase, and $PGF_{2\alpha}$, which induces luteolysis, allows the uterus to clear the infection. Thus, we conducted two experiments to determine whether Lutalyse, a $PGF_{2\alpha}$ analogue, alters the uterine immune response to bacterial challenge or whether oxytocin a uterine $PGF_{2\alpha}$-secretogue alters the uterine immune response to bacterial challenge. In Exp. 4, ewes (n = 6/group) were assigned to randomized treatments in a 2 x 2 factorial array; bacterial challenge and Lutalyse were main effects. In Exp 5, ewes (n=6/group) were assigned to randomized treatments in a 2 x 2 factorial array, bacterial challenge and oxytocin were the main effects. Vena caval blood was collected on d 6 through 11 of the estrous cycle. On d 6, each uterus received either 35 X 10^7 cfu of *Escherichia coli* and 75 X 10^7 cfu of *Arcanobacterium pyogenes* in PBS or PBS . On d 9 in Exp 4, ewes received two i.m. injections of either saline or Lutalyse (5 mg with a 5-h interval). On d 9 in Exp. 5, ewes received 200 USP units of oxytocin i.v. or 10 mL of saline. The lymphocyte blastogenic response to mitogens was determined to evaluate the immune status of the ewes, progesterone. Prostaglandin $F_{2\alpha}$ and PGE_2 were quantified, and white blood cells were counted. Uteri were collected at slaughter on d 11. The packed cell volume (PCV) in uterine flushings and the ability to culture *E. coli* and *A. pyogenes* were used to diagnosis uterine infections.

Introduction

Postpartum uterine infections are a major economic problem in the livestock industry. Progesterone usually from the CL seems to down-regulate the uterine immune system and predispose cows and sheep to uterine infections (Lander Chacin et al., 1990). Progesterone seems

to operate by reducing neutrophil and lymphocyte functions. Various prostanoids are known to be involved in regulating the activity of the immune cells.

Lutalyse and other $PGF_{2\alpha}$ analogues are currently the treatment of choice for uterine infections (Gilbert and Schwark, 1992). However, the mechanism of action of $PGF_{2\alpha}$ in treatment of uterine infections is not completely understood. In cows and sheep with active CL, $PGF_{2\alpha}$ induces luteolysis, which reduces progesterone and increases estradiol concentrations within 6 h of the treatment (Fogwell et al., 1978). Those changes in progesterone and estradiol should up-regulate the immune system and permit a cow to clear the uterine infection (Lander Chacin et al., 1990). However, $PGF_{2\alpha}$ is also beneficial in cows without active corpora lutea (Gilbert and Schwark, 1992). Lutalyse induces the uterus to secrete $PGF_{2\alpha}$ (Wade et al., 1996). The increase of endogenous $PGF_{2\alpha}$ following the dose of exogenous $PGF_{2\alpha}$ may lead to stimulation of immune cells and clearance of the infections.

Because Lutalyse causes regression of the CL in sheep it is impossible to determine if uterine infections are cleared as a direct result of injections of Lutalyse or indirectly through regression of the CL. If exogenous $PGF_{2\alpha}$ leads to the secretion of endogenous $PGF_{2\alpha}$, immune cell stimulation and clearance of uterine infections, it should not matter which compound stimulates the release of endogenous $PGF_{2\alpha}$. Oxytocin is a $PGF_{2\alpha}$ secretogue.

Therefore, this study was designed to 1) determine the effects of exogenous $PGF_{2\alpha}$ or oxytocin on immune cell function, and 2) determine if the endogenous release of $PGF_{2\alpha}$ leads to the clearance of uterine infections in sheep.

Materials and Methods

General. Ewes that were 2 to 5 y of age, produced at the Virginia Tech Sheep Center, and that had at least two consecutive estrous cycles of 14 to 17 d in duration were used for this study. All of the ewes were healthy and had no history of uterine infections.

Estrous Synchronization. Ten to 12 d after ewes were detected in estrus they received one half of a Syncro-Mate-B (Sanofi, Overland Park, KS) implant. Implants were removed 10 d after they were inserted. Ewes were penned twice daily with vasectomized rams to check for estrus after synchronization treatment. Ewes not detected in estrus within 60 h after implant removal were not assigned to the experiment. The onset of estrus was considered d 0.

Vena Caval Catheterization. The vena cava was catheterized 24 h before each ewe received intrauterine inoculations. For a full review of the procedure refer to Benoit and Daily (1991). Briefly, the saphenous vein, just dorsal to the right hock during the first estrous cycle and the left hock during the second estrous cycle, was exposed. A 90-cm-long catheter (inner diameter, 0.42 mm; outer diameter, 0.74 mm; ICO-Rally, Palo Alto, CA) was inserted into the vein through a small incision and then passed up through the saphenous vein into the vena cava. The catheters were marked 45, 50, 55, and 60 cm from the external end. One 4-mL blood sample was taken at each marked position on the catheter as it was passed up the vein, and then the catheter was positioned temporarily. Final catheter position was determined according to progesterone concentrations; the greatest progesterone concentration indicated the region where the uteroovarian blood entered the vena cava. The catheters were placed at that position.

Intrauterine Inoculations. For a full review of the laparoscopic procedure, refer to Ramadan et al. (1997) and Wulster-Radcliffe et al. (1999). Briefly, the uterus was visualized with a laparoscope and positioned with both uterine horns in view. The probe used for positioning the uterus was removed and replaced with a 17 g aspiration needle. The needle was

plunged into the uterine horn. A 12 mL syringe containing either PBS or bacterial treatments was attached to the end of the aspiration needle. A second person slowly depressed the plunger of the syringe until the syringe was empty. The needle and syringe were removed.

Inoculations. The strains of *A. pyogenes* and *E. coli* used for this study were isolated from a cow with endometritis at the Virginia Tech Dairy Center (Del Vecchio et al., 1992). Both strains of bacteria were purified and stored in skim-milk broth medium at -20° until they were used to prepare intrauterine inoculations. Inoculations prepared with bacteria produced endometritis in cows (Del Vecchio et al.,1992) and sheep (Ramadan et al.,1997).

The bacteria were cultured in brain-heart infusion broth (Difco, Detroit, MI) to obtain enough colony-forming units to prepare inoculations. The optical density of the cultures was measured periodically at 560 nm to determine bacterial growth. The optical density was used in a regression equation, that was derived from optical densities of cultures and direct counts of the number of colony forming units on blood agar plates after a sample of the cultures had been incubated for 24 h to estimate the number of colony forming units per 10 μL of medium (Ramadan et al., 1997 unpublished data). To prepare inoculations, an appropriate volume of culture medium was transferred into sterile culture tubes and centrifuged at 10 x g for 20 min at 4°C. The supernatant, which can act as a chemoattractant (Hunter et al., 1999 unpublished data) was removed, and the pellet was resuspended in 5 mL of sterile isotonic PBS. The inoculations were held on ice until they were used. A 5 mL volume of the suspension was injected into each uterine horn. On the basis of a dose response curve, each inoculation contained 75×10^7 cfu of *A. pyogenes* and 35×10^7 cfu of *E. coli.*

Lymphocyte Separation and Blastogenic Assay. Heparinized blood was centrifuged at 2,800 x g for 20 min at 4°C. The buffy coat was mixed with 4 mL of HBSS at pH 7.4 (Gibco

BRL, Grand Island, NY). The mixture was layered on top of 4 mL of Ficoll-Paque (Sigma Chemical, St. Louis, MO) and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte-containing portion was transferred to sterile culture tubes. The suspension was cenrifuged 1,000 x g for 10 min at room temperature. The supernatant was removed, and the lymphocytes were washed twice with HBSS. Then cells were suspended in complete RPMI-1640 medium (Gibco) that contained fetal bovine serum (.1 mL/mL; Gibco), pencillin (100 IU/mL; Sigma), and streptomycin (100 mg/mL; Sigma). The number of live lymphocytes were determined using a hemocytometer and a trypan blue dye exclusion procedure. The final concentration of live cells was adjusted to 1×10^6/mL of RPMI-1640.

The blastogenic assay was similar to the one described in Burrells and Wells (1977). Lymphocytes (1×10^5 live cells) from each vena caval sample were cultured in 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ). Mitogenesis was stimulated with Con A (stimulates T-lymphocytes; 1 μg/well; Sigma) or LPS (stimulates B lymphocytes; .5 μg/well; Sigma), or other cells were left unstimulated (100 μL of RPMI-1640/well) so that basal mitogenesis could be estimated. Incubation treatments were in triplicate.

The plates were held at 37° for 48 h in a humidified chamber with an atmosphere of 5% CO_2 in air. A tracer of 1 μCi of [^{3}H]thymidine (specific radioactivity 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in RPMI-1640 was added to each well, and the plates were held under the same conditions for another 16 h. At the end of culture, lymphocyte viability was determined with a trypan blue dye-exclusion procedure, and cells were transferred to fiberglass filters (Whatman, Madistone, England). Filter discs corresponding to each well were transferred to separate scintillation vials, and lymphocytes were solubilized to release [^{3}H]thymidine.

Disintegrations per minute were determined with a liquid scintillation counter and used to calculate the picomoles of [^{3}H]thymidine incorporated into newly synthesized DNA.

Immunoassays. Radioimmunoassays were used to measure progesterone, PGE_2 and $PGF_{2\alpha}$. For catheter positioning and measurement of sample, an [^{125}I]progesterone kit (Diagnostic Products, Los Angeles, CA) was used to quantify progesterone. All samples collected after final catheter positioning were evaluated in a single assay with a CV of 9.9%. The concentrations of PGE_2 and $PGF_{2\alpha}$ were determined using tritated tracers (Lewis et al., 1978). The PGE_2 intra- and interassay CV were 10.9 and 27.2% respectively. The intraassay and interassay CV for $PGF_{2\alpha}$ were 16.2 and 35.3%, respectively.

Slaughter. All ewes were slaughtered on d 11 of the estrous cycle, and the reproductive tracts were collected and examined for signs of infection. Clear uterine flushings with small amounts of sediment, no signs of endometrial inflammation, and the inability to culture *A. pyogenes* and *E. coli* indicated that the uterus was not infected. Cloudy or colored uterine flushings with large amounts of sediment, inflamed endometrium, and the ability to culture *A. pyogenes* and *E. coli* indicated that the uterus was infected.

Statistical Analysis. The General Linear Models procedures of the Statistical Analysis System (SAS, 1985) were used to analyze the data. The GLM model for Exp. 4 included the dependent variables: bacteria, Lutalyse, bacteria x Lutalyse, ewe nested within bacteria and Lutalyse, time, bacteria x time, Lutalyse x time, and bacteria x Lutalyse x time. Ewe nested within bacteria and Lutalyse was the main plot error term, and the residual was the subplot error term. The GLM model for Exp. 5 included the dependent variables: bacteria, oxytocin, bacteria x oxytocin, ewe nested within bacteria and oxytocin, time, bacteria x time, oxytocin x time, and bacteria x oxytocin x time. Ewe nested within bacteria and oxytocin was the main plot error

term, and the residual was the subplot error term. The variance associated with the main plot error term was used to calculate overall standard errors (SEM) associated with main plot variables. When appropriate, the PDIFF (i.e., a method for comparing all possible least squares means) or the Duncan's option in SAS was used to compare individual means.

The shapes of the response curves for basal lymphocyte blastogenic activity were determined for ewes in each treatment group, and then basal activity was used as a covariant for Con A- and LPS-stimulated lymphocytes. The incorporation data were reported after the adjustment for basal activity rather than reporting a stimulation index. The reason for this is because a stimulation index represents a percentage change from basal activity, and the index is based on the assumption that the relationship between basal activity and stimulated incorporation is linear, which was not always true for the data from this experiment. The prediction option in SAS was used to generate a new set of data that contained the adjusted values for Con A and LPS. The adjusted values were analyzed with the original GLM model.

Experiment 4: Does Lutalyse change the uterine immune response to infectious bacteria differ during the luteal phases of the estrous cycle in ewes?

Experimental Design. In Exp. 4, the effects of intrauterine bacterial inoculation of *A. pyogenes* and *E. coli* and Lutalyse on uterine immune function were evaluated. Bacterial inoculation and Lutalyse were main effects. Ewes (n=6/treatment) were assigned to one of four randomized treatments: 1) PBS-saline, 2) PBS-Lutalyse, 3) bacteria-saline, and 4) bacteria-Lutalyse.

Estrus was synchronized. Vena caval catheters were inserted on d 5 of the estrous cycle. Twelve h after inserting vena caval catheters, the uteri were infused with either saline or bacteria. More specifically, on d 6, uteri were inoculated with either 35×10^7 cfu of E. coli and 75×10^7

cfu of A. pyogenes in PBS (10 mL) or PBS. On d 9 in Exp 4, ewes received two i.m. injections of either saline or Lutalyse (5 mg with a 5-h interval).

Blood Sampling. Vena caval blood was collected twice daily between d 6 and 11. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12-mL sample was taken from the catheter placed caudal to the site where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple ewes, blood samples were kept in an ice-water bath until centrifuged for the collection of plasma and the buffy coat. Before centrifugation on d 6, 8, 10, a small portion of the heparinized sample was used for differential WBC. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for blastogenic assay. The lymphocyte blastogenic response to mitogens was measured. Plasma samples assayed for progesterone and for prostaglandins ($PGF_{2\alpha}$ and PGE_2). Blood samples were collected twice daily approximately 12 h apart. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin et al., 1994).

Experiment 5: Does Oxytocin change the uterine immune response to infectious bacteria differ during the luteal phases of the estrous cycle in ewes?

Experimental Design. In Exp. 5, the effects of intrauterine bacterial inoculation of *A. pyogenes* and *E. coli* and oxytocin on uterine immune function were evaluated. Bacterial inoculation and oxytocin were main effects. Ewes (n=6/treatment) were assigned to one of four randomized treatments: 1) PBS-saline, 2) PBS-oxytocin, 3) bacteria-saline, and 4) bacteria-oxytocin.

Estrus was synchronized. Vena caval catheters were inserted on d 5 of the estrous cycle. Twelve h after inserting vena caval catheters, the uteri were infused with either saline or bacteria. More specifically, on d 6, uteri were inoculated with either 35×10^7 cfu of *E. coli* and 75×10^7

cfu of *A. pyogenes* in PBS (10 mL) or PBS. On d 9 in Exp. 5, ewes received 200 USP units of oxytocin i.v. or 10 mL of saline.

Blood Sampling. Vena caval blood was collected twice daily between d 6 and 11. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12-mL sample was taken from the catheter placed caudal to the site where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple ewes, blood samples were kept in an ice-water bath until centrifuged for the collection of plasma and the buffy coat. Before centrifugation on d 6, 8, and 10, a small portion of the heparinized sample was used for differential WBC. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for blastogenic assay. The lymphocyte blastogenic response to mitogens was measured. Plasma samples were assayed for progesterone and for prostaglandins ($PGF_{2\alpha}$ and PGE_2). Blood samples were collected twice daily approximately 12 h apart. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin et al., 1994).

Results

Experiment 4.

Uterine Infections. A ewe was diagnosed with a uterine infection if the PCV of the uterine flushing obtained at slaughter was greater than 1%, and if we were able to culture *A. pyogenes* and *E. coli* from the uterine flush (Figure 1). All of the ewes inoculated with *A. pyogenes* and *E. coli* on d 6 of the estrous cycle developed uterine infections, but ewes that received bacteria on d 6 and Lutalyse on d 9 had less severe uterine infections than ewes that received bacteria on d 6 and saline on d 9 (PCV = 2 vs 18%; $P < .01$; Figure 1). None of the ewes inoculated with PBS on d 6 estrous cycle developed infection (Figure 1). Because ewes were not

slaughtered until the end of the estrous cycle, we do not have an accurate measure of the development of uterine infections after the bacterial inoculation, but before the administration of Lutalyse.

Lymphocyte Blastogenic Activity. Basal incorporation of [^{3}H]thymidine into newly formed lymphocytes was greater ($P < .01$) for ewes that received Lutalyse on d 9 than ewes that received saline (12.2 vs 1.3 pmol, SEM = .5). Lipolysaccharide-stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was greater ($P < .01$) for ewes that received Lutalyse on d 9 than ewes that received saline (8.8 vs 4.9 pmol, SEM = .5). Concanavalin-A stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was not different between treatments.

Differential White Blood Cell Counts. Treatment with Lutalyse increased neutrophils (33 vs 45/100 WBC; $P < .01$) and decreased lymphocytes (52 vs 36/ 100 WBC; $P < .01$). There were no differences observed with monocyte or eosinophil numbers.

Steroids and Prostaglandins. Progesterone concentrations were less ($P < .01$) after treatment with Lutalyse vs treatment with saline (3.2 vs .4 ng/mL; SEM = .8; Figure 2).

Vena caval $PGF_{2\alpha}$ increased in ewes treated with Lutalyse in comparison to ewes treated with saline (.35 vs .11 ng/mL; SEM=.07; Figure 3), and $PGF_{2\alpha}$ concentrations ($P < .01$) changed with time after inoculation (Figure 3). Neither the main effects nor the interactions affected vena caval concentrations of PGE_2.

Experiment 5.

Uterine Infections. A ewe was diagnosed with a uterine infection if the PCV of the uterine flushing obtained at slaughter was greater than 1%, and if we were able to culture *A. pyogenes* and *E. coli* from the uterine flush (Figure 4). All of the ewes inoculated with *A.*

pyogenes and *E. coli* on d 6 of the estrous cycle developed uterine infections, but ewes that received bacteria on d 6 and oxytocin on d 9 had less severe uterine infections than ewes that received bacteria on d 6 and saline on d 9 (PCV = 8% vs 18%; $P < .01$; Figure 4). None of the ewes inoculated with PBS on d 6 estrous cycle developed infection (Figure 4). Because ewes were not slaughtered until the end of the estrous cycle, we do not have an accurate measure of the development of uterine infections after the bacterial inoculation, but before the administration of oxytocin.

Lymphocyte Blastogenic Activity. Treatment with oxytocin did not change basal incorporation of [^{3}H]thymidine or Con A- or LPS- stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes.

Differential White Blood Cell Counts. Day of estrous cycle affected ($P < .05$; Figure 6) the numbers of neutrophils. Treatment with oxytocin increased neutrophils (Figure 6; $P < .01$) and decreased lymphocytes (Figure 6; $P < .01$).

Steroids and Prostaglandins. Neither treatment nor d of estrous cycle affected vena caval progesterone. Mean progesterone concentrations of ewes between d 6 and 12 was 4.2 ng/mL (SEM=.6).

Vena caval $PGF_{2\alpha}$ increased in ewes treated with Lutalyse compared to ewes treated with saline (.48 vs .08 ng/mL; SEM=.04), and $PGF_{2\alpha}$ concentrations ($P < .01$) changed with time after inoculation (Figure 5). Vena caval PGE_2 concentrations did not change with treatments.

Discussion

Treatment with Lutalyse results in the clearance of uterine infections in cattle with and without active CL. After luteal regression, most uterine infections are cleared. It is not known whether clearance of these infections is related to the concomitant decrease in progesterone

during luteolysis, an increase in endogenous $PGF_{2\alpha}$, or a combination of the two events. Clearance of uterine infections following treatment with Lutalyse in the absence of a CL suggests that exogenous $PGF_{2\alpha}$ changes uterine immune function independent of luteolysis.

Although sheep provide an inexpensive, ruminant model to study "typical" uterine infections and clearance of these infections, there are several physiological limitations associated with this model. In particular, it is difficult to study the effects of exogenous $PGF_{2\alpha}$ treatment independently of luteolysis, because exogenous $PGF_{2\alpha}$ causes luteolysis. To overcome these limitations, we adjusted our sheep model. Administration of Lutalyse results in an increase in endogenous $PGF_{2\alpha}$. If the increase in endogenous $PGF_{2\alpha}$ mediates changes in the immune response that lead to clearance of uterine infections independently of luteolysis, it should not matter how the endogenous release of $PGF_{2\alpha}$ is stimulated. Therefore, we administered oxytocin as a $PGF_{2\alpha}$ secretogogue to initiate pulsatile releases of endogenous $PGF_{2\alpha}$ without causing luteolysis.

Treatment of sheep with Lutalyse ad oxytocin resulted in the clearance or partial clearance of uterine infections in these experiments. In sheep treatment with Lutalyse caused regression of the CL, which was confirmed by decreases in vena caval progesterone concentrations and visual appraisal of the ovaries at slaughter. Treatment with oxytocin did not cause regression of the CL. Infections in sheep were completely cleared following administration of Lutalyse, but only partially cleared following administration of oxytocin.

Lutalyse and oxytocin administration resulted in increased concentrations of $PGF_{2\alpha}$ in vena caval blood in sheep. A recent study with sheep indicated that Lutalyse induced uterine secretion of $PGF_{2\alpha}$, which required an increase in the availability of free endometrial arachidonic acid (Wade and Lewis, 1996). Free arachidonic acid can be converted through the

cyclooxygenase pathway to various prostaglandins and through the lipoxygenase pathway to LTB4. In addition to the chemotactic effects of LTB4 and $PGF_{2\alpha}$, LTB4 is a potent activator of neutrophil function, and $PGF_{2\alpha}$ reduces intracellular cAMP concentrations. Reduced cAMP is associated with activated immune cells. The increased uterine secretion of $PGF_{2\alpha}$ may be an important component of the uterine defense mechanism.

Following administration of $PGF_{2\alpha}$, neutrophil numbers in circulation decreased regardless of bacterial inoculation. This further supports the chemotactic role of $PGF_{2\alpha}$ in the uterine lumen following administration of Lutalyse.

Vena caval lymphocytes from ewes that were administered Lutalyse had increased capacities for basal and LPS- stimulated incorporation of [3H]thymidine than did the lymphocytes from ewes that receive saline. Changes in vena caval blood lymphocyte population corresponded to the changes in lymphocyte activity. It is difficult to determine if changes in lymphocyte population are both related to treatment or if the decrease in one is related to an increase in the other.

In order to further evaluate the role of endogenous $PGF_{2\alpha}$ in the clearance of uterine infections we used a $PGF_{2\alpha}$ secrotogue that does not cause luteolysis. Following administration of oxytocin, progesterone concentrations did not decrease, suggesting that luteolysis did not occur. This was confirmed by observing the ovaries at slaughter. Ewes that received oxytocin treatment after intrauterine inoculation of bacteria had less severe infections. The infections were not completely cleared. This suggests that dose or administration protocols could be inadequate, or that luteolysis and the decrease in progesterone is a crucial portion of this mechanism. There was a decrease in neutrophils in vena caval blood, suggesting movement if the neutrophils toward the chemotactic eicosanoids. There were also increases in lymphocyte numbers in vena

caval blood. However, in this case, I believe that the increase in lymphocytes is an artifact of changes in the population of neutrophils, because oxytocin treatments did not increase basal, Con A- or LPS-stimulated blastogenesis. This suggests that the secretion of uterine $PGF_{2\alpha}$ in the response to bacteria or $PGF_{2\alpha}$ -secretogues is modulating clearance of uterine infections through an innate immune response.

Implications

Lutalyse decreases the severity of uterine infections in sheep. Typically, it is believed that Lutalyse exerts it effects by causing regression of the CL; however, benefit of the administration of Lutalyse in the absence of a CL suggest another mechanism. Treating uterine infections with Lutalyse may be possible at any point in the cycle.

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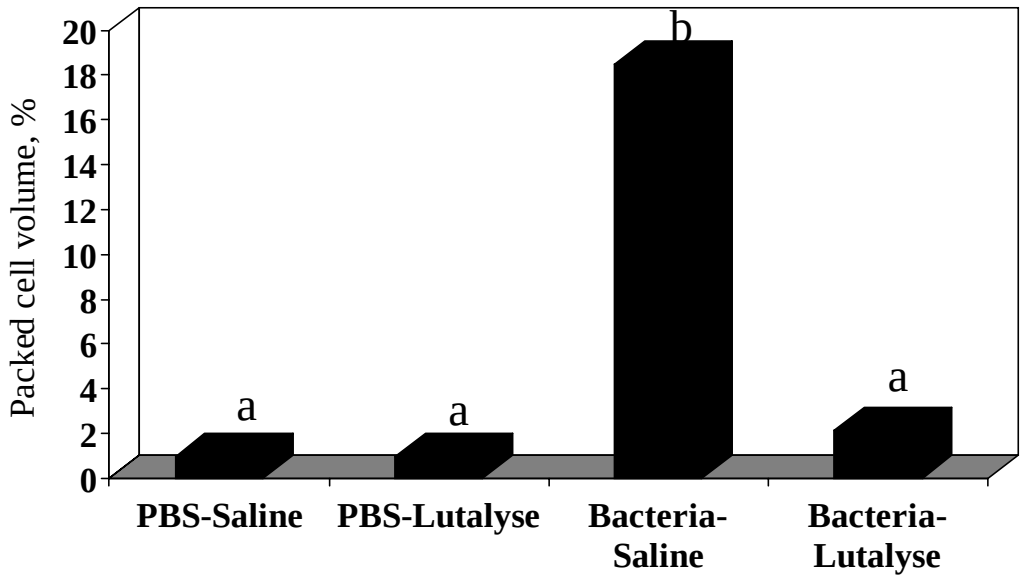

Figure 1. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 4. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or Lutalyse on d 9. Uteri were collected and flushed. Packed cell volume of the uterine flushings were measured. [a,b]Different superscripts indicate that means differed between treatment groups ($P < .01$), The SEM = 1.7, and there were six ewes per treatment.

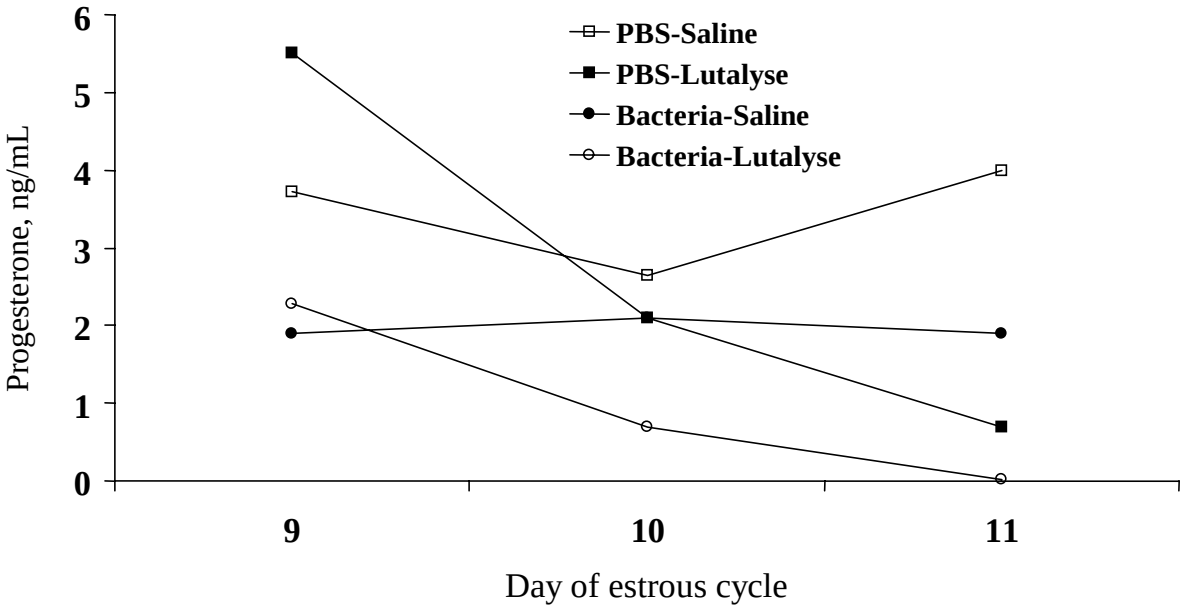

Figure 2. The effect of treatment on progesterone concentration on Exp. 4. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or Lutalyse on d 9. Samples were taken on d 9 to 11. There was a significant affect of Lutalyse ($P < .01$) on progesterone concentration. The SEM = .8, and there were six ewes per treatment.

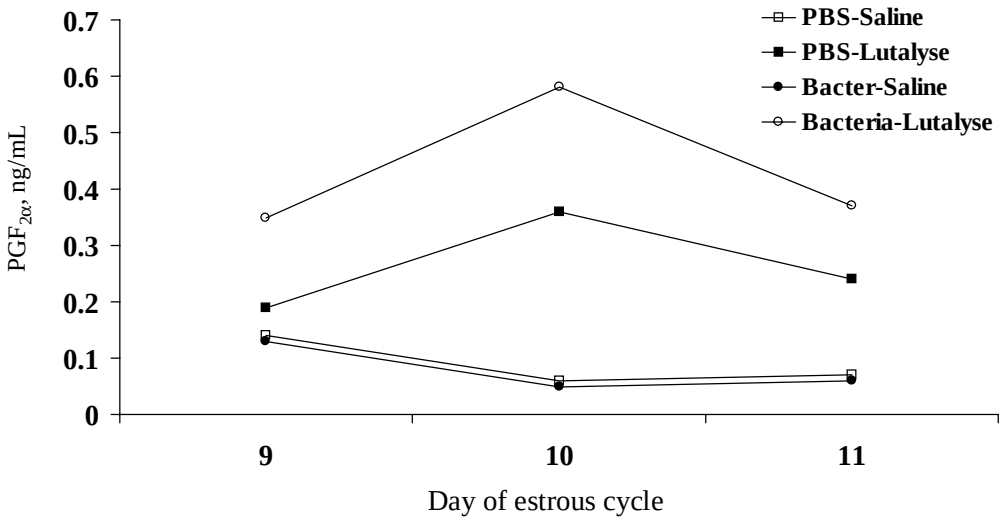

Figure 3. The effect of treatment on vena caval $PGF_{2\alpha}$ concentration on Exp. 4. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or Lutalyse on d 9. Samples were taken on d 9 to 11. There was a significant affect of Lutalyse ($P < .01$) on $PGF_{2\alpha}$ concentration. The SEM = .07, and there were six ewes per treatment.

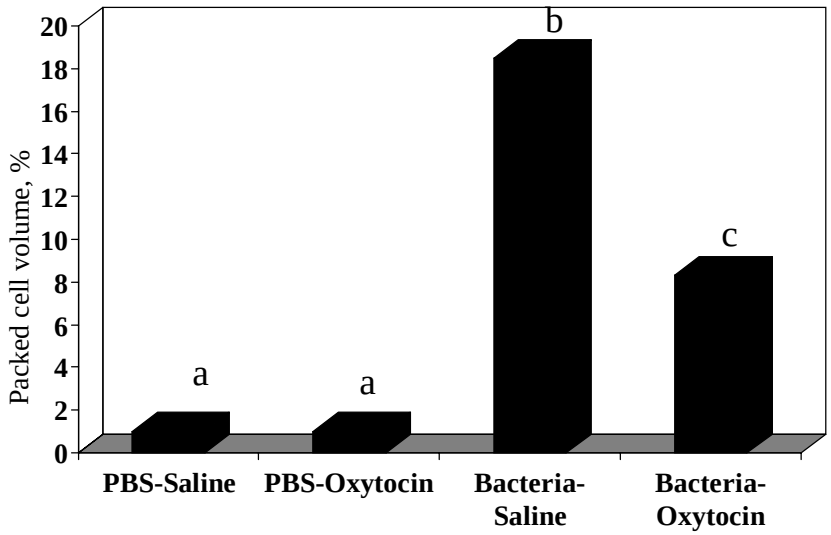

Figure 4. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 5. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or oxytocin on d 9. Uteri were collected and flushed. Packed cell volume of the uterine flushings were measured. [a,b]Different superscripts indicate that means differed between treatment groups ($P < .01$), The SEM = 1.3, and there were six ewes per treatment.

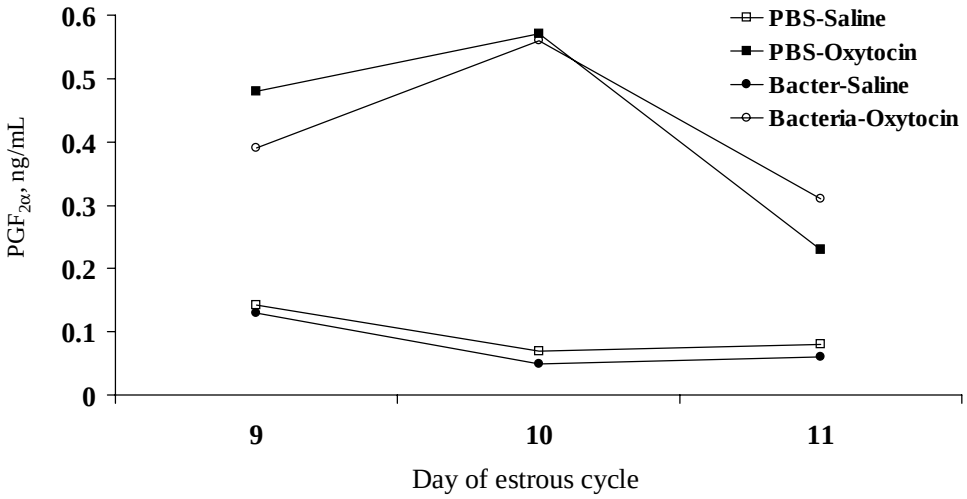

Figure 5. The effect of treatment on vena caval $PGF_{2\alpha}$ concentration on Exp. 5. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or oxytocin on d 9. Samples were taken on d 9 to 11. There was a significant affect of oxytocin ($P < .04$) on progesterone concentration. The SEM = .8, and there were six ewes per treatment.

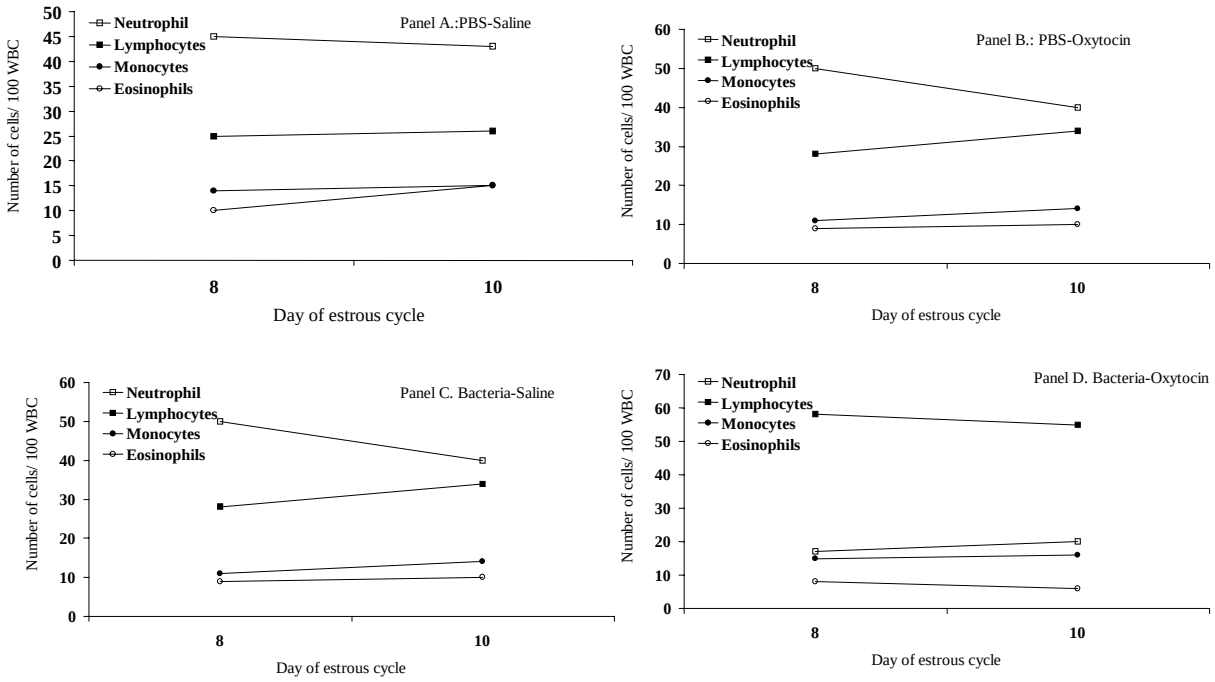

Figure 6. The effect of treatment on WBC on Exp. 5. Ewes were assigned to randomized treatments, estrous cycles were synchronized, and ewes received intrauterine inoculations (PBS or Bacteria) on d 6 and i.m. injections of saline or Lutalyse on d 9. Samples were taken on d 9 to 11. There was a significant affect of Lutalyse ($P < .01$) on WBC populations. There were six ewes per treatment. Neutrophils (SEM = 1.4); Lymphocytes (SEM = 1.2); Monocytes (SEM = .4); Eosinophils (SEM = .5)

Chapter VII

PROSTAGLANDIN $F_{2\alpha}$ CHANGES THE IMMUNE RESPONSE IN SOWS AFTER EXPOSURE TO INFECTIOUS BACTERIA

ABSTRACT: The uterus is susceptible to infection during the luteal phase, and $PGF_{2\alpha}$, allows the uterus to clear the infection. Thus, we conducted an experiment to determine whether Lutalyse, a $PGF_{2\alpha}$ analogue, alters the uterine immune response to bacterial challenge. In Exp. 6, sows (n = 6/group) were assigned to randomized treatments in a 2 x 2 factorial array; bacterial challenge and Lutalyse were main effects. Vena caval blood was collected on d 7 through 11 of the estrous cycle. On d 7, each uterus received either 70 X 10^7 cfu of *E. coli* and 150 X 10^7 cfu of *A. pyogenes* in PBS or PBS . On d 9 in Exp 6, sows received an i.m. injection of either saline or Lutalyse (10 mg). The lymphocyte blastogenic response to mitogens was determined to evaluate the immune status of the sows. Prostaglandin $F_{2\alpha}$ and PGE_2 were quantified, and white blood cells were counted. Uteri were collected at slaughter on d 11. The packed cell volume (PCV) in uterine flushings and the ability to culture *E. coli* and *A. pyogenes* were used to diagnosis uterine infections. Uterine infections were more severe in sows that did not receive Lutalyse; however, Lutalyse did not completely clear the infections. Progesterone concentrations did not change following administration of Lutalyse indicating that luteolysis did not occur. Lymphocyte blastogenesis did not change in response to treatments. However, following administration of Lutalyse, $PGF_{2\alpha}$ concentrations increased and neutrophil numbers changed. Clearance of uterine infections following administration of Lutalyse and in the absence of changes in progesterone in pigs is probablly mediated through a change in neutrophil rather than lymphocyte activity.

Introduction

The major determinant of lifetime productivity in female livestock is reproductive performance (Lewis, 1997). Reproductive disorders are the second most prevalent reason for culling breeding sows in intensive management systems in the United States (M. Estienne, personal communication). The incidence and type of reproductive disorders has not been categorized in the swine industry. The incidence of postpartum uterine infections and the subsequent culling rates in sows is unknown. Because, postpartum uterine infections are associated with periparturient hypogalactia syndrome, they are often classified as a lactation problem rather than a reproductive problem, therefore, biasing the limited statistics available on uterine infections in swine. The number of sows or gilts affected by periparturient hypogalactia syndrome and therefore exhibiting signs of uterine infections during a farrowing is highly dependent on environment and management system. From 10 to 100% of gilts or sows are affected during a farrowing (Martineau et al., 1999).

Progesterone, usually from the corpus luteum, seems to down-regulate the uterine immune system and predispose livestock to uterine infections. Progesterone seems to operate by reducing neutrophil and lymphocyte functions. Various prostanoids are known to be involved in regulating the activity of immune cells. Prostaglandin E_2, $PGF_{2\alpha}$ and LTB_4 are especially active; PGE_2 is a potent suppressor of neutrophil and lymphocyte activity, and $PGF_{2\alpha}$ and LTB_4 are potent activators.

To date, the research in our laboratory and much of the research already discussed in this dissertation has focused on the use of sheep as a model to study uterine infections in dairy cows. However, there are some inherent problems with this model. Regression of the CL, results in clearance of bacterial infections in sheep and cows. The absence of progesterone seems to up-regulate the uterine immune system leading to the clearance of infections. Injections of Lutalyse

during the luteal phase 3 to 5 d after intrauterine inoculation of bacteria leads to the clearance of uterine infections. Concurrently, Lutalyse causes activation of immune cells and regression of the CL in sheep. In a sheep model it is impossible to separate the direct effects of Lutalyse on immune cells from the direct effects of Lutalyse on the CL. Infections may be being cleared as a result of activation of immune cells, a reduction in progesterone, and(or) a combination of these two effects.

To study the direct effects of $PGF_{2\alpha}$ on uterine infections, we must develop a model in which Lutalyse can be used in vivo without causing regression of the CL. The simplest method for doing this is to begin studying uterine infections in other species. For instance, injections of Lutalyse into sows or gilts before d 12 of the estrous cycle does not cause regression of the CL. Using sows or gilts to study interactions between prostaglandins and uterine infections allows us to study the effects of $PGF_{2\alpha}$ independently of regression of the CL.

In cows, sheep, and rabbits the resistance to uterine infections is greatest at estrus (Black et al., 1953a,b; Rowson et al., 1953; Ramadan et al., 1997). It has been speculated that in pigs some bacteria are able to survive this phase and multiply, leading to infections during the luteal phase associated with increased embryonic death (Scofield et al., 1974).

This study was conducted with sows to evaluate 1) the immune response to bacterial challenge and Lutalyse during the luteal phase of the estrous cycle, 2) the ability of $PGF_{2\alpha}$ to modulate in vivo primed lymphocytes in vitro, and 3) the value of pigs for detailed studies of uterine infections.

Materials and Methods

Experiment 6: Does Lutalyse change the uterine immune response to infectious bacteria during the luteal phases of the estrous cycle in sows?

General. Second and third parity sows, produced at the Virginia Tech Swine Center were used for this study. All of the sows were healthy and had no history of uterine infections.

Estrous Synchronization. Estrous cycles were synchronized at weaning. Sows were penned twice daily with boars beginning at weaning. Following second estrous cycle post weaning, gilts were assigned to treatment. The onset of estrus was considered d 0.

Experimental Design. In Exp. 6, the effects of intrauterine bacterial inoculation of *A. pyogenes* and *E. coli* and Lutalyse on uterine immune function were evaluated. Bacterial inoculation and Lutalyse were main effects. Sows (n=6/treatment) were assigned to one of four randomized treatments: 1) PBS-saline, 2) PBS-Lutalyse, 3) bacteria-saline, and 4) bacteria-Lutalyse.

Estrus was synchronized. Vena caval catheters were inserted on d 7 of the estrous cycle. Twelve h after inserting vena caval catheters, the uteri were infused with either saline or bacteria. More specifically, on d 7, uteri were inoculated with either 70 x 10^7 cfu of *E. coli* and 150 x 10^7 cfu of *A. pyogenes* in PBS (10 mL) or PBS. On d 9 in Exp 6, sows received an i.m. injections of either saline or Lutalyse (10 mg).

Blood Sampling. Vena caval blood was collected twice daily between d 7 and 11. At each blood collection, a 2-mL sample was taken from each catheter and discarded. Immediately after, a 12 mL sample was taken from the catheter placed caudal to the site where uteroovarian blood enters the vena cava. Blood was collected into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). During collection from multiple sows, blood samples were kept in an ice-water bath until centrifuged for the collection of plasma and the buffy coat. Before centrifugation, a small portion of the heparinized sample was used for differential WBC. Plasma samples were stored at -20° until they were assayed. Lymphocytes were separated from the buffy coat for

blastogenic assay. The lymphocyte blastogenic response to mitogens was measured. Plasma samples assayed for steroid hormones (progesterone and estradiol-17β) and for prostaglandins ($PGF_{2\alpha}$ and PGE_2). Blood samples were collected twice daily approximately 12 h apart. Plasma from the samples was pooled at equal volumes to provide an average daily concentration (Fortin et al, 1994).

Vena Caval Catheterization. The vena cava was catheterized 12 h before each sow received intrauterine inoculations. For a full review of the procedure refer to Benoit and Daily, 1991. Sows were anesthetized using a combination of xylazine (2mg/mL of BW) and Telazol (3mg/kg). The right rear leg was anesthetized dorsal and lateral to the hock with 6 to 10 mL of 2% lidocaine hydrochloride. The skin was incised approximately 3 cm dorsal to the hock and 2 cm lateral to the Achilles tendon, the subcutaneous fat was separated, and the saphenous vein was exposed (Benoit and Daily, 1991). A 180-cm-long catheter (inner diameter, 0.42 mm; outer diameter, 0.74 mm; ICO-Rally, Palo Alto, CA) was inserted into the vein through a small incision and then passed up through the saphenous vein into the vena cava. The catheters were marked 50, 55, 60, and 65 cm from the internal end. The external end of the catheter was positioned within the subcutaneous fat of the hind leg, the rump, and the back. A trocar and cannula were used to "tunnel" under the skin. The external end of the catheter was ultimately placed in a velcro pocket glued to the center of the sows' back to ensure that the sows would not pull out catheter. One 4-mL blood sample was taken at each marked position on the catheter as it was passed up the vein, then the catheter was positioned temporarily. The final catheter position was determined according to progesterone or estradiol-17β concentrations depending on which d of the estrous cycle that the surgery was performed; the greatest progesterone or estradiol-17β

concentration indicated the region where the uteroovarian blood entered the vena cava. The catheters were placed at that position.

Intrauterine Inoculations. For a full review of the laparoscopic procedure refer to Ramadan et al. (1997) or Wulster-Radcliffe et al. (1999). Sows were anesthetized with the same combination of drugs as was used for inserting vena caval catheters. Briefly, sows were placed at a 45° angle in the dorsal recumbent position. A 2-cm incision was made in between the first and second most dorsal mammary glands on each side of the sow. The subcutaneous fat was blunt dissected away from the body wall. After visualizing the body wall, a trocar and cannula were stabbed through the body wall in each of the incisions. The abdominal cavity was insulflated with CO_2. The trocars were removed and replaced with a laparoscope and a probe. The uterus was visualized with a laparoscope and positioned with both uterine horns in view. The probe used for positioning the uterus was removed and replaced with a 17 g aspiration needle. The needle was plunged into the uterine horn. A 20 mL syringe containing either PBS or bacterial treatments was attached to the end of the aspiration needle. A second person slowly depressed the plunger of the syringe until the syringe was empty. The needle and syringe were removed.

Inoculations. The strains of *A. pyogenes* and *E. coli* used for this study were isolated from a cow with endometritis at the Virginia Tech Dairy Center. Both strains of bacteria were purified and stored in skim-milk broth medium at -20° until they were used to prepare intrauterine inoculations. Inoculations prepared with bacteria produced endometritis in cows (Del Vecchio et al., 1992) and sheep (Ramadan et al.,1997). The effectiveness of bacteria and dose for pigs was evaluated before the study began (Wulster-Radcliffe et al., unpublished data).

The bacteria were cultured in brain-heart infusion broth (Difco, Detroit, MI) to obtain enough colony-forming units to prepare inoculations. The optical density of the cultures was

measured periodically at 560 nm to determine bacterial growth. The optical density was used in a regression equation, which was derived from optical densities of cultures and direct counts of the number of colony forming units on blood agar plates after a sample of the cultures had been incubated for 24 h to estimate the number of colony forming units per 10 μL of medium. To prepare inoculations, an appropriate volume of culture medium was transferred into sterile culture tubes and centrifuged at 10 x g for 20 min at 4°C. The supernatant, which can act as a chemoattractant (Hunter et al., unpublished data), was removed, and the pellet was resuspended in 10 mL of sterile isotonic saline. The inoculations were held on ice until they were used. Five mL of the suspension was injected into each uterine horn. On the basis of a dose response curve, each inoculation contained 150 x 10^7 cfu of *A. pyogenes* and 70 x 10^7 cfu of *E. coli.*

Lymphocyte Separation and Blastogenic Assay. Heparinized blood was centrifuged at 2,800 x g for 20 min at 4°C. The buffy coat was mixed with 4 mL of HBSS at pH 7.4 (Gibco BRL, Grand Island, NY). The mixture was layered on top of 4 mL of Ficoll-Paque (Sigma Chemical, St. Louis, MO) and centrifuged at 400 x g for 30 min at room temperature. The lymphocyte containing portion was transferred to sterile culture tubes. The suspension was centrifuged 1,000 x g for 10 in at room temperature. The supernatant was removed, and the lymphocytes were washed twice with HBSS. The cells were then suspended in complete RPMI-1640 medium (Gibco) that contained fetal bovine serum (.1 mL/mL; Gibco), pencillin (100 IU/mL; Sigma), and streptomycin (100 mg/mL; Sigma). The number of live lymphocytes were determined using a hemocytometer and a trypan blue dye exclusion procedure. The final concentration of live cells was adjusted to 1 x 10^6/mL of RPMI-1640.

The blastogenic assay was similar to the one described in Burrells and Wells (1977). Lymphocytes (1 x 10^5 live cells) from each vena caval sample were cultured in 96-well

microtiter plates (Becton Dickinson, Lincoln Park, NJ). Mitogenesis was stimulated with Con A (stimulates T-lymphocytes; 1 μg/well; Sigma) or LPS (stimulates B lymphocytes; .5 μg/well; Sigma), or other cells were left unstimulated (100 μL o RPMI-1640/well) so that basal mitogenesis could be estimated. Incubation treatments were in triplicate.

The plates were held at 37° for 48 h in a humidified chamber with an atmosphere of 5% CO_2 in air. [^{3}H]Thymidine (1 μCi; specific radioactivity 4 Ci/mmol; ICN Radiochemicals, Irvine, CA) in RPMI-1640 was added to each well, and the plate were held under the same conditions for another 16 h. At the end of culture, lymphocyte viability was determined with a trypan blue dye-exclusion procedure, and cells were transferred to fiberglass filters (Whatman, Madistone, England). Filter discs corresponding to each well were transferred to separate scintillation vials, and lymphocytes were solubilized to release [^{3}H]thymidine. Disintegrations per minute were determined with a liquid scintillation counter and used to calculate the pmol of [^{3}H]thymidine incorporated into newly synthesized DNA.

Immunoassays. Radioimmunoassays were used to measure progesterone, estradiol-17β, and PGE_2. For catheter positioning and measurement of sample, a [^{125}I]progesterone and [^{125}I]estradiol-17β RIA kits (Diagnostic Products, Los Angeles, CA) were used. All samples for progesterone and estradiol 17-β collected after final catheter positioning were evaluated for both studies in a single progesterone and single estradiol-17β assay with a CV of 7.6 and 8.7%, respectively. The concentration of PGE_2 was determined using tritated tracer (Lewis et al., 1978). The PGE_2 intra- and interassay CV for Exp. 6 were 16.2 and 21.4% respectively. The PGE_2 intra- and interassay CV for Exp. 2 were 9.7 and 21.4% respectively.

The concentrations of $PGF_{2\alpha}$ were determined with an EIA (Del Vecchio et al., 1992; Fortin et al., 1994). The intraassay and interassay CV for $PGF_{2\alpha}$ for Exp. 6 were 9.9 and 27.2%, respectively.

Slaughter. All sows were killed at the end of the experiment. Sows were anesthetized with sodium pentobarbital (65 mg/mL) and than electrocuted using a 220 V electrocution set. Immediately after slaughter, the reproductive tracts were collected and examined for signs of infection. Clear uterine flushings with small amounts of sediment, no signs of endometrial inflammation, and the inability to culture *A. pyogenes* and *E.coli* indicated that the uterus was not infected. Cloudy or colored uterine flushings with large amounts of sediment, inflamed endometrium, and the ability to culture *A. pyogenes* and *E.coli* indicated that the uterus was infected. Ovaries were also observed to confirm stage of the estrous cycle.

Statistical Analysis. The General Linear Models procedures of the Statistical Analysis System (SAS, 1985) were used to analyze the data. The GLM model for Exp. 6 included the dependent variables: bacteria, Lutalyse, bacteria x Lutalyse, sow nested within bacteria and Lutalyse, time, bacteria x time, Lutalyse x time, and bacteria x Lutalyse x time. Sow nested within bacteria and Lutalyse was the main plot error term, and the residual was the subplot error term. The variance associated with the main plot error term was used to calculate overall standard errors (SEM) associated with main plot variables. When appropriate, the PDIFF (i.e., a method for comparing all possible least squares means) or the Duncan's option in SAS was used to compare individual means.

The shapes of the response curves for basal lymphocyte blastogenic activity were determined for sows in each treatment group, and then basal activity was used as a covariant for Con A- and LPS-stimulated lymphocytes. The incorporation data were reported after the

adjustment for basal activity rather than reporting a stimulation index. The reason for this is because a stimulation index represents a percentage change from basal activity, and the index is based on the assumption that the relationship between basal activity and stimulated incorporation is linear, which was not always true for the data from this experiment. The prediction option in SAS was used to generate a new set of data that contained the adjusted values for Con A and LPS. The adjusted values were analyzed with the original GLM model.

Results

Experiment 6.

Uterine Infections. A sow was diagnosed with a uterine infection if the PCV of the uterine flushing obtained at slaughter was greater than 5%, and if we were able to culture *A. pyogenes* and *E. coli* from the uterine flush (Figure 1). All of the sows inoculated with *A. pyogenes* and *E. coli* on d 7 of the estrous cycle developed uterine infections, but sows that received bacteria on d 7 and Lutalyse on d 9 had less severe uterine infections than sows that received bacteria on d 7 and saline on d 9 (PCV = 25 vs 67%; $P < .01$; Figure 1). None of the sows inoculated with PBS on d 7 estrous cycle developed infection (Figure 1). Because sows were not slaughtered until the end of the estrous cycle, we do not have an accurate measure of the development of uterine infections after the bacterial inoculation, but before the administration of Lutalyse.

Lymphocyte Blastogenic Activity. Basal incorporation of [^{3}H]thymidine into newly formed lymphocytes was greater ($P < .01$) for sows that received Lutalyse on d 9 than sows that received saline (10.2 vs 2.3 pmol, SEM = .8). Concanavalin-A stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was not different between treatments. Lipolysaccharide-stimulated incorporation of [^{3}H]thymidine into newly formed lymphocytes was

greater (P < .01) for sows that received Lutalyse on d 9 than sows that received saline (6.8 vs 2.9 pmol, SEM = .5).

Differential White Blood Cell Counts. Treatment with Lutalyse increased neutrophils (65 vs 84/100 WBC; P < .01) and decreased lymphocytes (28 vs 16/ 100 WBC; P < .01). There were no differences observed with monocyte or eosinophil numbers.

Steroids and Prostaglandins. Neither progesterone (64 ng/mL) nor estradiol-17β (less than 1 ng/mL) concentrations were affected by Lutalyse or bacteria.

Vena caval $PGF_{2\alpha}$ increased in sows treated with Lutalyse in comparison to sows treated with saline (.44 vs .28 ng/mL; SEM=.1; Figure 2), and $PGF_{2\alpha}$ concentrations (P < .01) changed with time after inoculation (Figure 2). Neither the main effects nor the interactions affected vena caval concentrations of PGE_2.

Discussion

Treatment with Lutalyse results in the clearance of uterine infections in cattle with and without active CL. Treatment of pigs with Lutalyse resulted in the clearance or partial clearance of uterine infections in this experiments. Treatment with Luteolysis did not change progesterone concentration or cause luteolysis in sows. Pigs that received Lutalyse had less severe infections than pigs that did not receive Lutalyse, but the infections were not cleared completely (Figure 1). The amount of Lutalyse necessary to treat uterine infections in pigs has not been established, and it is possible that a larger dose or multiple doses of Lutalyse would have resulted in complete clearance. However, extremely large doses of Lutalyse or multiple doses of Lutalyse can result in luteolysis, so in order to retain the integrity of our model it will be necessary to carefully evaluate dose. This may also indicate that $PGF_{2\alpha}$ is a “weaker” treatment when luteolysis and the subsequent up-regulation of the uterine immune system do not accompany it. Lutalyse

administration resulted in increased concentrations of $PGF_{2\alpha}$ in vena caval blood in pigs (Figure 2).

Following administration of $PGF_{2\alpha}$, neutrophil numbers in circulation decreased regardless of bacterial inoculation. This further supports the chemotactic role of $PGF_{2\alpha}$ in the uterine lumen following administration of Lutalyse.

Vena caval lymphocytes from ewes that were administered Lutalyse had increased capacities for basal and LPS- stimulated incorporation of [^{3}H]thymidine than did the lymphocytes from ewes that receive saline. Changes in vena caval blood lymphocyte population corresponded to the changes in lymphocyte activity. It is difficult to determine if changes in lymphocyte population are both related to treatment or if the decrease in one is related to an increase in the other.

Implications

Lutalyse decreases the severity of uterine infections in pigs without causing luteolysis. Typically, it is believed that Lutalyse exerts it effects by causing regression of the CL; however, benefit of the administration of Lutalyse in the absence of a CL suggest another mechanism.

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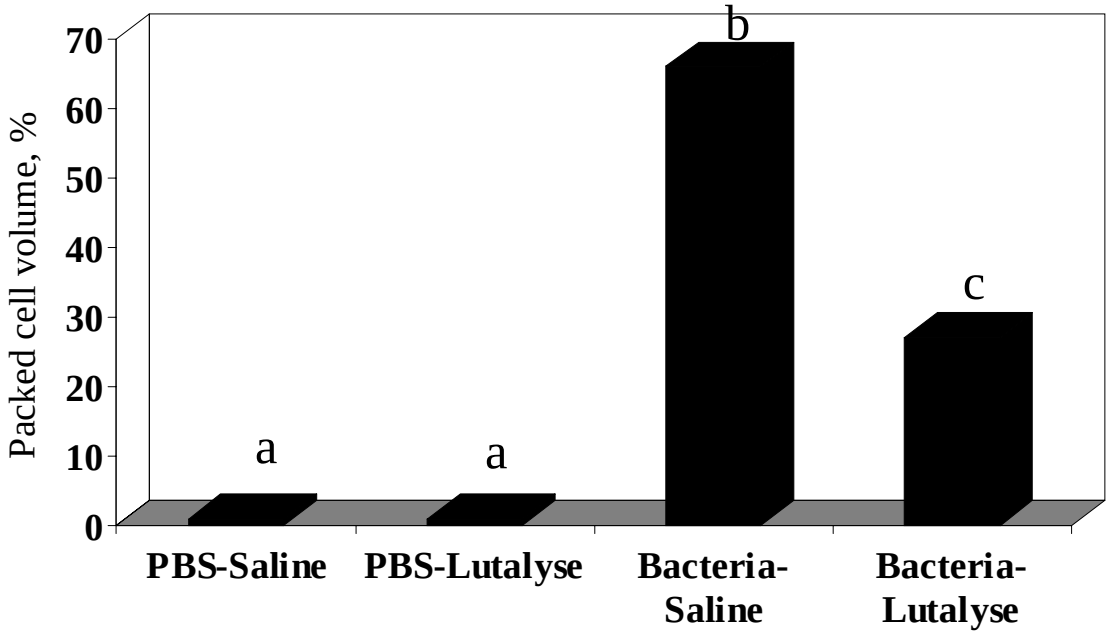

Figure 1. The effect of treatment on packed cell volume of uterine flushings at slaughter in Exp. 6. Sows were assigned to randomized treatments, estrous cycles were synchronized, and sows received intrauterine inoculations (PBS or Bacteria) on d 7 and i.m. injections of saline or Lutalyse on d 9. Uteri were collected and flushed. Packed cell volume of the uterine flushings were measured. ^{a,b,c}Different superscripts indicate that means differed between treatment groups ($P < .01$), The SEM = 3.1, and there were six sows per treatment.

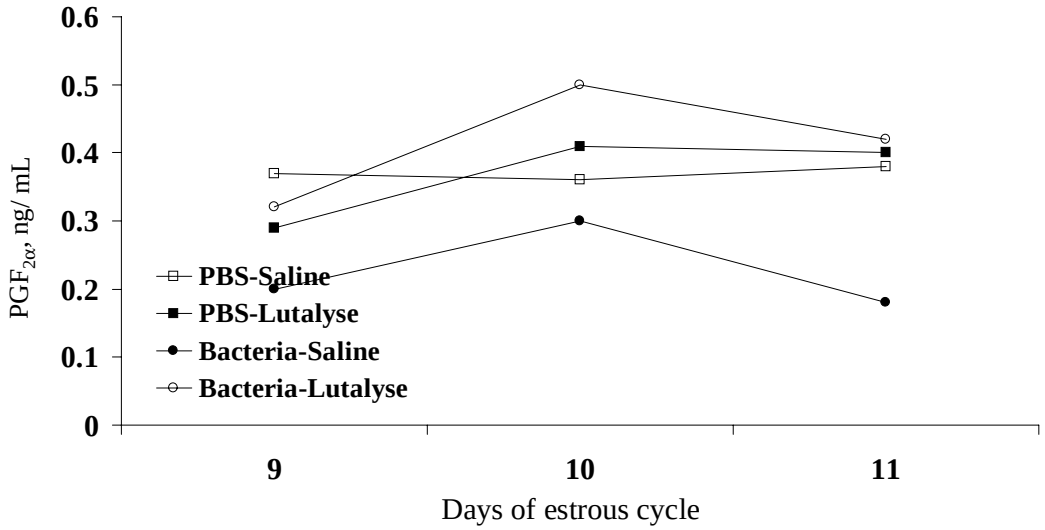

Figure 2. The effect of treatment on vena caval $PGF_{2\alpha}$ concentration on Exp. 6. Sows were assigned to randomized treatments, estrous cycles were synchronized, and sows received intrauterine inoculations (PBS or Bacteria) on d 7 and i.m. injections of saline or Lutalyse on d 9. Samples were taken on d 9 to 11. There was a significant affect of Lutalyse ($P < .01$) on $PGF_{2\alpha}$ concentration. The SEM = .1, and there were six sows per treatment.

Chapter VIII

SUMMARY

Introduction. Postpartum uterine infections are a major economic problem in the livestock industry. In most species, appropriate treatments are inexpensive and available; however, the infections are not necessarily treated appropriately. Instead, many postpartum uterine infections are treated with a variety of "traditional" treatments that may not be efficacious or safe. Lutalyse is the most cost effective and efficacious treatment available, but the biochemical mechanisms associated with the clearance of uterine infections following administration of Lutalyse have not been elucidated. Thus, the "true" mechanism of action of Lutalyse in eliminating uterine infections is not known. Therefore, the objective of this dissertation and associated research was to determine the mechanism of action of Lutalyse in the clearance of uterine infections. To achieve this objective, a new animal model was developed to study uterine infections, aspects of our cyclic sheep uterine infection model were reevaluated, and the role of Lutalyse and oxytocin, a $PGF_{2\alpha}$ secretagogue, in the clearance of uterine infections and in the associated uterine immune responses was examined.

Objective. In Chapter 2, a series of questions and objectives were posed. In this chapter, answers to those questions are offered.

Proposed Mechanism. The general relationship among immune function, hormone environment, and uterine infections was described many years ago for cattle, but these relationships have not been adequately described for other species. In addition, the biochemical mechanisms of action have not been elucidated, primarily because of the expense and difficulty of developing repeatable experimental models in cattle (Black et al., 1953; Rowson et al., 1953; Lander Chacin et al., 1990). Previous studies indicate that sheep are an economical and reliable

substitute for cattle in studies to understand the regulation of uterine immune function. However, the sheep model has not been adequately characterized (Hawk et al., 1961; Ramadan et al., 1997), and, in some cases, it does not provide an adequate physiological model. In particular, Lutalyse, which is a $PGF_{2\alpha}$ analogue that may have direct effects on immune function, causes luteolysis when administered during the luteal phase in ruminants. This causes a decrease in progesterone concentrations, and progesterone can have major effects on immune function. Therefore, it is impossible to separate the effects of a decrease in progesterone from the effects of Lutalyse on the uterine immune system. Lutalyse does not cause luteolysis in pigs before d 12 of the estrous cycle, so it is possible to use pigs to investigate the role of Lutalyse in the function of the uterine immune system without having to account for changes in progesterone. However, the uterine immune response to bacterial infections is poorly characterized in pigs. To address that void in our knowledge of uterine infections, it was first necessary to evaluate the effects of stage of the estrous cycle and progesterone dominance on the uterine immune response in pigs.

QUESTIONS AND ANSWERS

Question 1 (unpublished data; Chapter IV, Exp. 1 and 2; Chapter VII, Exp. 6): Do the bacterial isolates that we use in sheep cause uterine infections in female pigs?

Question 2 (unpublished dose response curve): What is the optimal dose of *A. pyogenes* and *E. coli* to cause an infection in female pigs similar to the infections that we generate in sheep?

Question 3 (Chapter IV, Exp. 1 and 2): Do female pigs provide a good model for detailed studies of uterine infections?

Question 4 (Chapter IV, Exp. 1 and 2): Using female pigs, can we consistently produce animals that are susceptible to or resistant to infections?

Question 5 (Chapter IV, Exp. 1): Does the uterine immune response to infectious bacteria differ during the follicular and luteal phases of the estrous cycle in female pigs?

Question 6 (Chapter IV, Exp. 1 and 2): If there are differences in the uterine immune response to infectious bacteria during the follicular and luteal phases of the estrous cycle, are these changes steroid hormone dependent?

Question 7 (Chapter IV, Exp. 2): Does progesterone mediate the changes in uterine immune response to infectious bacteria during the estrous cycle in female pigs?

> **Question 7a (Chapter IV, Exp. 2):** Do exogenous and endogenous progesterone increase susceptibility to uterine infections via the same mechanism?
>
> **Question 7b (Chapter IV, Exp. 2):** If female pigs are ovariectomized during the follicular phase, a period when the uterus is not susceptible to infection, do they maintain their resistance to uterine infections?

The results of Exp. 1 and Exp. 2 indicate that the uterine immune response to bacteria in pigs under the influence of progesterone or estrogen is similar to the response in sheep and cattle. Specifically, under the influence of progesterone, pigs develop uterine infections following bacterial exposure; whereas, when progesterone is decreased and estrogen is increased, pigs are not susceptible to uterine infections following bacterial inoculations.

The changes in vena caval WBC populations and the changes in the response of lymphocytes to in vitro stimuli help explain resistance and susceptibility to infection. Vena caval lymphocytes from gilts inoculated with either bacteria or PBS on d 0 of the estrous cycle had greater capacities for basal and Con A- and LPS-stimulated incorporation of [^{3}H]thymidine than did the lymphocytes from gilts inoculated on d 8. Also, vena caval blood from gilts on d 0 contained more lymphocytes, fewer neutrophils, and greater concentrations of $PGF_{2\alpha}$ than did vena caval blood from gilts inoculated on d 8. The difference in immune function between the follicular (d 0) and luteal (d 8) phase gilts probably explains the difference in susceptibility to uterine infections between these two groups.

In all studies with sheep or pigs, animals under the influence of progesterone that were inoculated with bacteria had infections at the time of slaughter; whereas, animals that were under the influence of estrogens or were not exposed to progesterone were resistant to the infections.

Immune function in gilts seems to be up-regulated during the follicular phase. Several previous studies indicate that uterine immune function is enhanced during the follicular phase of ewes and cattle. In particular, estrogen treatment enhances uterine immune function in ovariectomized ewes, mares, and cows (Hawk et al., 1961; Roth et al., 1983; Lander Chacin et al., 1990; Ramadan et al., 1997). Thus, the results from the studies with gilts are consistent with studies with other species.

The in vitro lymphocyte response to mitogens was consistent with the changes associated with lymphocyte concentration in circulation at different phases of the estrous cycle following bacterial inoculation. When the number of vena caval lymphocytes was increased in association with increases in estrogen and decreases in progesterone, the basal blastogenic ability and the ability of T and B lymphocytes to respond to mitogens was increased.

Even though immune function seemed to be up-regulated when estrogens were increased, it is difficult to determine whether increased estrogens during the follicular phase induced the up-regulation or whether up-regulation was due to the removal of the suppressive effects of progesterone. To unravel this relationship, one would have to supplement the gilts with progesterone during the follicular phase following intrauterine inoculation of bacteria and examine the uterine immune response.

It was much easier to identify the suppressive effects of progesterone. In the present series of experiments, immune function was decreased during the luteal phase or in the presence of an exogenous source of progesterone, when vena caval progesterone was increased and vena caval estrogens were decreased. In fact, the down-regulation of the immune system was more pronounced in gilts with higher concentrations of vena caval progesterone (i.e., the gilts with

intact ovaries and receiving progesterone treatments) and less severe in gilts receiving progesterone supplementation without an endogenous source.

The distribution of WBC in vena caval blood changed with the stage of the estrous cycle and type of inoculation. The changes in lymphocytes and neutrophils seem to govern the distribution; the changes in monocytes and eosinophils were minor in comparison. Gilts inoculated with bacteria on d 8 or in the presence of exogenous progesterone developed infections, and numbers of vena caval neutrophils from these gilts decreased with time after inoculation. The numbers of lymphocytes in gilts inoculated on d 8 increased during the same period. The reduction of neutrophils may be because they leave the circulation and travel to the site of infection (Tizard, 1996). In theory, the vena caval blood that was collected during this experiment had passed through the uterus providing neutrophils with an opportunity to leave circulation and move to the site of infection (Wade and Lewis, 1996). The increases in numbers of vena caval lymphocytes in response to bacterial challenge on d 8 may reflect the movement of immunocompetent lymphocytes from the secondary lymphoid tissues into the blood lymphocyte pool. Movement into the blood lymphocyte pool moves the lymphocytes toward the mucosa-associated lymphoid tissue in the uterus and the lymph nodes in the uterus. That response should increase humoral and cell mediated immunity.

Intrauterine inoculation with bacteria increased vena caval $PGF_{2\alpha}$ concentrations, regardless of the stage of the cycle when bacteria were introduced. This increase in $PGF_{2\alpha}$ is consistent with increases with postpartum cows (Del Vecchio et al., 1992), cyclic sheep (Ramadan et al., 1997), and postpartum sheep (Seals et al., 2000 unpublished data). In sheep, the uterus is the major source of $PGF_{2\alpha}$ in vena caval blood (Wade and Lewis, 1996). This is also true in pigs (Benoit and Daily, 1991). The increase in $PGF_{2\alpha}$ is probably a result of the

inflammation associated with infections (Tizard, 1996). However, the exact role of increased concentrations of $PGF_{2\alpha}$ in uterine infections is unknown. In vitro experiments indicate that $PGF_{2\alpha}$ and other arachidonic acid cascade products act as neutrophil chemoattractants, and this may indicate that the bacteria-induced increase in $PGF_{2\alpha}$ is a signal for neutrophils to migrate into the uterus.

In gilts, intrauterine inoculation with bacteria decreased vena caval PGE_2 concentrations. This has not been observed in our sheep model. However, the role of PGE_2 as an immune signaling compound is well documented. It is possible that PGE_2 acts as an inhibitory molecule of various cytokines, and, following bacterial exposure, PGE_2 suppression may remove its inhibitory effects from other immune signaling cells. The decrease in PGE_2 may also relate to the generation of systemic immune effects and, therefore, to the severity of infection.

Our initial experiments with pigs used all gilts, and the above changes in uterine immune function in response to bacterial inoculation and hormonal environment were evaluated in animals with similar reproductive backgrounds from closely related genetic lines. Unfortunately, there is no guarantee that the mechanisms are similar in animals with different reproductive backgrounds. In fact, we have noticed differences between the uterine immune response to bacterial inoculations in nulliparous and multiparous sheep.

The definitive factors that predispose livestock to postpartum uterine infections are unknown. There are many clinical situations that increase the likelihood of an animal developing a uterine infection, but a single cause has not been elucidated. It appears that aberrant prepartum and postpartum immune function contributes to susceptibility, but it is not known why some animals develop uterine infections while others in the same herd under similar management systems do not. A contributing factor may be previous bacterial exposure, which relates to parity.

If a ewe lambs, one may assume that her uterine environment has been exposed to bacteria a minimum of twice, once at breeding and once during parturition. The uteri of ewes that have not lambed have not been exposed to bacteria to the same degree as parous ewes. Even if they have been bred, but did not lamb, bacterial exposure would have been at estrus, when the uterine immune system is up-regulated (Ramadan et al., 1997).

Typically, the sheep at Virginia Tech that were used for these experiments were of varying ages and parity. This may be problematic. Recent studies indicate that older cows seem more resistant to uterine infections than younger cows, indicating that some degree of active immunity is acquired naturally (Watson et al., 1990). Therefore, a group of nulliparous ewes that had never been exposed to rams was used to evaluate the effects of multiple intrauterine inoculations with the same bacteria on uterine immune response and susceptibility to infection. This experiment also provided the opportunity to confirm that responses to intrauterine bacterial inoculation during the luteal phase were similar in nulliparous and multiparous ewes. We were able to compare the responses following the first set of inoculations in the nulliparous ewes with the responses from multiparous ewes in previous experiments. Although the degree of response differed between the groups, the trends were similar.

Question 8 (Chapter V, Exp. 3): Does the uterine immune response change after multiple exposures to infectious bacteria in nulliparous ewes?

Question 9 (Chapter V, Exp. 3): If the uterine immune response changes after multiple exposures to infectious bacteria, is it quantifiable?

Ewes that received multiple inoculations of bacteria developed less severe infections after the second inoculation of bacteria than did ewes that only received a single bacterial inoculation. Unfortunately, our diagnosis of uterine infections is based on PCV of uterine flushings and the ability to regrow bacteria from uteri collected at slaughter. This prevented? a definite diagnosis

during the first bacterial challenge. Therefore, we do not know whether these ewes developed less severe infections or whether the uterine immune system was able to clear the infection faster, because the uterine response could not be compared between the first and second inoculations. In the future, this will be an important distinction to make. Making this distinction would allow us to begin evaluating the primary immune response associated with uterine infections in livestock. In particular, "Is clearance of uterine infections in livestock mediated by innate or acquired immune responses?" To date, this is the first experiment that we have conducted that begins to address this issue. Typically in experiments, our measures have been measures of acquired immunity, which may or may not be the primary immune response involved in the clearance of uterine infections in livestock.

Vena caval lymphocytes from ewes that were inoculated with multiple doses of bacteria on d 6 had greater capacities for basal and Con A- and LPS- stimulated incorporation of [^{3}H]thymidine than did the lymphocytes from ewes inoculated with a single dose of bacteria or saline. Changes in vena caval blood lymphocyte population corresponded to the changes in lymphocyte activity. However, neutrophil numbers increased in the vena caval circulation after the second inoculation with bacteria. This seems counter intuitive. Frequency of inoculations should not increase the rate of the innate immune response, and it certainly should not decrease it. In the event of a rapid innate immune response to a bacterial pathogen, neutrophils leave circulation, enter the tissue, and are transported to the site of infection; therefore, neutrophil numbers in circulation usually decrease.

The increase in neutrophil numbers in circulation 48 h after inoculation with a second dose of bacteria may be an artifact of sampling times. Neutrophil migration to the uterus in response to chemotactic agents is extremely fast in sheep. The neutrophil pool in circulation is

significantly less after 2 h (Hunter et al., 1998 unpublished data). Therefore, to detect movement of neutrophils out of circulation might have needed a different sampling frequency.

In ewes that received multiple bacterial inoculations, concentration of $PGF_{2\alpha}$ increased within 24 h after the inoculation. This may relate to our problems tracking neutrophil migration. Prostaglandin $F_{2\alpha}$ is a potent neutrophil chemoattractant, and increased concentrations accumulating in the uterine lumen in response to the bacteria may have resulted in rapid migration of neutrophils to the uterine lumen and the site of infection.

Discussions of rapid neutrophil involvement infer a rapid innate immune response. However, changes in the presence of Ab in the serum of ewes inoculated with bacteria confirm the development of an acquired immune response.

Basal and Con A- and LPS-stimulated incorporation of [^{3}H]thymidine increased following the first bacterial exposure, and they remained increased in these ewes. Before the second inoculation, ewes that received bacteria during the first inoculation had a greater capacity for unstimulated and stimulated blastogenesis, suggesting that the B and T lymphocytes had remained activated. Perhaps a longer “rest” period was needed between inoculations to evaluate the effect of each inoculation independently.

Differences in the duration of activation times of these cells may relate to development of postpartum uterine infections. During uterine involution, bacteria invade the uterus. The composition of the uterine flora fluctuates constantly during the puerperal period. Essentially the uterus seems to become contaminated repeatedly, to clear the organisms, and to become re-contaminated until uterine involution is complete. The majority of animals spontaneously resolve the mild nonpathological endometritis that results during this period of bacterial recycling, but,

others do not. It may be possible that immune cells are in a constant state of up-regulation or that they are perpetually activated at this point, and therefore, can resolve these infections quickly.

To evaluate the efficacy of intrauterine inoculations of bacteria to confer immunity in nulliparous or high risk livestock it will be necessary to further evaluate the involvement of the innate immune response and the acquired immune response in clearance of uterine infections. A method of examining this would be to repeat the above experiment. However, rather than inoculating the ewes during the luteal phase when infections will become established, the ewes should be inoculated at estrus when the uterine immune system is up-regulated, and the ewes should be restedinoculated again during the subsequent luteal phase to evaluate the susceptibility of these ewes to uterine infections. If ewes were resistant to infections, this would also be a safer method for inoculating the ewes against uterine infections, because the risk of the ewe developing an infection would be reduced.

Question 10 (Chapter VI, Exp. 4): Does Lutalyse increase the rate of clearance of uterine infections?

Question 11 (Chapter VII, Exp. 6): Does Lutalyse increase the rate of clearance of uterine infections alone or in conjunction with luteolysis?

Question 12 (Chapter VI, Exp. 4): Does Lutalyse increase the rate of clearance of uterine infections alone or in conjunction with an endogenous increase in $PGF_{2\alpha}$?

Treatment with Lutalyse results in the clearance of uterine infections in cattle with and without active CL. Treatment of pigs and sheep with Lutalyse resulted in the clearance or partial clearance of uterine infections in these experiments. In sheep, treatment with Lutalyse caused regression of the CL, which was confirmed by decreases in vena caval progesterone concentrations and visual appraisal of the ovaries at slaughter. Treatment with Lutalyse did not change progesterone concentration or cause luteolysis in sows. Infections in sheep were

completely cleared following administration of Lutalyse. Pigs that received Lutalyse had less severe infections than pigs that did not receive Lutalyse, but the infections were not cleared completely. The amount of Lutalyse necessary to treat uterine infections in pigs has not been established, and it is possible that a larger dose or multiple doses of Lutalyse would have resulted in complete clearance. However, extremely large doses of Lutalyse or multiple doses of Lutalyse can result in luteolysis, so, to retain the integrity of our model, it will be necessary to carefully evaluate dose. This may also indicate that $PGF_{2\alpha}$ is a "weaker" treatment when luteolysis and the subsequent up-regulation of the uterine immune system do not accompany it.

Lutalyse administration resulted in increased concentrations of $PGF_{2\alpha}$ in vena caval blood in sheep and pigs. A recent study with sheep indicated that Lutalyse induced uterine secretion of $PGF_{2\alpha}$, which required an increase in the availability of free endometrial arachidonic acid (Wade and Lewis, 1996). Free arachidonic acid can be converted through the cyclooxygenase pathway to various prostaglandins and through the lipoxygenase pathway to LTB_4. In addition to the chemotactic effects of LTB_4 and $PGF_{2\alpha}$, LTB_4 is a potent activator of neutrophil function, and $PGF_{2\alpha}$ reduces intracellulr cAMP concentrations. Reduced cAMP is associated with activated immune cells. The increased uterine secretion of $PGF_{2\alpha}$ may be an important component of the uterine defense mechanism.

Following administration of $PGF_{2\alpha}$, neutrophil numbers in circulation decreased regardless of bacterial inoculation. This further supports the chemotactic role of $PGF_{2\alpha}$ in the uterine lumen following administration of Lutalyse.

Vena caval lymphocytes from ewes that were administered Lutalyse had increased capacities for basal and Con A- and LPS- stimulated incorporation of [^{3}H]thymidine than did the lymphocytes from ewes that received saline. Changes in vena caval blood lymphocyte

population corresponded to the changes in lymphocyte activity. It is difficult to determine whether changes in lymphocyte population are related to treatment or if the decrease in one is related to an increase in the other.

Question 13 (Chapter VI, Exp. 5): Does oxytocin increase the rate of clearance of uterine infections in sheep?

Question 14 (Chapter VI, Exp. 5): Does oxytocin increase the rate of clearance of uterine infections via changes in prostaglandin secretion?

In order to further evaluate the role of endogenous $PGF_{2\alpha}$ in the clearance of uterine infections, we used a $PGF_{2\alpha}$ secrotagogue that does not cause luteolysis. Without our knowledge at the start of these studies, oxytocin is already used to treat uterine infections in cows and pigs. Oxytocin is the preferred treatment for some types of mastitis in cattle and PHS in swine. Oxytocin is administered in both cases to increase milk output and to "flush" the infection from the mammary tissue. However, in swine, researchers report a decrease in the severity of the associated uterine infections following treatment with oxytocin. It is also possible that clearance of the bacterial infection in the mammary gland is a result of immunomodulation by oxytocin rather than the physical force of increasing milk let down.

Following administration of oxytocin, progesterone concentrations did not decrease, suggesting that luteolysis did not occur. This was confirmed by observing the ovaries at slaughter. Similar to the response to Lutalyse in pigs, ewes that received oxytocin treatment after intrauterine inoculation of bacteria had less severe infections. The infections were not completely cleared. Again, this suggests that dose or administration protocols could be inadequate or that luteolysis and the decrease in progesterone is a crucial portion of this mechanism. Administration of oxytocin caused increased concentrations of vena caval progesterone. And there was a corresponding decrease in neutrophils in vena caval blood, suggesting movement of the

neutrophils toward the chemotactic eicosanoids. There was also increase in lymphocyte numbers in vena caval blood. However, in this case, I believe that the increase in lymphocytes is an artifact of changes in the population of neutrophils, because oxytocin treatments did not increase basal or Con A- or LPS-stimulated blastogenesis. This suggests that the secretion of uterine $PGF_2\alpha$ in the response to bacteria or $PGF_{2\alpha}$ secretagogues is modulating clearance of uterine infections through an innate immune response.

Conclusions

1. During periods of estrogen dominance, the uterine immune system is up-regulated, and therefore, infections do not develop after intrauterine inoculation with bacteria.

2. During periods of progesterone dominance, the uterine immune system is down-regulated, and, therefore, infections develop after intrauterine inoculation with bacteria.

3. Stimulation of the uterus with $PGF_{2\alpha}$ or oxytocin independently of luteolysis up-regulates the uterine immune system enough to combat the down-regulation associated with progesterone dominance.

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