ROLE OF THE ENDOCRINE AND IMMUNE SYSTEMS IN THE DEVELOPING AND REGRESSING CORPUS LUTEUM

DISSERTATION

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By

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* * * * *

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ABSTRACT

Chronic administration of a GnRH agonist to cattle beginning when the CL is developing or fully functional increases basal LH secretion, subsequent plasma progesterone concentrations, and size of the CL. An objective of the first study was to determine if administration of GnRH analogs alters the abundance of mRNAs encoding for StAR, or the steroidogenic enzymes, P450_scc, or 3β-HSD. It was also to be determined if the cell size or ratio of small to large luteal cells was altered as a result of treatment. Heifers received a GnRH agonist beginning on Day 3 (Day 0 = estrus) to Day 16 (D3) or Day 12 to Day 16 (D12) via osmotic pumps. An antagonist of GnRH was administered daily by injection on Day 3 to Day 16 (SB-75). Neither progesterone nor size of the CL was altered with SB-75 treatment. Progesterone concentrations were greater in the agonist-treated females as compared with the control group. Size and weight of the CL was greater in D3-treated heifers, however, size and ratio of the small and large luteal cells were not altered. The steady-state amount of StAR mRNA was greater in GnRH-agonist treated heifers as compared with control heifers, but the relative abundance of P450_scc and 3β-HSD remained unchanged with treatment. Enhanced luteal function was likely the result of the increased amount of StAR protein as indicated by the greater amount of mRNA for this protein.
Dendritic cells are antigen-presenting cells important for the initiation of T cell immune responses. Dendritic-like cells have been described in the CL, but further characterization has not been reported. The objective of the second study was to determine if dendritic cells are present in the CL by examining for gene expression and localization of CD83, a cell surface molecule widely used as a marker of mature dendritic cells. The CD83 mRNA was detected at all stages of the luteal phase and during regression. The temporal expression of CD83 in luteal tissue is coincident with the presence of cytokines that are known to upregulate CD83. Activation of dendritic cells is regulated by CD40/CD40 ligand interaction. The amount of CD40 mRNA increased from early to midcycle and remained elevated during luteal regression. Localization of CD83 could not be determined by *in situ* hybridization or immunohistochemistry. It could not be determined if CD83 expression was localized to dendritic cells or another cell type within the CL.

The events that surround the demise of the corpus luteum (CL) appear to involve a cell-mediated immune response. Near the time of luteolysis an increase in the number of $\alpha\beta$ T cells in the CL has been reported. Moreover, luteal cells are capable of eliciting T cell proliferation *in vitro*. The objective of the third study was to determine which types of T cells were stimulated to proliferate and if cytolytic or helper cytokines were produced. Which T cell proliferated was assessed by flow cytometry. The $\gamma\delta$ T cells that were identified for the first time in the bovine CL in the present study were the prominent T cell population that were stimulated by luteal cells. In contrast, the number of CD4$^+$ T cells was less in
luteal cell-T cell cocultures in the presence of staphylococcal enterotoxin B (SEB) as compared with T cells cultured alone. There was no effect of the luteal cells on the number of CD8$^+$ T cells. Interferon (IFN)-$\gamma$ and interleukin (IL)-10 were both produced by T cells cultured with luteal cells. Interleukin-10 was likely produced to suppress the proinflammatory actions of IFN-$\gamma$. In summary, luteal cells primarily stimulated $\gamma$δ T cells and a proinflammatory T cell response.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

DEVELOPMENT OF THE CORPUS LUTEUM

Ovulation

Ovulation is a highly regulated process that has been described as a localized inflammatory reaction (Espey, 1980). The ovulatory process is initiated by the preovulatory LH surge, which is the stimulus that induces the cascade of events that result in rupture of the ovarian follicle (for review, Brännström and Enskog, 2002). Because there are some species differences in what genes are expressed and the timing of gene expression, the primary focus of this Literature Review will be the domestic ruminant species, the cow and ewe. In cattle, ovulation occurs about 24 hours after the preovulatory surge release of LH (Dieleman et al., 1983).

During the preovulatory period, the follicle begins to undergo morphological and biochemical changes that are necessary for corpus luteum (CL) development. In response to the LH surge, the preovulatory follicle produces inflammatory mediators. The prostaglandins of the E and F series are both elevated in the wall of the preovulatory follicle after the LH surge (Murdoch et al.,
1981). The concentration of intra-follicular PGF$_{2\alpha}$ remains elevated until ovulation, while the increase of PGE$_2$ is only transient (Murdoch et al., 1986). The cyclooxygenase (COX) enzyme is the rate-limiting factor in the conversion of arachidonic acid to prostaglandins. The inducible COX-2 isoform is increased in the preovulatory follicle 24 hours after the natural LH surge (Liu et al., 1997), after the administration of GnRH (Tsai et al., 1996), or an ovulatory dose of hCG (Sirois, 1994). The presence of COX-2 protein is not only time dependent, but also specific to the granulosal cells (Liu et al., 1994; Sirois, 1994). Prostaglandins are essential for ovulation. The requirement of prostaglandins for ovulation but not luteinization was demonstrated decades ago (Armstrong and Grinwich, 1972; Tsafriri et al., 1973). Ewes treated with indomethacin, a COX inhibitor, failed to ovulate. However, this inhibition can be overcome with exogenous PGF$_{2\alpha}$ (Murdoch et al., 1986).

An inflammatory reaction involves the migration of immune cells to the site of inflammation. Neutrophils are the first immune cells that invade the preovulatory follicle after the LH surge (Cavender and Murdoch, 1988; Brännström et al., 1993; 1994). Macrophages are the second and major population of immune cells that are recruited to the site of inflammation, or in this circumstance, the preovulatory follicle (Brännström et al., 1993). Recruitment of neutrophils and macrophages is dependent on chemoattractants secreted by inflamed tissue. Murdoch and McCormick (1989) performed an experiment to test the ability of follicular-conditioned medium to attract immune cells. Conditioned medium from tissues collected at 24 and 36 hours after the LH surge attracted
neutrophils, but monocytes only migrated with the conditioned medium of tissues collected at 36 hours.

Tumor necrosis factor-α was first described as a tumoricidal cytokine produced by activated macrophages (Carswell et al., 1975). Preovulatory follicles of many species secrete TNF-α (Terranova, 1997). The sources of TNF-α in preovulatory follicles are the thecal endothelial cells (Murdoch et al., 1997) and the oocyte-cumulus cell complex (Johnson et al., 1999). Tumor necrosis factor-α is synthesized as a membrane bound precursor protein. The mature soluble form of TNF-α is activated by the protease, plasmin. Plasmin releases TNF-α from the endothelial cells (Murdoch et al., 1999). Plasmin activity increases 12 hours after the gonadotropin surge (Dow et al., 2002), 8 hours prior to when soluble TNF-α is localized to the thecal endothelial cells of the preovulatory follicle (Murdoch et al., 1997). Preovulatory follicles injected with TNF-α antiserum fail to ovulate (Murdoch et al., 1997; Gottsch et al., 2000).

Murdoch et al., (1997) proposed that TNF-α functions to weaken the apical surface of the preovulatory follicle. There is a greater loss of collagen within the apical surface as compared with the basal portion of the ovulatory follicle (Murdoch and McCormick, 1992). Degradation of collagen is through the actions of collagenases that are induced by TNF-α in the preovulatory follicle. Antibodies against TNF-α and the transcription inhibitor actinomycin D abolish collagenolysis in the preovulatory follicle (Johnson et al., 1999; Gottsch et al.,
Both of these treatments diminish matrix metalloproteinase (MMP)-2 activity in the follicle (Gottsch et al., 2000).

The matrix metalloproteinases (MMPs) are enzymes responsible for remodeling the extracellular matrix by degrading the proteinaceous components in tissues, including the ovary (for review, Smith et al., 1999). Remodeling of the tissue depends on the ratio between the MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Matrix metalloproteinase-2 and MMP-14 have been identified in follicular tissue. Matrix metalloproteinase-2 mRNA is localized to the thecal layer and the amount of mRNA does not change during the peri-ovulatory period (Bakke et al., 2002). However, MMP-2 is necessary for ovulation. Neutralization of MMP-2 with intra-follicular injections of an anti-MMP-2 results in an unruptured luteinizined follicle (Gottsch et al., 2002). In contrast, MMP-14 mRNA increases after the LH surge where the mRNA is localized to the thecal and granulosal layers as well as the surrounding stroma (Bakke et al., 2002). The MMPs are involved in degrading the extracellular matrix at the apical surface of the pre-ovulatory follicle to allow rupture of the follicle.

**Formation of the Corpus Luteum (CL)**

Luteinization is the transition of the ovarian follicle into the CL. While there are many biochemical and morphological changes that occur during the peri-ovulatory period to prepare the follicle for ovulation, just as many changes are simultaneously beginning to take place to ensure that the ruptured follicle develops into a fully functional CL. Ironically, tissue degradation is required for
ovulation yet the extent of the destruction cannot be so extensive that formation of the CL is impaired.

In response to the LH surge, there is a shift in the steroidogenic pathway from estradiol being the primary steroid synthesized by the follicle to progesterone production in the developing CL (Dieleman et al., 1983; Fortune and Hansel, 1985). The thecal and granulosal cells both actively participate to produce estradiol. In the widely accepted two-cell two-gonadotropin theory, the thecal cells produce the enzymes to convert cholesterol to the androgens (Fortune and Armstrong, 1978). The androgens are converted to estradiol by the granulosal cells that produce aromatase, the enzyme that converts androgens to estradiol. The mRNAs for cytochrome P450-17α and cytochrome P450-aromatase, responsible for the conversion of pregnenolone to the androgens and androgens to estrogens, respectively, both dramatically decrease (95%) after the LH surge (Voss and Fortune, 1993). The enzymes responsible for the conversion of cholesterol to progesterone, cytochrome P450 side chain cleavage (P450scc) and 3β hydroxysteroid dehydrogenase (3β-HSD) increase during the development of the CL (Rodgers et al., 1986; Juengel et al., 1994), as does the steroidogenic acute regulatory protein (StAR; Pescador et al., 1996).

The follicle is composed of two steroidogenic cell types, the granulosa and the theca. The avascular granulosal layer is enveloped by a basal lamina that separates this layer from the vascular thecal layer. Within a few hours of ovulation, the basal lamina becomes discontinuous and almost completely disappears within 24 hours (O’Shea et al., 1980). The breakdown of the basal
lamina is caused by the actions of MMPs (Gottsch et al., 2002). The disruption of the basal lamina allows for the migration of the thecal cells into the deeper portions of the tissue where the granulosal cells reside (O'Shea et al., 1980). This mixing of the thecal and granulosal cells is part of the major structural remodeling that occurs during formation of the CL.

Thecal and granulosal cells of the follicle luteinize as two distinct cell types in the CL of the cow, small luteal cells (SLC) and large luteal cells (LLC), respectively (Donaldson and Hansel, 1965; Alila and Hansel, 1984). In the preovulatory follicle, the increased estradiol concentrations stimulate mitotic activity of the granulosal cells (Drummond and Findlay, 1999). After the preovulatory gonadotropin surge, the granulosal cells no longer proliferate (McClellan et al., 1975; O'Shea et al., 1980). As stated previously, estradiol production decreases after the surge release of LH. Mitotic activity of the granulosal cells is suppressed when estradiol is diminished (Drummond and Findlay, 1999). The increase in LH that induces luteinization causes a reduction in cyclin D2 and induces the cell cycle inhibitor p27Kip1 (Robker and Richards, 1998). Cyclin D2 is necessary for granulosal cell proliferation and cyclin D2 mRNA is nearly undetectable within 4 hours of hCG administration to rats (Robker and Richards, 1998). In support of the belief that granulosal cells stop proliferating, the number of granulosal cells is equivalent to the number of LLC in the CL (O'Shea et al., 1987).

The CL is one of the fastest growing tissues in the body. In a few days there is an approximately five-fold increase in the size of the developing CL (Jablonka-
Shariff et al., 1993). This increase in weight is attributed to hyperplasia and hypertrophy of the luteal cells. In contrast to the mitotic suppression of granulosal cells, the thecal cells begin to undergo mitosis after ovulation (Rao et al., 1978; O'Shea et al., 1980). The growth of the CL is also the result of hypertrophy of LLC that is stimulated by LH (Donaldson and Hansel, 1965; Farin et al., 1986).

Prior to ovulation the granulosal layer of the follicle is avascular. After ovulation the basement membrane becomes discontinuous and the capillaries in the thecal layer begin to migrate into the granulosal layer (Cavender and Murdoch, 1988). Sprouting of the capillaries into the avascular areas during angiogenesis is controlled by the angiogenic factors, vascular endothelial growth factor and angiopoietins (for review, Stouffer et al., 2001). The endothelial cells are the most abundant population of proliferating cells in the developing CL (Jablonka-Shariff et al., 1993). The rapid and extensive growth of the luteal vasculature exceeds that of most other tissues. This is important because the CL has a blood flow rate and metabolic demand greater than other tissues (for review, Niswender et al., 2000).

**Luteal Cell Types**

The CL consists primarily of four distinct luteal cell types: 1) small steroidogenic luteal cells, 2) large steroidogenic luteal cells, 3) capillary endothelial cells, and 4) fibroblasts (for review, Wiltbank, 1994). Endothelial cells and fibroblasts are the non-steroidogenic luteal cells. The capillary endothelial cells only comprise 10% of the total volume of the CL, yet are the most abundant of all cells, constituting 50% of the total cells of the CL (O'Shea et al., 1989). The
total volume of the vasculature, including endothelial cells and the capillary lumen is between 14 and 23% (Niswender et al., 1976; Rodgers et al., 1984). The large volume capacity of the vasculature allows close apposition of the steroidogenic cells to the capillaries. Fibroblasts that provide structure to the CL only constitute 10% of the total cells in the CL of cows (O’Shea et al., 1989) and less than 25% in the CL of ewes (Farin et al., 1986).

Steroidogenic luteal cells are identified based on structural characteristics and their ability to respond to LH. Size of the cells is a distinct structural difference used for identification. The LLC are 22 to 50 μm in diameter and SLC are approximately half the size, 10 to 20 μm (Koos and Hansel, 1981; O’Shea et al., 1989; Lei et al., 1991; Fields and Fields, 1996). Large luteal cells are characterized by the presence of an abundant number of secretory granules (Koos and Hansel, 1981; Fields et al., 1992). These secretory granules contain the oxytocin peptide. The presence of the granules are stage dependent with a lesser abundance on day 3 of the estrous cycle, an increase on days 7 and 11, and the least number of granules on days 17 and 19 of the estrous cycle (Fields et al., 1992). Steroidogenic cells also have an abundance of cytoplasmic lipid droplets (Fields and Fields, 1996). Common to both the LLC and SLC is the abundance of endoplasmic reticulum and mitochondria. Both of these organelles are required for steroidogenesis.

As previously mentioned, the SLC and LLC are of follicular origin. Large luteal cells and the SLC are derived from granulosal and thecal cells, respectively (Alila and Hansel, 1984; O’Shea et al., 1987). Donaldson and Hansel (1965)
were the first to report that the SLC are derived from thecal cells in the CL of cows. The use of monoclonal antibodies against surface antigens of granulosa and thecal cells of cows allowed for determination of the follicular origin of the LLC and SLC, respectively (Alila and Hansel, 1984) that had been proposed years earlier (Donaldson and Hansel, 1965).

After identification of the origin of the steroidogenic luteal cells, studies were published that identified the ratio of these cells to one another and the speculation that SLC differentiate into LLC. Large luteal cells constitute 40% of the total volume of the CL but are only 4% of the total number of cells in the bovine CL (O’Shea et al., 1989). Small luteal cells are only 17% of the CL volume, but 28% of the total cell number (O’Shea et al., 1989). These percentages of the steroidogenic luteal cells are fairly consistent with what has been reported for steroidogenic luteal cells of sheep (Farin et al., 1986).

The steroidogenic luteal cells also differ in response to hormone stimulation. Luteinizing hormone is important for normal luteal function in cows and sheep. Even though nearly 80% of secreted progesterone is synthesized by LLC (Niswender et al., 1985), response of LLC to LH is minimal. Similar numbers of receptors for LH are present on SLC and LLC of ewes (Harrison et al., 1987) and cows (Chegini et al., 1991). However, Fitz et al. (1982) found greater numbers of receptors for LH on SLC compared with LLC. The number of receptors for LH does not reach a maximum until the midluteal phase (Day 10) of the estrous cycle of sheep (Diekman et al., 1978). Luteinizing hormone receptor mRNA has been isolated in LLC and SLC of the sheep CL (Smith et al., 1996). Contrary to
what has been reported in sheep (Smith et al., 1996), LH receptor mRNA has only been detected in luteinized thecal cells of cattle (Mamluk et al., 1998a,b).

Large luteal cells contain greater amounts of progesterone as compared with SLC, yet in vitro, SLC respond more dramatically to LH as compared with LLC (Ursely and Leymarie, 1979; Koos and Hansel, 1981). Luteinizing hormone-stimulated SLC secrete progesterone at a greater rate (2 – 40 fold) than LLC (Fitz et al., 1982). To explain the differences in response of SLC and LLC to LH, researchers began to investigate the second messenger system of these cells and their ability to respond to LH stimulation. Luteinizing hormone stimulates SLC to secrete progesterone through the cyclic adenosine monophosphate (cAMP) second messenger system and activation of protein kinase A (PKA; Hoyer and Niswender, 1986). Small luteal cells and LLC contain similar amounts of PKA (Wiltbank et al., 1989) however, LLC do not respond to LH binding to its receptor nor to stimulation by PKA as do SLC (Hoyer and Niswender, 1986).

PROGESTERONE PRODUCTION

Luteinizing Hormone Dependent Progesterone Production

Forty years ago, Simmons and Hansel (1964) proposed that LH is the primary luteotropin in cattle. It is now accepted that development and function of the CL is LH-dependent in domestic ruminants. When sheep are hypophysectomized during the luteal phase, serum progesterone concentrations dramatically decline (Kaltenbach et al., 1968; Denamur et al., 1973).
Altering LH secretion by the use of GnRH analogs has provided further insight for the requirement of LH for luteal progesterone production. Administration of a GnRH antagonist to cattle two days prior to and after an induced LH surge reduced the size of and progesterone production by the subsequent CL (Quintal-Franco et al., 1999). When a GnRH antagonist is administered to cattle from days 2-7 or days 7-12 of the estrous cycle, LH pulses are suppressed and plasma progesterone concentrations are less as compared with control cows (Peters et al., 1994). Interestingly, chronic administration of a GnRH agonist from days 3 – 21 or days 12 – 21 of the estrous cycle (behavioral estrus = day 0) increased plasma progesterone concentrations, likely due to increased basal LH secretion (Davis et al., 2003). However, the effects of the LH increase on the CL to increase progesterone production were not determined.

Contrary to what has been reported in cattle, administration of a GnRH antagonist to ewes during the early luteal phase of the estrous cycle does not influence progesterone secretion (McNeilly and Fraser, 1987). Progesterone secretion in ewes is independent of LH pulse stimulation (McNeilly and Fraser, 1987; McNeilly et al., 1992), but basal secretion of LH is required to maintain progesterone release in ewes (Karsch et al., 1971). This is different than cattle, which require episodic stimulation by LH at least through Day 12 of the estrous cycle for normal progesterone production (Peters et al., 1994).

As stated previously, LLC are not as responsive to LH as are SLC, yet LLC are the source for more than 80% of the progesterone secreted by the CL (Fitz et al., 1982; Niswender et al., 1985). Luteinizing hormone stimulates the cAMP/PKA
pathway. The LH-stimulated increase in cAMP concentrations increases progesterone secretion by SLC (Hoyer et al., 1984). Adenylate cyclase activity can be stimulated by forskolin in LLC, yet progesterone secretion is not altered (Hoyer et al., 1984; Wiltbank et al., 1993). Diaz et al. (2002) concluded that through the cAMP/PKA pathway, SLC are stimulated to synthesize progesterone and LLC do not require hormonal stimulation because of the already abundant amount of cAMP/PKA.

**Cholesterol – The precursor of steroids**

Brown and Goldstein (1986) received the Nobel Prize for their elucidation of the importance of cholesterol derived from circulating lipoproteins as a substrate for cellular functions. Sources of cholesterol for progesterone synthesis by luteal cells include uptake of lipoproteins from circulation and *de novo* synthesis from acetate (Strauss et al., 1981; Grummer and Carroll, 1988). *De novo* synthesis of cholesterol by steroidogenic luteal cells is unable to provide enough substrate for progesterone synthesis in typical amounts by the fully functional CL. The maximum rate of *de novo* synthesis can only provide approximately 10% of the cholesterol to meet the amount of substrate needed for typical CL function in the rat (Strauss et al., 1981).

The primary source of cholesterol for progesterone synthesis by the CL is circulating lipoproteins. *In vitro*, bovine luteal cells utilize both low-density (LDL) and high-density lipoproteins (HDL; Pate and Condon, 1982; Bao et al., 1997). Addition of lipoproteins to cultured bovine luteal cells increases progesterone production and luteal cells are stimulated to a greater extent with HDL as
compared with LDL (Pate and Condon, 1982; Wiltbank et al., 1990). The finding that HDL stimulates progesterone production more so than LDL might not be so surprising considering HDL is the most abundant lipoprotein in cattle blood plasma and is rich in cholesterol esters (Grummer and Carroll, 1988).

Lipoprotein uptake by cells is a receptor-mediated event. Mechanisms of uptake for LDL are better known than for HDL. The receptor for LDL binds two proteins, apolipoprotein B and apolipoprotein E. Li et al. (1998) have provided evidence for the presence of a HDL receptor in rat thecal cells and steroidogenic cells of the CL. Administration of hCG to rats when a CL is present increases the amount of the HDL receptor mRNA. In cultured bovine granulosal cells, the increase in steroid production stimulated by dibutyryl cAMP (dbcAMP) is parallel to an increase in LDL binding (Savion et al., 1981).

**Conversion of Cholesterol to Progesterone**

After the internalization of the lipoproteins into the cell, cholesterol is cleaved from the lipoprotein within the lysosome. The first step of progesterone synthesis, side chain cleavage of cholesterol to pregnenolone, occurs at the inner mitochondrial membrane. The rate-limiting step of steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane (Crivello and Jefcoate, 1980; Privalle et al., 1983). The availability of cholesterol to the inner mitochondrial membrane is dependent on tropic hormone stimulation by a cycloheximide-sensitive action (Privalle et al., 1983).

Clark et al. (1994) proposed that the protein that responds to tropic hormone stimulation to deliver cholesterol from the outer to inner mitochondrial membrane
is the Steroidogenic Acute Regulatory protein (StAR). The role of StAR in
steroidogenesis became more evident when it was shown that humans with
congenital lipoid adrenal hyperplasia (CAH) have impaired synthesis of steroid
hormones (Lin et al., 1995). The abnormal steroidogenic capacity of the patients
was attributed to mutated and nonfunctional StAR protein. The development of
mice null for the StAR gene have the same characteristic symptoms of lipoid
CAH, severe defects in adrenal and gonadal steroidogenesis and an abundance
of lipid deposits within the interstitium of the adrenal cortex and testis (Caron et
al., 1997). Even though StAR appears to be essential for acute tropic regulation
of steroid synthesis, StAR does not need to be imported into mitochondria to
stimulate steroid production (Arkane et al., 1996). Instead StAR is closely
associated with the peripheral-type benzodiazepine receptor (PBR) at the
mitochondrial membrane (West et al., 2001). West et al. (2001) proposed that
StAR transfers the cholesterol to the PBR, which facilitates cholesterol transfer to
the inner mitochondrial membrane.

Luteinizing hormone stimulates progesterone synthesis by increasing the
mRNA encoding for StAR (Juengel et al., 1995). The mRNA and protein are
highly correlated (Pescador et al., 1996). In cattle, StAR mRNA is in greater
abundance in the mid- and late luteal phase CL as compared with the developing
CL (Pescador et al., 1996). The StAR mRNA does not increase between Days 4
and 15 of the sheep estrous cycle (Juengel et al., 1995).

Once cholesterol is transported to the inner mitochondrial membrane by
StAR, cholesterol is converted to pregnenolone through the actions of
cytochrome P450 side chain cleavage enzyme (P450<sub>scc</sub>; Hanukogiu et al., 1992). The P450<sub>scc</sub> enzyme catalyzes the first step of progesterone synthesis in a reaction of two hydroxylations and cleavage of the side chain of cholesterol (Hanukogiu et al., 1992). The conversion of pregnenolone to progesterone by 3β-HSD occurs at the smooth endoplasmic reticulum.

The P450<sub>scc</sub> and 3β-HSD enzymes do not appear to be as acutely regulated by LH as does StAR, even though P450<sub>scc</sub> gene expression is cAMP dependent (Lauber et al., 1993). The induction of P450<sub>scc</sub> mRNA is cAMP/PKA regulated in granulosal cells of rat preovulatory follicles (Goldring et al., 1987). Transcription of P450<sub>scc</sub> mRNA is further enhanced by the LH surge (Hickey et al., 1989). Once luteinization occurs, P450<sub>scc</sub> activation in the CL is cAMP independent and is maintained in a constitutive manner (Goldring et al., 1987; Hickey et al., 1989; Oonk et al., 1990).

Steady-state concentrations of P450<sub>scc</sub> mRNA and 3β-HSD mRNA increase no more than two-fold, whereas serum progesterone concentrations can increase as much as 25-fold in ewes during the estrous cycle (Juengel et al., 1994). Concentrations of P450<sub>scc</sub> mRNA (Rodgers et al., 1986; Juengel et al., 1994) and 3β-HSD mRNA (Juengel et al., 1994) in luteal tissue increase from the early to mid-luteal phase. Large luteal cells have greater concentrations of mRNA for P450<sub>scc</sub> and 3β-HSD as compared with SLC (Wiltbank et al., 1993; Juengel et al., 1994). The difference in mRNA for P450<sub>scc</sub> and progesterone production might be due to the greater amount of mitochondria per unit volume in LLC as compared with SLC (Kenny et al., 1989).
In summary, luteinizing hormone is the luteotropic hormone that stimulates progesterone production. Progesterone production by the SLC is dependent on LH-stimulated increases in cAMP concentrations, while progesterone production by the LLC is independent of elevated cAMP concentrations induced by LH. Luteinizing hormone also regulates transfer of cholesterol from the outer to the inner mitochondrial membrane by StAR, while LH does not acutely regulate P450\textsubscript{scC} and 3β-HSD enzymes.

**LUTEOLYSIS**

**Uterine Prostaglandin \textit{F}_2\textalpha**

Prostaglandins are produced from the fatty acid, arachidonic acid (Bergström et al., 1964). Arachidonic acid is released from phospholipids by phospholipase A2 (Lands et al., 1968; reviewed by Balsinde et al., 2002). Arachidonic acid is converted to the intermediate, prostaglandin G/H, by prostaglandin H synthase, also called cyclooxygenase (COX). The COX enzyme exists as two isoforms, COX-1 that is responsible for constitutive, basal prostaglandin production, whereas COX-2 is the inducible isoform (reviewed by, Smith et al., 2000). Prostaglandin F synthase stimulates the conversion of PGH to PGF\textsubscript{2α} (Watanabe et al., 1985).

Luteolysis is the regression of the CL. This event occurs at the end of the estrous cycle when females have not conceived and another cycle will ensue as a result of this process. The influence of the uterus on regression of the CL was first observed in the guinea pig (Loeb, 1923). Later, Wiltbank and Casida (1956)
reported that after removal of the uterus the CL was maintained for 100 and 154 days in the ewe and cow, respectively. Cows returned to estrus 33 and 49 days after partial removal of the uterus. The uterine luteolytic factor, PGF$_{2\alpha}$, was finally identified in 1972 (McCracken et al., 1972).

Concentrations of prostaglandin F$_{2\alpha}$ are significantly increased in the endometrium late in the estrous cycle of the ewe as compared with concentrations during the early and midcycle (Wilson et al., 1972). During luteolysis, pulses of PGF$_{2\alpha}$ are released from the uterus with pulses being approximately 1 h in duration and being released at a 6 to 9 h interval (Barcikowski et al., 1974). The lungs rapidly metabolize PGF$_{2\alpha}$ after it enters the circulation (Piper et al., 1970). After just one passage through the lungs 65% of injected PGF$_{2\alpha}$ is metabolized in cattle (Davis et al., 1985), whereas 99% of PGF$_{2\alpha}$ is metabolized with a single passage through the lungs of sheep (Davis et al., 1980). The rapid clearance of PGF$_{2\alpha}$ from systemic circulation suggests the effect of PGF$_{2\alpha}$ on the CL as being local. Autotransplantation of the ovary to the neck of the sheep with vascular anastomosis of the ovarian artery into the carotid prolongs the luteal phase to an average greater than 100 days (McCracken et al., 1971). This separation of the ovary from the uterus indicated that luteal regression is mediated through a local effect. The countercurrent transfer of [${}^3$H]PGF$_{2\alpha}$ from the utero-ovarian vein to the ovarian artery was first demonstrated by McCracken et al. (1971). Later studies that implemented sectioning of the broad ligament (Hixon and Hansel, 1974) or partial
hysterectomies and surgical anastomosis (for review, Ginther, 1974) are consistent with a local transfer of PGF$_{2\alpha}$ from the uterus to the ovarian artery.

McCracken et al. (1999) proposed that luteolysis begins as a result of down-regulation of the progesterone receptor in the hypothalamus and endometrium. This was hypothesized to be a predetermined event. A premature increase of progesterone during the estrous cycle shortens cycle length in ewes (Ottobre et al., 1980) and cows (Garrett et al., 1988). During the luteal phase, progesterone inhibits the actions of estradiol, however, during luteolysis this inhibition is removed. It is believed that estradiol from the preovulatory follicle stimulates the release of hypophysial oxytocin and upregulates its own receptor in the endometrium. The pulses of oxytocin stimulate a small release of PGF$_{2\alpha}$ from the endometrium that will trigger release of oxytocin from the CL. Large luteal cells are the source of luteal oxytocin in sheep (Rodgers et al., 1983) and cows (Fields et al., 1992). The oxytocin released from the CL amplifies the release of PGF$_{2\alpha}$ from the uterus at the onset of luteolysis, but the contribution of luteal oxytocin to the release of PGF$_{2\alpha}$ decreases as luteolysis progresses (McCracken et al., 1999).

There is evidence that luteal oxytocin is not involved in the positive feedback loop of PGF$_{2\alpha}$ release from the uterus. Oxytocin is most abundant in luteal tissue during the mid portion of the estrous cycle, while late in the estrous cycle near the onset of luteolysis, on Days 17 and 19 (Day 0 = estrus), only 5% of LLC contain oxytocin (Fields et al., 1992). Luteal oxytocin concentrations are undetectable on Day 17 of the estrous cycle in medium collected by microdialysis.
(Shaw and Britt, 2000). Furthermore, plasma oxytocin concentrations are greatest at midcycle and decline during luteolysis in the ewe (Sheldrick and Flint, 1981). It has also been reported that reduction or near depletion of luteal oxytocin does not prevent luteolysis from occurring (Sheldrick and Flint, 1983; Kotwica and Skarzynski, 1993; McCracken et al., 2000). Removal of approximately 70% of the granulosal cells from a preovulatory follicle; the source of luteal oxytocin, decreases progesterone concentrations, but does not alter length of the estrous cycle (Milvae et al., 1991). Therefore, the contribution of oxytocin in luteal regression remains unclear.

Luteolysis in domestic ruminants requires uterine PGF$_{2\alpha}$ release. It has been postulated that PGF$_{2\alpha}$ functions locally via a counter-current exchange mechanism. The CL itself is a source of PGF$_{2\alpha}$ that may be involved in its own demise. Luteal PGF$_{2\alpha}$ is regulated by progesterone and cytokines (Pate, 1996), which will be discussed later. The end result of the luteolytic process is functional and structural regression of the CL.

**Acquisition of Luteolytic Capacity**

In cattle and sheep, the CL is resistant to the luteolytic effects of PGF$_{2\alpha}$ before Day 5 of the estrous cycle (Rowson et al., 1972; Henricks et al., 1974; Braun et al., 1988; Tsai and Wiltbank, 1998; Silva et al., 2000). The term coined by Dr. Milo Wiltbank for the period when the CL becomes responsive to PGF$_{2\alpha}$ is the ‘acquisition of luteolytic capacity’. The lack of response of the CL before Day 5 to PGF$_{2\alpha}$ is not due to the lack of PGF$_{2\alpha}$ receptors (Rao, 1975; Wiltbank et al., 1995; Tsai et al., 1996) or to a decrease in binding affinity of the receptors.
(Wright et al., 1980). Interestingly, prostaglandin secretion by the CL is greatest during the early luteal phase when the CL is non-responsive to the luteolytic effects of PGF$_{2\alpha}$ (Milvae and Hansel, 1983; Rodgers et al., 1988). The CL has greater amounts of COX-2 mRNA on Day 4 of the estrous cycle as compared with Day 13 when COX-2 mRNA is undetectable (Silva et al., 2000). An injection of PGF$_{2\alpha}$ to cows on Day 4 does not decrease serum progesterone concentrations, but does cause a decrease in the COX-2 mRNA concentrations (Tsai and Wiltbank, 1998). In contrast, serum progesterone concentrations decline and COX-2 mRNA is increased in response to a luteolytic dose of PGF$_{2\alpha}$ on Day 11 of the estrous cycle (Tsai and Wiltbank, 1998). The mRNA for, and activity of, prostaglandin dehydrogenase, the enzyme that converts PGF$_{2\alpha}$ to its inactive metabolite PGFM, is greater in the CL on Day 4 as compared with Day 11 (Silva et al., 2000). The CL might protect itself from uterine or intra-luteal PGF$_{2\alpha}$ by having the capacity to rapidly metabolize PGF$_{2\alpha}$ before it has any detrimental effect on the structure or the function of the CL.

**Functional Demise of the Corpus Luteum**

During luteolysis, the CL loses its ability to synthesize progesterone prior to tissue destruction. Prostaglandin F$_{2\alpha}$ binds to a G-protein coupled receptor (Sakamoto et al., 1994) and rapidly increases inositol triphosphate and intracellular Ca$^{2+}$ (Davis et al., 1987). Intracellular Ca$^{2+}$ and PKC are increased in LLC in response to PGF$_{2\alpha}$ (Wiltbank et al., 1989). Treatment of bovine luteal cells with PGF$_{2\alpha}$ activates the MAP kinases (Chen et al., 1998) and induces
expression of c-fos and c-jun downstream of the MAP kinases (Chen et al., 2001). Fos and jun activate the transcription factor AP-1, however, it has not been shown what genes of this pathway are transcribed in response to AP-1 in the luteal cells. Induction of COX-2 by PGF$_{2\alpha}$ in ovine luteal cells is not regulated through the MAPK pathway, but rather it is induced through the PKC pathway (Wu and Wiltbank, 2001).

Prostaglandin F$_{2\alpha}$ could exert its effects to decrease progesterone production through a number of different mechanisms involved in progesterone synthesis. The mRNA encoding for the LH receptor declines rapidly after ewes are treated with a luteolytic dose of PGF$_{2\alpha}$ (Smith et al., 1996). Binding to the LH receptor does not decrease, however, until after serum progesterone concentrations decline (Diekman et al., 1978; Rao et al., 1984). The decrease in binding is due to a decrease in receptor number, not binding affinity (Rao et al., 1984). Although neither receptor number nor affinity is affected immediately following PGF$_{2\alpha}$ treatment, membrane fluidity is decreased in the CL of rats (Carlson et al., 1984; Sawada et al., 1991) and cows (Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982) as a result of treatment with PGF$_{2\alpha}$. A decrease in membrane fluidity could prevent the interaction of bound LH receptors that might be important in the signal transduction processes (Carlson et al., 1982; Riley and Carlson, 1988; Roess and Smith, 2003).

Luteal cells require cholesterol from circulating lipoproteins for progesterone synthesis. Deprivation of lipoproteins to the luteal cells might be a cause of PGF$_{2\alpha}$-induced progesterone decline. Prostaglandin F$_{2\alpha}$ inhibits lipoprotein-
stimulated progesterone production in cultured ovine (Wiltbank et al., 1990) and bovine luteal cells (Pate and Nephew, 1988; Pate and Condon, 1989; Grusenmeyer and Pate, 1992). However, PGF$_{2\alpha}$ does not affect cellular uptake of lipoprotein or intracellular cholesterol concentrations (Grusenmeyer and Pate, 1992). The luteolytic effects exerted by PGF$_{2\alpha}$ are not due to a lack of substrate for progesterone production.

It was also shown in the previous study that PGF$_{2\alpha}$ does not inhibit progesterone synthesis when a diffusible cholesterol derivative is used in culture. Therefore, the authors concluded that PGF$_{2\alpha}$ inhibits progesterone production after cholesterol reaches the mitochondria, but before the side-chain of cholesterol is cleaved to produce pregnenolone. The protein responsible for this action is StAR. The mRNA encoding for StAR decreases within 4 h after a luteolytic injection of PGF$_{2\alpha}$ in ewes (Juengel et al., 1995; 2000) and cows (Pescador et al., 1996; Tsai and Wiltbank, 1998). Plasma progesterone concentrations begin to decline before 4 h of PGF$_{2\alpha}$ administration, however, there is no report of whether StAR mRNA declines within this period. It would be expected that the amount of StAR mRNA begins to decrease prior to 4 h of PGF$_{2\alpha}$ treatment. The decline in serum progesterone concentrations parallels a decrease in StAR (Juengel et al., 1995). Furthermore, the protein is undetectable in regressed CL (Pescador et al., 1996). Unlike StAR, the steroidogenic enzymes P450$_{scc}$ and 3β–HSD are not as sensitive to the effects of PGF$_{2\alpha}$. A sub-luteolytic dose (3 mg) of PGF$_{2\alpha}$ reduces the amount of StAR mRNA, but has no effect on
P450scc and 3β–HSD mRNA concentrations (Juengel et al., 2000). Treatment of luteal cells with PKC or PMA does not alter the activity of P450scc (Wiltbank et al., 1993; Belfoire et al., 1994) or 3β–HSD (Wiltbank et al., 1993). Gene expression for 3β–HSD has been shown to decrease after treatment of cows with an injection of PGF$_{2\alpha}$ (Tsai and Wiltbank, 1998). In contrast, P450scc mRNA concentrations are not decreased at 12 h after PGF$_{2\alpha}$ treatment (Pescador et al., 1996).

Prostaglandin F$_{2\alpha}$ inhibits LH-stimulated progesterone production. It is not surprising that the effects of PGF$_{2\alpha}$ are exerted at the rate-limiting step of steroidogenesis, StAR. A disruption of LH stimulation would be expected to cause a decline in StAR, which is acutely regulated by LH. It is, however, not surprising that P450scc and 3β–HSD are not as sensitive to PGF$_{2\alpha}$ inhibition of LH stimulated progesterone production because LH acutely regulates neither.

**Structural Regression of the Corpus Luteum**

Although luteolysis is sometimes not thought to occur through separate processes, it can be described as two distinct mechanisms. Functional regression or the loss of the ability to synthesize progesterone was discussed in the previous section. This section will focus on the structural regression of the CL during luteolysis.

One of the contributing factors of PGF$_{2\alpha}$-induced luteolysis is a change in luteal blood flow. Following PGF$_{2\alpha}$ treatment, blood flow within the CL initially increases between 30 minutes and 2 hours and then decreases (Acosta et al.,
2002). There is a correlation between the decline in blood flow to the CL-containing ovary and serum progesterone concentrations (Nett et al., 1976). The decline in blood flow occurs in PGF$_{2\alpha}$-responsive CL, but not in Day 4 non-responsive CL (Acosta et al., 2002). The decrease in blood flow can be attributed to a decrease in the prevalence of blood vessels (Nett et al., 1976; O'Shea et al., 1977; Gaytan et al., 1999) and to the occlusion of the vessels with cellular debris (O'Shea et al., 1977; Modlich et al., 1996). Some of the cellular debris is composed of endothelial cells that have become detached from the basement membrane of degrading vessels (Modlich et al., 1996). Although volume of the luteal vasculature decreases during luteolysis, there is some functional vasculature maintained in the regressing CL (Gaytan et al., 1999).

Endothelial cells appear to be the first cells that are affected by the luteolytic effects of PGF$_{2\alpha}$, followed by the steroidogenic cells and fibroblasts (Sawyer et al., 1990). During luteolysis, LLC decrease in size, but there is no change in the size of SLC (Braden et al., 1988), whereas in regressing CL, SLC are preferentially lost before LLC (Schwall et al., 1986; Braden et al., 1988). In sheep and cattle, the loss of luteal cells seems to be the result of apoptosis (Sawyer et al., 1990; Juengel et al., 1993; 2000) via Fas-mediated signaling (Pru et al., 2002). However, apoptotic and necrotic luteal cells have been identified in regressing CL in the marmoset monkey (Young et al., 1997; Fraser et al., 1999).

**The Role of Immune Cells During Luteolysis**

After loss of function, luteal tissue undergoes involution that might be a function of immune cells. Macrophages which are capable of phagocytosis, have
been identified in luteal tissue from numerous species. Macrophages are present at all stages of development and regression in the CL, but increase significantly during luteolysis (Paavola, 1979; Lobel and Levy, 1968; Bagavandoss et al., 1988; Penny et al., 1999; Bauer et al., 2001). Paavola (1979) described macrophages that contained luteal cell fragments, supporting the hypothesis that macrophages function to remove regressed luteal tissue.

Bauer et al. (2001) reported that the increase in number of macrophages is due to proliferation. However, the increase in macrophages might also be the result of influx from the peripheral circulation due to chemoattractants. Monocyte chemoattractant protein-1 (MCP-1) directs monocytes/macrophages into sites of inflammation. There is an increase in MCP-1 associated with luteolysis and the accumulation of macrophages (Tsai et al., 1997; Haworth et al., 1998; Penny et al., 1998; Townson et al., 2002). Vascular endothelial cells of the bovine CL contain MCP-1 (Townson et al., 2002). Cytokines, not PGF$_{2\alpha}$, induce MCP-1 gene expression in luteal-derived endothelial cells (Cavicchio et al., 2002). Intracellular-adhesion molecule-1 (ICAM-1) gene expression and monocyte/macrophage accumulation occurs as a result of prolactin-induced luteolysis in rats (Olson et al., 2000; 2001). Similar to MCP-1, the amount of ICAM-1 is not altered by PGF$_{2\alpha}$ (Olson et al., 2001). The accumulation of macrophages is the result of chemoattraction by ICAM-1 and MCP-1 and not regulation by PGF$_{2\alpha}$ (Olson et al., 2001; Cavicchio et al., 2002).

T lymphocytes have also been identified in the CL. Similar to macrophages, CD4$^+$ and CD8$^+$ T cells are present in the bovine CL during the luteal phase and
luteolysis (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002). The CD4⁺ T helper cells are less abundant and there are conflicting reports as to whether CD4⁺ cells increase during luteal regression (Bauer et al., 2001; Townson et al., 2002) or to what extent numbers of these cells vary with luteal development and regression (Penny et al., 1999). Unlike CD4⁺ T cells, CD8⁺ cytolytic T cells are more abundant within the CL and increase during the late stages of the estrous cycle in non-pregnant animals (Penny et al., 1999; Townson et al., 2002), most likely by infiltration (Bauer et al., 2001).

Petroff et al (1997) reported that bovine luteal cells stimulate T cell proliferation in vitro. However, it has yet to be reported which T cell subset, CD4⁺ or CD8⁺ T cells, is stimulated to proliferate. Another T cell subset, gamma/delta T cells, has not been identified in the bovine CL. A study presented in this dissertation will address which T cell subset is stimulated to proliferate when co-cultured with luteal cells and what cytokines are produced by the T cells when stimulated. T cell stimulation and cytokine production will be discussed in a later section.

The Involvement of Cytokines During Luteolysis

Tumor necrosis factor-α was first described as a tumoricidal factor produced by macrophages (Carswell et al., 1975). Tumor necrosis factor-α has been suggested to have a role in luteal regression (for review; Pate, 1995; Davis and Rueda, 2002). Tumor necrosis factor-α mRNA has been detected at all stages of the estrous cycle in CL of cattle, however, the concentration does not vary even after PGF₂α-induced luteolysis (Petroff et al., 1999; Sakumoto et al., 2000).
Tumor necrosis factor-α protein has also been reported to be present in the CL (Shaw and Britt, 1995).

The primary source of TNF-α in the porcine CL is macrophages (Zhao et al., 1998). However, endothelial cells are also a potential source of TNF-α (Hehnke-Vagnoni et al., 1995; Zhao et al., 1998). Immunoreactive TNF-α has been localized in the cytoplasm of human granulosa-lutein cells (Roby et al., 1990). Two TNF receptors have been identified (Hohmann et al., 1989). Signaling through the type I receptor induces proliferation of cytotoxic T cells (Tartaglia et al., 1991) and is associated with apoptosis (Wong and Goeddel, 1994), whereas signaling through the type II receptor induces cell survival and proliferation (Tartaglia et al., 1991). The type I receptor has received the most attention in the study of the CL (Friedman et al., 2000; Sakumoto et al., 2000) most likely because the signaling effects of this receptor are associated with luteolysis. The amount of TNF receptor protein does not change throughout the estrous cycle in the bovine CL (Sakumoto et al., 2000).

The presence of TNF-α and its receptor in the CL suggests that TNF-α might contribute to luteal function. A TNF-α dose-dependent inhibition of LH-stimulated progesterone production occurs in long-term culture of bovine luteal cells (Benyo and Pate, 1992). In rats, administration of TNF-α inhibits the hCG- and prolactin-induced increase in StAR mRNA (Chen et al., 1999). However, this effect of TNF-α inhibition of progesterone synthesis could be an indirect effect through the actions of PGF$_{2\alpha}$.
Treatment of bovine luteal cells in vitro with TNF-α causes a dose-dependent increase in PGF$_{2\alpha}$ synthesis and a tendency for progesterone concentrations to decline (Benyo and Pate, 1992). The TNF-α-induced PGF$_{2\alpha}$ production by luteal cells requires RNA and protein synthesis (Townson and Pate, 1996). The addition of a phospholipase A$_2$ (PLA$_2$) inhibitor to the cultures inhibits TNF-α-induced PGF$_{2\alpha}$ production, but not basal PGF$_{2\alpha}$ production. Townson and Pate (1996) concluded that TNF-α stimulates PGF$_{2\alpha}$ synthesis by bovine luteal cells through PLA$_2$ activation. Prostaglandin production depends on expression of the COX gene. Treatment of bovine luteal cells in vitro with TNF-α increases the amount of COX-2 mRNA (Sandeen, 2003). Amounts of COX-2 protein and PGF$_{2\alpha}$ release are increased when bovine brain microvessel endothelial cells are treated with TNF-α (Mark et al., 2001). The COX gene promoter contains a nuclear factor kappa-B (NF-κB) binding site. Tumor necrosis factor-α increases binding of the transcription co-activator NF-κB to the COX-2 promoter (Deng et al., 2003).

Interferon-gamma (IFN-γ) has also been implicated to have a role in luteolysis (Pate, 1995; Pate and Keyes, 2001). Interferon-γ was first identified through its antiviral activity (Wheelock, 1965), but has since been recognized to regulate many other cellular processes. In luteal cell cultures, IFN-γ has similar effects to TNF-α. Luteinizing hormone (Fairchild and Pate, 1991) or hCG-induced (Fukuoka et al., 1992; Best et al., 1995) progesterone production is inhibited by
IFN-γ. Inhibition of progesterone production is greater if granulosa-lutein cells are treated with a combination of TNF-α and IFN-γ (Fukuoka et al., 1992).

Interferon-γ not only causes a reduction in progesterone production, but also cell viability. IFN-γ alone decreases cell viability, however, cell death is greater if luteal cells are cultured with IFN-γ and TNF-α (Benyo and Pate, 1992; Jo et al., 1995; Petroff et al., 2001). Inhibitors of COX, lipooxygenase, PLA₂, as well as nitric oxide synthase do not protect luteal cells from cytokine-induced cell death (Petroff et al., 2001).

Similar to TNF-α, long-term treatment with IFN-γ stimulates prostaglandin production in cultured luteal cells (Fairchild and Pate, 1991). Treatment with interferon-γ increases the amounts of COX-2 protein and PGE₂ production in mouse peritoneal macrophages (Blanco et al., 2000). Interferon-γ functions as an activator of the COX-2 gene through interferon regulatory factor-1 (IRF-1) transcription factor and IFN-stimulated responsive element (ISRE; Blanco et al., 2000). To date, only the STAT1/3 proteins have been determined to be involved in IFN-γ signaling in bovine luteal cells (Suter et al., 2001). Fairchild and Pate (1991) reported that treatment of cultured luteal cells with the COX inhibitor, indomethacin, does not prevent the IFN-γ-induced inhibition of progesterone production, yet exogenous progesterone abolishes IFN-γ-induced prostaglandin synthesis. Therefore, inhibition of progesterone production by the luteal cells is not the result of increased PGF₂α induced by IFN-γ.
Interferon-γ is a known regulator of the major histocompatibility complex (MHC) molecules that present peptide antigens to T cells. Luteal cells have both MHC class I and II molecules (Fairchild and Pate, 1989; Khoury and Marshall, 1990; Kenny et al., 1991). Treatment with IFN-γ for 72 hours increases the number of MHC class I and II molecules on bovine luteal cells (Fairchild and Pate, 1989). The elevation of MHC class II molecules is attenuated with LH treatment. Freshly dissociated luteal cells collected throughout the luteal phase of the estrous cycle, luteolysis, and on day 18 of pregnancy vary in the amount of MHC class II (Benyo et al., 1991). Major histocompatibility complex class II molecules increase during luteal development with fewer being present during the midcycle as compared with Day 18 of the estrous cycle. The percentage of luteal cells with MHC class II molecules also increases during PGF<sub>2α</sub>-induced luteolysis. Interestingly, luteal cells isolated from CL collected on Day 18 of pregnancy have fewer MHC class II molecules as compared with luteal cells obtained from animals on Day 18 (non-pregnant) of the estrous cycle.

At all stages of luteal development and regression, MHC class I is constitutively present (Benyo et al., 1991). Treatment of bovine luteal cells with IFN-γ increased the percentage of MHC class I positive cells (Fairchild and Pate, 1989). Peptides that are presented via MHC class I are processed intracellularly by the proteasome. The proteasome consists of multiple subunits including LMP7 and LMP10 that are IFN-γ induced (Groettrup et al., 1996; Nandi et al., 1996). Both LMP7 and LMP10 mRNA has been detected in luteal tissue by Northern blot analysis and LMP10 protein is present in SLC (Cannon and Pate, 2003).
Steady-state amounts of LMP7 mRNA do not change while LMP10 mRNA increases from early to mid and the later stages of the estrous cycle. Therefore, it is conceivable that the CL is capable of processing peptides and presenting these peptides via MHC class I.

Luteal cells are able to stimulate T cell proliferation in a MHC class II dependent manner (Petroff et al., 1997). It has not been determined if luteal cells have the ability to stimulate T cell proliferation in a MHC class I dependent manner. Furthermore, stimulation of T cells via MHC class I or class II are specific for either CD8⁺ or CD4⁺ T cells, respectively. A specific aim of the research presented in this dissertation addresses whether luteal cells are able to stimulate T cells via MHC class I and class II and which cells are stimulated.

OTHER IMMUNE CELLS

CD4⁺ T Helper Cells

Naïve CD4⁺ T cells have the potential to differentiate into two subsets, T₄₁ or T₄₂ (Mosmann et al., 1986). These subsets are based upon the pattern of cytokine secretion. The T₄₁ cells are important for cell-mediated immunity and produce IFN-γ, whereas T₄₂ cells produce IL-4, IL-5, and IL-10 that promote humoral immunity (Abbas et al., 1996). Differentiation of naïve T cells depends upon many signals including T cell receptor and peptide antigen recognition, co-stimulatory molecules, cytokine signaling, and induction of key transcription factors.
Activation of T cells requires two signals, T cell receptor occupancy and a co-stimulatory signal (Mueller et al., 1989). The first signal is the interaction of the T cell receptor with the antigen-presenting cell. CD4+ T cells form a complex with antigen-presenting cells presenting antigen via the MHC class II molecule (Doyle and Strominger, 1987). The CD4+ co-receptor increases adhesion between the T cell and antigen-presenting cell (Sung et al., 1986) by increasing the avidity to which a T cell binds to the antigen-presenting cell (Marrack et al., 1983; Moretta et al., 1984). Anti-CD4 inhibits CD4+ cell functions that are specific for the MHC class II molecules (Krensky et al., 1982; Meuer et al., 1982; Biddison et al., 1982) by inhibiting the interaction of the cell surface molecules and subsequent increase in intracellular Ca2+ concentrations (Tite et al., 1986; Ledbetter et al., 1987).

In the absence of a co-stimulatory signal, T cell clones fail to proliferate (Jenkins and Schwartz, 1987). The co-stimulatory pathway is directed through the CD28 molecule on T cells that interacts with CD86 on antigen presenting cells (Harding et al., 1992; Jenkins et al., 1991). The CD28 co-stimulation increases transcription and stability of IL-2 mRNA (Fraser et al., 1991), the cytokine that induces T cell proliferation.

The cytokine environment has a role in commitment of naïve CD4+ T cells to either Th1 or Th2. The classic cytokine that has a role as a Th1-inducing factor is IL-12 (Hsieh et al., 1993; Seder et al., 1993; Manetti et al., 1993; 1994). Interleukin-12 is a heterodimer consisting of a p35 and p40 subunit (Presky et al., 1996). Interleukin-12 has pleiotropic functions that include inducing cell
proliferation, enhancing IFN-γ production by T cells and NK cells, and inducing T_H1 differentiation (Watford et al., 2003). Naïve CD4^+ T cells lack the IL-12 receptor, but upon activation the IL-12 receptor β_1 chain and β_2 chain are upregulated on T_H1 cells but suppressed on T_H2 cells (Szabo et al., 2003). Binding of IL-12 to its receptor activates the transcription factor STAT4 (Trinchieri et al., 1998; Nishikomori et al., 2002). The importance of STAT4 for T_H1 response is evident in STAT4 deficient mice. In STAT4^−/− mice, IFN-γ production is abolished along with T cell proliferation (Thierfelder et al., 1996) and there is a shift from a T_H1 to a T_H2 phenotype (Kaplan et al., 1996).

Naïve CD4^+ T cells are directed toward a T_H2 phenotype by IL-4. CD4^+ cells primed with IL-4 acquire the capacity to secrete the T_H2 cytokines, IL-4 and IL-5 (Swain et al., 1990; Seder et al., 1992). Cells primed with IL-4 secrete minimal amounts of IFN-γ, but in the absence of IL-4 the cells differentiate to the T_H1 cell phenotype and produce IFN-γ (Seder et al., 1992). While STAT4 appears to be important for a T_H1 cell response, the STAT6 transcription factor is involved in T_H2 cellular differentiation. Mice deficient for STAT6 have impaired T_H2 cellular differentiation (Zhu et al., 2001).

Cytokine regulation of transcription factors has received a lot of attention in T_H1 versus T_H2 cellular differentiation. The transcription factor, T-bet is present in T_H1 cells but absent in T_H2 cells (Szabo et al., 2000). The absence of T-bet in CD4^+ T cells causes a marked reduction in IFN-γ production (Szabo et al., 2002). Interestingly, T-bet^−/− CD4^+ T cells fail to differentiate into the T_H1 cell type and default to the T_H2 phenotype (Szabo et al., 2002). T-bet has a role in epigenetic
regulation by inducing structural changes in chromatin at the IFN-γ locus (Mullen et al., 2001). The induction of T-bet is not dependent on IL-12/STAT4 (Mullen et al., 2001) but this has been shown to be important for Th1 cellular differentiation. Mullen et al. (2001) concluded that T-bet functions as a secondary stimulus to prolong IFN-γ synthesis.

The other Th helper specific transcription factor, GATA-3 is believed to be specific for Th2 cells. Expression of the GATA-3 gene results in the induction of a Th2 phenotype and suppresses the Th1 phenotype (Zhang et al., 1997; Ouyang et al., 1998). If GATA-3 gene expression is proportionately greater than T-bet gene expression, naïve CD4^+ T cells commit to the Th2 phenotype based on their cytokine production (Cousins et al., 2002; Chaker et al., 2003).

**CD8^+ Cytolytic T Cells**

Although CD4^+ and CD8^+ T cells contain a similar αβ T cell receptor, the CD4^+ and CD8^+ cells recognize different MHC-antigen complexes (Meuer et al., 1982). The CD8^+ T cells recognize antigens presented via MHC class I (Swain, 1981; Meuer et al., 1982). The interaction of the CD8 co-receptor with MHC class I augments a cytolytic T cell response.

Unlike CD4^+ T cells that are major sources of cytokines that regulate a humoral or cell-mediated response, CD8^+ T cells synthesize and secrete pro-inflammatory cytokines. One of the biological functions of CD8^+ T cells is the synthesis and secretion of IFN-γ (Prystowsky et al., 1982; Guerne et al., 1984; Boehm et al., 1997) and TNF-α (Vassalli et al., 1992).
The primary function of CD8\(^+\) T cells is lymphocyte-mediated cytotoxicity. Two independent pathways have been demonstrated to exist in the elimination of target cells by CD8\(^+\) T cells. The secretory pathway, or perforin-dependent model, involves the secretion of cytolytic granules (for review, Kägi et al., 1996). Upon release from the T cell, the perforin undergoes a conformational change and inserts itself into the target cell membrane. Eventually the cell membrane becomes permeable to water and other ions that leads to death of the target cell (for review, Catalfamo and Henkart, 2003).

Target cell lysis in perforin-deficient mice proceeds via the exocytosis independent pathway, the Fas pathway (Simon et al., 2000). Fas-mediated apoptosis of target cells is via a receptor-mediated pathway. Upon T cell activation, Fas ligand is upregulated on T cells. The Fas ligand present on T cells interacts with Fas that is present on the surface of the target cells (Kägi et al., 1994; Lowin et al., 1994). The secretion of IFN-\(\gamma\) by T cells up-regulates Fas on target cells (Müllbacher et al., 2002).

During an immune response, CD8\(^+\) T cells can kill target cells through activation of the perforin-dependent pathway or by Fas-Fas ligand interaction. Cytolysis of target cells involves fragmentation of target cell DNA (Russell et al., 1980) as well as membrane damage (Müllbacher et al., 2002). Through the actions of lymphocyte-mediated cytotoxicity, tumor cells are eliminated. Luteal regression might be considered a model of lymphocyte-mediated cytotoxicity. The increase in CD8\(^+\) cell numbers in the regressing CL suggests these cells have some function during luteolysis. It has been reported that the Fas-Fas
ligand interaction mediates apoptosis in the regressing CL (Roughton et al., 1999; Taniguchi et al., 2002). The Fas-mediated apoptotic pathway, therefore, might contribute to regression of the CL.

**Gamma/Delta T Cells**

Gamma/delta (γδ) T cells undergo the same V-D-J rearrangement as CD4⁺ and CD8⁺ αβ T cells, however the function or purpose of this lineage of T cells remains an enigma. Interestingly, there are age and species differences in the numbers of γδ cells. In humans, there are age-related changes in the different V delta subsets (Parker et al., 1990). The γδ cells are a minor population in the adult human and mouse (Kronenberg, 1994). An age-related decrease in γδ cells also occurs in cattle (Hein and Mackay, 1991). Nearly 70% of the T cells in circulation are γδ cells in calves and the percentage of γδ positive cells decreases in adult cattle (Hein and Mackay, 1991; Wyatt et al., 1994; Davis et al., 1996). The reason for the difference in number of these cells among species remains unknown.

Two subpopulations of γδ T cells have been identified in cattle (Wyatt et al., 1994; Davis et al., 1996; MacHugh et al., 1997). The major population of γδ cells in blood is WC1⁺ CD2⁻ CD4⁻ CD8⁻, while the major population in the spleen is WC1⁻ CD2⁺ CD8⁺ (MacHugh et al., 1997). Gamma/delta cells positive for CD8 contain the α-chain, not the β-chain (MacHugh et al., 1997).

Workshop cluster 1 (WC1) is a scavenger receptor with cysteine-rich repeats (Wijngaard et al., 1992; Ahn et al., 2002). This receptor is specific for a subset of
γδ cells in ruminants, although a WC1-like sequence is also present in humans and mice (Wijngaard et al., 1992). The significance of WC1 is unknown.

The γδ T cell receptor is similar to immunoglobulins (Rock et al., 1994). Antigen recognition by γδ T cells is different than that by αβ T cells (Schild et al., 1994). Alpha/beta T cells recognize antigenic peptides present in complex with MHC class I or II molecules, but γδ cells recognize antigens in a MHC independent manner (Rock et al., 1994; Schild et al., 1994; Morita et al., 1995). Antigen processing is not required for recognition by γδ T cell receptors (Schild et al., 1994; Morita et al., 1995). Because antigen uptake, processing, and presentation are not by the classical MHC pathway, γδ T cell surveillance of antigens and response is more rapid. One such circumstance is the recognition of mycobacterial antigens, isopentenyl pyrophosphates (Tanaka et al., 1995; for review, Hayday, 2000).

Gamma/delta T cells recognize stress-induced self-antigens termed MHC class I-related chains (MICA/B; Groh et al., 1998; 1999; Wu et al., 2002). The MICA/B antigens are expressed on intestinal epithelial cells (Groh et al., 1996; 1998) and many carcinomas including lung, breast, ovarian, and kidney (Groh et al., 1999). The γδ cells recognize the MICA/B via the Vd1 region of the receptor (Groh et al., 1996; 1998). Binding of MICA provides both a T cell receptor-dependent signal and the natural killer receptor (NKG2D) co-stimulatory signal (Wu et al., 2002). The NKG2D is a receptor for MICA detected by Vd1 γδ T cells (Bauer et al., 1999). The MICA/NKG2D interaction results in an anti-tumor
function. Besides MICA, Vd1 γδ T cells recognize antigen-presenting cells expressing the CD1 antigen-presenting molecule (Leslie et al., 2002).

Similar to CD4⁺ αβ T cells, γδ T cells discriminate with respect to cytokine stimulation and differentially produce T helper cytokines (Ferrick et al., 1995). Unlike CD4⁺ αβ T cells, γδ T cells default to the TH1 phenotype (Uin et al., 2000).

**Dendritic Cells (DC)**

Dendritic cells are antigen-presenting cells and are believed to be the most potent antigen-presenting cell in the body. *In vivo*, it is speculated that one dendritic cell can effectively interact with as many as 1,000 target cells (Banchereau and Steinman, 1998). Similar to the other immune cells, DC have a myeloid lineage. Bone marrow derived stem cells are stimulated to differentiate into dendritic cells in the presence of granulocyte-macrophage colony-stimulating factor (Inaba et al., 1992; Scheicher et al., 1992). Interleukin-4 also has a role in differentiation of DC from myeloid precursor cells (Zhou and Tedder, 1996). Dendritic cells can also be derived from a lymphoid precursor population (Ardavin et al., 1993; Saunders et al., 1996).

Dendritic cells go through two phases of development and function. The primary function of immature DC is antigen processing. Through the process of phagocytosis, the immature dendritic cell internalizes apoptotic bodies and other cellular contents and processes the peptides to be presented by the MHC complex (for review, Banchereau et al., 2000). After the immature DC have processed the antigen, DC relocate from the peripheral tissue to lymph tissues where immature DC will undergo maturation and stimulate naïve T cells (for
review, Banchereau et al., 2000). For mature DC to be able to function as antigen-presenting cells and stimulate naïve T cells, the DC must possess the critical cell surface molecules required for antigen presentation. After terminal differentiation or maturation, there is an increase in MHC class II molecules, CD80, CD86, and CD83 (Zhou and Tedder, 1995; Zhou et al., 1996). Activation of T cells requires two signals, T cell receptor/MHC antigen complex interaction and CD28/CD80/CD86 co-stimulation. Dendritic cells possess the cell surface molecules to provide both signals for T cell activation.

The cell surface molecule CD83 appears to be exclusively present on mature DC (Kozlow et al., 1993; Zhou and Tedder, 1995; Twist et al., 1998). The CD83 molecule was originally termed HB-15 and belongs to the immunoglobulin superfamily (Zhou et al., 1992). It is speculated that CD83 is an adhesion molecule that has a role in regulating DC-mediated immune responses (Scholler et al., 2001). Fujimoto et al. (2002) reported that CD83 is crucial for CD4+ T cell development. The presence of CD83 on DC is upregulated by TNF-α (Morse et al., 1997; Berchtold et al., 1999), CD40 ligation (Caux et al., 1994), and prostaglandins (Steinbrink et al., 200). The presence of CD83 on the cell surface of DC has been accepted to be a marker of maturation, however, the presence of CD83 is not indicative of activation and function. Interleukin-12 is the primary cytokine secreted by mature DC. Mosca et al. (2000) found CD83 to be upregulated on the cell surface of DC within 4 hours of stimulation in vitro, yet, secretion of IL-12p40 slowly increased until 24 hours after stimulation. The
authors concluded that mature DC contained the CD83 protein, but presence of the cell surface molecule does not directly correlate with activity of DC.

Dendritic and T cells are dependent on each other for activation. Dendritic cell maturation and activation requires CD40 ligation. The interaction between the CD4+ T cells that contain the CD40 ligand and the DC that have the receptor for CD40L provide this terminal differentiation signal. When immature DC are stimulated with an agonistic CD40 antibody, this stimulation induces an increase in CD86, CD40, MHC class I and class II cell surface molecules (Schuerhuis et al., 2000). The CD40-ligated DC induce expansion of CD4+ and CD8+ T cells with the majority of the CD4+ cells producing IFN-γ (Terheyden et al., 2000). In the absence of CD40 ligation, DC predominantly stimulate T_{H2} cells (Terheyden et al., 2000). The CD40-activated DC ‘help’ to activate CD8+ cytolytic T cells (Bennett et al., 1998; Schoenberger et al., 1998; Zhou and Seder, 1998) and mature but not immature DC are able to stimulate a cytolytic response (Jonuleit et al., 2000). For CD8+ T cells to effectively induce a cytolytic response, these cells sometimes require help or priming from CD4+ T cells (Keene and Forman, 1982). The ability of CD4+ T cells to activate DC that then prime cytolytic T cells is termed cross-priming (Heath and Carbone, 1999), and depends on antigen recognition by the helper and cytolytic T cell (Keene and Forman, 1982; Bennett et al., 1998).
GONADOTROPIN-RELEASING HORMONE

GnRH Regulated Cell Signaling

Gonadotropin-releasing hormone (GnRH) has an important role in regulating reproductive function and has become not only the focus of treatment of reproductive disorders, but also of gonadal steroid-dependent cancers. Drs. Schally and Guillemin were jointly awarded the Nobel Prize in Physiology for determining the GnRH decapptide sequence ([pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂] Matsuo et al., 1971; Schally et al., 1971; Burgus 1972). Gonadotropin-releasing hormone is released from the stalk median eminence in an episodic manner into the hypophysial portal circulation to be transported to the anterior pituitary where it binds to a high affinity receptor and functions to stimulate production and release of the gonadotropins, LH and FSH (Urbanski et al., 1988; Haisenleder et al., 1994).

The GnRH receptor (GnRHR) is a member of the seven-transmembrane receptor family that is coupled to the G_{q/11} G-proteins (Hsieh and Martin, 1992). Binding of GnRH to the GnRHR stimulates an increase in phosphoinositide turnover, diacylglycerol concentrations and protein kinase C activation (PKC; Hsieh and Martin, 1992). Intracellular Ca^{2+} concentrations are also increased when GnRH binds to its receptor stimulating L-type voltage sensitive Ca^{2+} channels and inositol 1,4,5-triphosphate-induced release of Ca^{2+} from the intracellular Ca^{2+} stores (Naor, 1990; Tse and Hille, 1992). Saunders et al. (1998) showed that treatment of GGH₃ cells with 8-bromo-cAMP enhanced activity of all the gonadotropin subunit gene promoters and to a greater extent with the shared
α-subunit. However, the GGH₃ cell line consists of rat somatomammotrophic tumor cells that have been transfected with the GnRHR expression vector (Kuphal et al., 1994). Earlier work showed that the cAMP pathway is not activated in the immortalized mouse pituitary cell line, αT3-1 (Horn et al., 1991). It has since been shown that GnRH stimulates cAMP production in LβT2 pituitary cells by coupling to the Gₛ protein (Lui et al., 2002) and influences protein kinase A (PKA) subunits in αT3-1 cells (Garrel et al., 1997). Though there is promiscuity of the GnRH receptor coupling to the G-proteins, Ca²⁺ mobilization and PKC activation through the G₉/11 protein appears to be the preferred pathway of GnRH-stimulated cell signaling.

**Differential GnRH Regulation**

Occupancy of the GnRHR by GnRH regulates synthesis of the common α-subunit and the unique β-subunits of LH and FSH and release of the gonadotropins from a single cell type. Other factors such as gonadal steroids regulate the release of LH and FSH, with the primary regulation of LH release residing at the hypothalamus. Luteinizing hormone is released from the gonadotropes of the anterior pituitary in an episodic manner in cattle (Rahe et al., 1980) and sheep (Clarke and Cummins, 1982). The pulse frequency of LH release is concomitant with the episodic pulse frequency of GnRH in cattle (Rodriquez and Wise, 1989) and sheep (Clarke and Cummins, 1982). Secretion of FSH occurs independently of GnRH stimulation and does not require GnRH pulses (Clarke et al., 1986; Hamernik and Nett, 1988).
Removal of GnRH stimulation, either through hypothalamic-pituitary disconnection (Hamernik and Nett, 1988) or administration of a GnRH antagonist (Perheentupa and Huhtaniemi, 1990), decreases the amount of gonadotropin mRNA in the pituitary. Vizcarra et al. (1997) reported that continuous infusion of GnRH to cows did not influence concentrations of the common α-subunit and FSHβ-subunit mRNA, but did decrease the amount of LHβ-subunit mRNA. The manner that GnRH is delivered to the anterior pituitary differentially regulates transcription of the gonadotropin subunits. Whereas continuous infusion of GnRH down-regulates transcription of the LHβ-subunit (Vizcarra et al., 1997), an episodic GnRH pulse signal stimulates transcription of all three subunit mRNAs (Haisenleder et al., 1991).

Using an *in vivo* model (Haisenleder et al., 1991) or an *in vitro* superfusion model (Kaiser et al., 1997) it has been determined that pituitary cells are differentially stimulated with GnRH pulses. Gonadotropin-releasing hormone pulse frequency selectively alters transcription of the gonadotropin subunits. Transcription of the α- and LHβ-subunit is maximally stimulated when frequent pulses (30-min intervals) of GnRH are administered (Haisenleder et al., 1991; Kaiser et al., 1997). In contrast, slower GnRH pulses at two-hour intervals increase the transcription rate of the FSHβ-subunit (Haisenleder et al., 1991; Kaiser et al., 1997). Duration of GnRH pulses also alters transcription of gonadotropin subunit mRNAs. When GnRH pulses are given to rats, all three subunit mRNAs are increased after 1 and 4 hours. However, only the mRNA of
the $\alpha$-subunit remains elevated for a 24-hour period and the LH$\beta$- and FSH$\beta$-subunit mRNA decline at 24 hours (Haisenleder et al., 1991).

Gonadotropin-releasing hormone differentially regulates the amount of mRNA for the gonadotropin subunits and secretion of the gonadotropins. More frequent pulses are effective in stimulating synthesis of the $\alpha$- and LH$\beta$-subunit and LH release. Though FSH secretion does not require GnRH pulses, FSH$\beta$ gene expression is stimulated by less frequent GnRH pulses.

**Development of GnRH Analogs**

Since the isolation and determination of GnRH in 1971, over 3000 GnRH analogs have been synthesized (for review, Schally, 1999). Analogs of GnRH are being used for gynecology applications such as *in vitro* fertilization programs, treatment of reproductive disorders, precocious puberty, as well as chemotherapeutic agents for gonadal steroid-dependent cancers (Schally and Comaru-Schally, 1997). Gonadotropin-releasing hormone antagonists were developed as potential non-steroidal contraceptives. Many analogs developed were never used clinically because of toxic effects induced when administered to rats.

Proline$^9$ is a conserved residue involved in receptor binding. Modification of this amino acid in analogs is tolerated but it is usually not substituted with another amino acid because of conformational differences and a decrease in activity of the peptide after such substitution (Sealfon et al., 1997). However, Gly$^{10}$ in the C-terminal domain can be substituted without any toxic effects. Replacement of Gly$^{10}$ with D-Ala increases the potency over native GnRH (for
review, Karten and Rivier, 1986). Substitution of Gly$^{10}$ with ethylamide also increases the activity of the agonist (Coy et al., 1975), specifically greater ovulation-inducing potency (Fujino et al., 1972). The glycine residue that resides in the sixth position is frequently targeted for modification in GnRH agonists. This amino acid is thought to have a role in the conformation of the peptide for binding to the receptor. Substitution of Gly$^{6}$ with the hydrophobic amino acid alanine enhances binding of the agonist to the GnRHR. When Gly$^{6}$ is replaced with tryptophan, the bulky aromatic ring of tryptophan is 144 times more biologically potent than alanine. Furthermore, [D-Trp$^{6}$]GnRH has a much longer half-life than the native peptide.

Fewer antagonist of GnRH have been developed because of the toxic effects that rats experienced when administered many of the antagonists, but also because GnRH agonists have proven to be more useful than antagonists for gynecological procedures. The efficacy of an antagonist depends on the ability of the antagonist to inhibit ovulation. As stated previously, a single injection of a GnRH antagonist suppresses LH release and suppresses the preovulatory surge release of this hormone. The first antagonist developed that suppressed ovulation was [D-Phe$^{2}$, D-Ala]GnRH (Karten and Rivier, 1986). Later it was discovered that 4-Cl-D-Phe$^{2}$ increased the anti-ovulatory potency of the synthetic peptide (Karten and Rivier, 1986). Bajusz et al. (1988) designed an antagonist to GnRH with the criteria that a single injection would have greater biological potency by inhibiting ovulation and not inducing edematogenic effects. Finally, Dr. Schally’s laboratory group succeeded in synthesizing [Ac-D-Nal(2)$^{1}$, D-
Phe(4Cl)², d-Pal(3)³, d-Cit⁶, d-Ala¹⁰]GnRH, commercially recognized as cetrorelix (SB-75). The penta-substitution of D-amino acids may confer greater resistance to enzyme degradation. Cetrorelix or SB-75 is currently being extensively tested in clinical trials to treat prostate cancer.

Effects of GnRH Treatment on Luteal Function

Administration of a GnRH agonist to rats (Harwood et al., 1980; Jones and Hsueh, 1980) interferes with luteal progesterone production. The anti-steroidogenic effects of GnRH appear to be direct because specific high affinity GnRHR were identified in a preparation of isolated rat luteal cells (Clayton et al., 1979). Human chorionic gonadotropin-stimulated progesterone production is inhibited in dissociated rat luteal cells treated with a GnRH agonist (Harwood et al., 1980). Treatment of these cells with dibutyryl cAMP in the presence of a GnRH agonist does not alter progesterone production. Inhibition of progesterone production by a GnRH agonist in rat luteal cells appears to be mediated through a specific GnRHR that disrupts hCG-stimulated cAMP production.

For many years it was believed that GnRHR did not exist in the bovine CL (Brown and Reeves, 1983). Recently, Ramakrishnappa et al. (2001) isolated the mRNA for GnRHR in midcycle luteal tissue. It remains unclear if GnRH can act directly at the CL in cattle or if the actions of GnRH and its analogs are indirect. Treatment of cows with GnRH or its agonist exerts paradoxical effects depending on the stage of the estrous cycle. Treatment of cows with 100 µg of GnRH on Days 2 and 8 (Martin et al., 1990) or Days 2 and 10 (Rodger and Stormshak, 1986) had no effect or decreased serum progesterone concentrations,
respectively. Luteal slices from GnRH-treated cows produced greater concentrations of progesterone as compared with control slices in vitro (Martin et al., 1990). The control slices responded to LH, whereas the slices collected from GnRH-treated cows did not. Therefore, GnRH treatment might have altered the in vitro response of the luteal slices to LH because treatment with GnRH rendered the luteal cells refractory to further LH stimulation or it might be a result of SLC to LLC differentiation. Conti et al. (1976) reported that treatment of rats with hCG can induce a state of desensitization to LH by loss of the LH receptor in luteinized ovarian tissue, while treatment of ewes with pharmacological concentrations of LH causes differentiation of SLC to LLC (Farin et al., 1988). Administration of GnRH on either Days 2 and 8 or Days 2 and 10 (estrus = Day 0) did not alter the duration of the estrous cycle.

Administration of GnRH or its agonist to cows can be luteotropic. Repeated injections of GnRH to cows during the midluteal phase increase LH secretion and subsequently circulating progesterone concentrations (Kittok et al., 1973; Milvae et al., 1984). Continuous treatment with a GnRH agonist induces a desensitized state of the pituitary and decreased LH secretion. Continuous treatment of heifers with a GnRH agonist beginning on Day 3 of the estrous cycle when the CL is developing increases basal LH secretion and plasma progesterone concentrations (Davis et al., 2003). Plasma progesterone concentrations increase on Day 16 and 17 of the estrous cycle when GnRH is administered beginning on Day 12, probably as the result of increased basal LH secretion (Davis et al., 2003). However, the effects of repeated or continuous treatment
with GnRH on luteal tissue remain unknown. It could be hypothesized that either
luteal cell differentiation occurs so there are greater numbers of LLC that produce
greater amounts of progesterone as compared with SLC, or the increase in LH
causes an increase of LH-regulated factors required for progesterone synthesis.

In summary, administration of GnRH or its agonist during the estrous cycle
can have no effect, suppress, or enhance progesterone production. Single
injections of GnRH seem to have no effect or be detrimental to progesterone
production. Yet, when GnRH or its agonist is administered by multiple injections
or continuously, GnRH either exerts a direct or indirect luteotropic effect.

**SUMMARY**

Within a few days the CL develops from an ovulated follicle, undergoes
extensive growth, and if pregnancy does not occur the CL regresses.
Development of the CL in the cow is dependent on pulses of LH. If LH secretion
is suppressed or enhanced by treatment with GnRH analogs, subsequent
progesterone production by the CL is altered. An objective of a study presented
in this dissertation was to determine if the change of plasma progesterone
concentrations that occurred when cattle were administered a GnRH analog to
cattle was the result of the differentiation of small luteal cells to large luteal cells
or a change in the amount of the mRNAs for StAR, P450\textsubscript{scC}, or 3\beta-HSD.

The endocrine system is not the only system to contribute to the
development and demise of the CL. In recent years, research has been
conducted to determine the role of the immune system in the ovary, with the most focus on ovulation and luteal regression. The immune system might contribute to the demise of the CL in two ways: secretion of cytokines by the immune cells, T cells and macrophages; or by inducing apoptosis and removing the regressed tissue from the ovary. Treatment of bovine luteal cells with pro-inflammatory cytokines inhibits LH-stimulated progesterone production. Near the onset of and during luteolysis there is an increase in the number of T cells and macrophages in the CL. It has been reported that bovine luteal cells are able to stimulate T cell proliferation *in vitro*. It seems that the immune system has an important role in luteal regression. This is supported by the presence of T cells and macrophages in the luteal tissue as well as the effects on progesterone production by luteal cells *in vitro* when luteal cells are treated with cytokines.

T cells have been identified in the CL and luteal cells are capable of stimulating proliferation of these cells. It has yet to be determined which T cell is stimulated to proliferate by the luteal cells. A study presented in this dissertation was designed to address the question of which T cell was stimulated by bovine luteal cells. An objective of the same study was to determine if γδ T cells are present in the bovine CL and if these cells were stimulated to proliferate by luteal cells. Dendritic cells, which are potent stimulators of T cells, have not been identified in the CL, but cells that have been described as dendritic-like have been reported in the mouse CL (Jasper et al., 2000). Therefore, it was to be determined if dendritic cells were present in the CL by identifying the presence of the cell surface marker, CD83, that is considered specific for dendritic cells.
CHAPTER 2

CHRONIC GnRH ANALOG TREATMENT OF HEIFERS: LUTEAL CELL POPULATIONS AND STEADY-STATE AMOUNTS OF mRNA FOR THE STEROIDOGENIC ENZYMES

INTRODUCTION

Administration of gonadotropin-releasing hormone (GnRH) analogs during the estrous cycle of cattle alters luteal function. Treatment with GnRH agonist from Days 9 through 12 (Day 0 = estrus) increases progesterone concentrations and lengthens the estrous cycle of cattle (Milvae et al., 1984). Administration of GnRH agonist on Days 2 and 8 of the estrous cycle, however, did not increase serum progesterone concentrations (Martin et al., 1990). Chronic administration of a GnRH agonist beginning on Day 3 or Day 12 of the estrous cycle of cows increases CL size and circulating progesterone concentrations (Davis et al., 2003). Administration of a GnRH antagonist from Day 2 through 7 and Day 7 through 12 of the estrous cycle of cattle decreases luteal function (Peters et al., 1994). In contrast, plasma progesterone does not decrease when a GnRH antagonist is administered from Day 12 through 17 (Peters et al., 1994) as compared with administration of a GnRH agonist from Day 12 through 21 of the estrous cycle when plasma progesterone is increased (Davis et al., 2003).
During luteinization, thecal and granulosal cells develop into small and large luteal cells, respectively (Alila and Hansel, 1984). As the CL develops there is an increased size and number of large and small luteal cells (Schwall et al., 1986). Schmitt et al. (1996) reported that administration of human chorionic gonadotropin (hCG) did not alter the number of small and large luteal cells of the CL of cattle, but did increase the size of small luteal cells. Using a monoclonal antibody against antigens of thecal and granulosal cells, Alila and Hansel (1984) reported that small luteal cells can differentiate into large luteal cells. Small luteal cells can be induced to differentiate into large luteal cells by administration of a pharmacological dose of LH to sheep (Farin et al., 1988). It has been reported that large luteal cells can produce nearly 80% of the progesterone from the CL (Niswender et al., 1985), therefore, greater differentiation of small to large luteal cells would be expected to increase circulating progesterone concentrations.

The key components that regulate progesterone synthesis are steroidogenic acute regulatory (StAR) protein and the enzymes, cytochrome P450 side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β−HSD). The mRNA encoding for StAR (Pescador et al., 1996), P450scc (Rodgers et al., 1986), and 3β−HSD (Couët et al., 1990) have all been shown to increase in the CL of the cow from early to the mid portion of the estrous cycle. The StAR protein is acutely regulated by LH stimulation (Clarke et al., 1994) and withdrawal of endogenous LH by hypophysectomy decreases luteal content of StAR mRNA and serum progesterone concentrations (Juengel et al., 1995). Replacement of
LH in hypophysectomized ewes increases StAR mRNA and serum progesterone concentrations (Juengel et al., 1995).

The use of GnRH agonist and antagonist has previously been shown to alter the secretory profile of LH and plasma progesterone concentrations (Davis et al., 2003; Peters et al., 1994). The objectives of the present study were to determine if the change in LH secretion with administration of GnRH analogs to heifers alters luteal morphology or the messages encoding for StAR, P450_{scC}, and 3β−HSD that are required for progesterone synthesis. The first hypothesis of the present study was that administration of a GnRH agonist beginning when the CL was developing would induce differentiation of small luteal cells to large luteal cells and circulating progesterone concentrations would be increased during the luteal phase. The second hypothesis was that administration of a GnRH antagonist would inhibit adequate luteotropic support, therefore, the ratio of small to large luteal cells would be less than CL collected from controls or GnRH-agonist-treated heifers. The third hypothesis of the present study was that the mRNA for StAR, P450_{scC}, and 3β−HSD would be greater in CL collected from GnRH agonist-treated heifers as compared with control and GnRH-antagonist-treated heifers.

MATERIALS AND METHODS

Reagents

Mannitol was purchased from Sigma-Aldrich (St. Louis, MO). Eosin Y, Gill's hematoxylin, and NaCl were all purchased from Fisher Scientific (Fair Lawn, NJ).
Prefer fixative was purchased from Anatech (Battle Creek, MI). Trizol and SuperScript II were purchased from Invitrogen Life Technologies (Carlsbad, CA). The T easy vector, RNasin, and dNTPs were all from Promega (Madison, WI). Random hexamer primers were purchased from Amersham Pharmacia Biotech Inc. (Buckinghamshire HP7 9NA England) and DyNamo SYBR green was purchased from MJ Research (Waltham, MA). The Coat-A-Count Progesterone radioimmunoassay kit was purchased from Diagnostic Products Corporation (Los Angeles, CA).

**Experimental Design**

The Institutional Animal Care and Use Committee at The Ohio State University approved all procedures used in this experiment. The Food and Drug Administration approved the administration of the GnRH analogs, deslorelin and SB-75, under INAD10-911 and INAD10-912, respectively. Beef heifers (n = 21) ranging in age from 18 to 24 months of age were used in this experiment. These heifers were randomly assigned to one of four treatment groups: untreated controls (Cont; n = 6), Day 3 GnRH agonist (D3; n = 6), Day 12 GnRH agonist (D12; n = 6), or Day 3 GnRH antagonist (SB-75; n = 3). The GnRH agonist used in the present study was deslorelin ([D-Trp$^6$-Pro$^9$-des-Gly$^{10}$] GnRH ethylamide). The GnRH antagonist used was cetrorelix (SB-75; [Ac-d-Nal(2)$^1$, d-Phe(4Cl)$^2$, d-Pal(3)$^3$, d-Cit$^6$, d-Ala$^{10}$] GnRH) synthesized by Dr. Gautam Sarath (University of Nebraska, Lincoln, NE).

Heifers were administered the GnRH agonist, deslorelin, at a dose of 1 $\mu$g/kg BW/day using Alzet osmotic pumps (model 2ML2, ALZA Corp., Palo Alto, CA).
Prior to implantation, the deslorelin-filled pumps were placed in 0.15 M NaCl at 37°C overnight so the agonist would be delivered at the onset of insertion. Osmotic pumps were surgically inserted through a small incision near the seventh rib under local anesthetic (lidocaine) on Day 3 (D3 group) or D12 (D12 group) of the estrous cycle (Day 0 = behavioral estrus). Osmotic pumps were removed on Day 16 of the estrous cycle under the same conditions following removal of the CL. The GnRH antagonist, SB-75, was dissolved in 5% mannitol and administered by daily injections at a dose of 10 µg kg body weight\(^{-1}\) day\(^{-1}\) beginning on Day 3 and ending on Day 15.

Heifers were observed three times daily for behavioral estrus. Ovaries were examined daily beginning on Day 1 (Day 0 = estrus) using a 7.5-MHz transrectal linear probe and ultrasonographic monitor to measure area (cm\(^2\)) of the CL. An estimated value of the area of the CL was determined at the time of ultrasonographic evaluation using the built-in area measurement function. Daily jugular blood samples were collected in heparin coated tubes and immediately placed on ice. Blood samples were centrifuged at 1500 x g for 15 min, the plasma decanted and stored at –20°C until assayed for progesterone. Concentrations of plasma progesterone were quantified using the Coat-A-Count assay kit. Intra- and interassay coefficients of variation were less than 13%.

**Determination of Cell Number and Cell Size**

Corpora lutea were removed transvaginally on Day 16 of the estrous cycle from the heifers in all treatment groups. Corpora lutea were weighed and immediately sliced into smaller portions and two portions placed in Prefer fixative
(Anatech; Battle Creek, MI) for 48 h until the sections were processed and embedded in paraffin. Tissues were sectioned at a 5 µm thickness and every tenth section was mounted to ensure that large luteal cells that average 22 to 50 µm would not appear in two sections and be counted twice. Luteal sections were stained with eosin and counterstained with Gill’s hematoxylin. Digital images using an Olympus BX51 microscope equipped with the Olympus MagnaFire camera were acquired from two portions of the CL, two sections from each portion, and six views from the sections for a total of 24 areas of luteal tissue analyzed per animal. The number of large luteal cells and small luteal cells were counted based on the following criteria; size (SLC ≤ 22µm, LLC > 22µm), pattern of heterochromatin, and presence of nuclei. Three independent observers determined numbers of luteal cells. Size of the steroidogenic luteal cells was determined by measuring the greatest diameter of the cell using Image Pro Plus software (version 4.5). To determine the size of cells, two separate portions of the CL were analyzed with five views from each section. A total of 100 cells were measured per CL.

**Isolation and Quantification of mRNA**

Remaining luteal tissue was immediately snap frozen in liquid nitrogen for RNA isolation using Trizol. Integrity of the RNA was determined by gel electrophoresis. Specific primers (Table 2.1) were designed according to published bovine sequences for P450<sub>scC</sub>, 3β-HSD, and glyceraldehyde 3 phosphate dehydrogenase (G3PDH). The G3PDH primers were designed and validated prior to the present study by the laboratory technician (Jodi Winkler).
Primers specific for bovine StAR were previously published (Tsai and Wiltbank, 1998; Table 2.1). Each primer pair was first validated to ensure amplification of the proper size fragment and that no amplification of DNA occurred. The PCR products for each sequence of interest were purified and subcloned into pGem T-easy vector. The plasmids were sequenced at the OARDC Molecular and Cellular Imaging Center to confirm amplification of the correct gene of interest.

First-strand cDNA was synthesized from 2 μg of RNA in a final volume of 20 μl (10 ng/μl random hexamer primers, 0.5 mM dNTPs, 1X First-Strand Buffer, 10 mM dithiothreitol [DTT], 40 units Rnasin, 200 units SuperScript II RT). Real-time PCR was performed using the DNA Engine Opticon 2 System (MJ Research) to determine relative abundance of mRNA in the luteal tissue. A cycle test and temperature gradient test (50 – 60°C) was performed for each pair of primers. Briefly, 10% of the reverse transcription reaction was used for a final 20 μl reaction volume (1X DyNamo SYBR Green Master Mix, 0.4mM forward and reverse primers). The PCR conditions were: 95°C, 30sec; 58.5°C (StAR and P450scc)/59.3°C (3β-HSD and G3PDH), 30sec; 72°C, 1min for 25 cycles (P450scc, 3β-HSD, and G3PDH) or 26 cycles (StAR). Standard curves that were generated from purified PCR product were included within every PCR reaction. The standard curve for each reaction consisted of four serial dilutions (1:10) that were adjusted so amplification of the unknown samples would lie within the range of values that were used to develop the curve. All samples were run as duplicates, standardized to G3PDH, and expressed as relative values. Amplification of a
single fragment of the correct size for every sample was verified by gel electrophoresis.

Statistical Analysis

Means for progesterone concentrations and CL area were determined by repeated measures using the MIXED procedure of SAS (version 8.1, Cary, NC). Treatment, day, and treatment X day interaction were included in the model. Analysis of variance using the MIXED procedure of SAS was used to compare the differences in luteal weights, cell numbers, cell sizes, and mRNA amounts. Differences in the LSmeans were determined using the PDIFF statement of SAS.

RESULTS

Luteal Function and Size

Treatment with deslorelin beginning on Day 3 of the estrous cycle increased plasma progesterone concentrations on Day 7 compared with control concentrations (P < 0.05; Figure 2.1). Plasma progesterone concentrations remained elevated in the D3 group through Day 16 of the estrous cycle (P < 0.01). Desorelin treatment increased progesterone concentrations on Days 14, 15, and 16 for the D12 group compared with control heifers (P < 0.01). Daily administration of SB-75 had no effect on plasma progesterone concentrations.

Area of the CL (cm²) as determined by ultrasonography was only altered in the D3 group compared with control heifers. Area of the CL was larger from Day 7 through Day 16 of the estrous cycle for the D3 group (P < 0.05; Figure 2.2). Neither treatment with deslorelin on Day 12 nor SB-75 changed the size of the
CL. The results were similar for treatment effect on weight of the CL. Corpora lutea collected from D3 heifers (8.77 ± 1.00 g) were larger as compared with CL from control heifers (4.67 ± 0.56 g; P < 0.001). Corpora lutea collected from D12 deslorelin (6.28 ± 0.59 g) and SB-75 treated heifers (4.12 ± 0.52 g) were similar in weight as those for the control group.

**Determination of Cell Size and Ratio**

Diameter of small and large luteal cells did not vary among any of the treatment groups (Table 2.2). One CL from the SB-75 group was destroyed during tissue processing; therefore, results included data from two CL. Treatment with SB-75 was associated with a decrease in small luteal cell numbers and an increase in large luteal cell numbers (Table 2.2; Figure 2.3). There was a decrease in small to large luteal cell ratio in luteal tissue removed from the SB-75-treated heifers compared with the heifers of the control group (Table 2.2).

**Steroidogenic Capacity**

Steady-state amounts of mRNA encoding for StAR were greater on Day 16 of the estrous cycle in luteal tissue from D3 deslorelin-treated heifers compared with heifers of the control group (P < 0.05; Figure 2.4). Deslorelin treatment beginning on Day 12 tended to increase (P = 0.09) the relative amount of StAR mRNA in luteal tissue, while there was no effect of SB-75 treatment on StAR mRNA concentrations. Relative amounts of mRNA for P450scc and 3β-HSD were not changed with either deslorelin or SB-75 treatment.
DISCUSSION

Treatment of heifers with deslorelin, a GnRH agonist, increased plasma progesterone concentrations, CL area, and CL weight when administered beginning on Day 3 of the estrous cycle. When deslorelin treatment began on Day 12 of the estrous cycle, only plasma progesterone concentrations were increased. There was no effect of treatment on size or weight of the CL in the D12 group. The enhanced luteal function and size in the present study was similar to what was previously reported when the GnRH agonist, nafarelin, was administered to cows (Davis et al., 2003). It was also reported in the previous study, that basal LH secretion was greater in the GnRH agonist-treated cows as compared with the cows of a control group. The goal of the present study was to determine what effects increased basal LH secretion induced by GnRH agonist treatment might have to enhance progesterone secretion.

Because large luteal cells are responsible for nearly 80% of the progesterone production (Niswender et al., 1985), it was hypothesized that the increase of plasma progesterone concentrations in the D3 group was the result of small luteal cells differentiating to large luteal cells. However, the relative proportion of small and large luteal cells did not differ with deslorelin treatment. This is in contrast to what has been previously reported in the ewe when administration of a pharmacological dose of pulses of LH to ewes decreased the small to large luteal cell ratio (Farin et al., 1988). In contrast, chronic GnRH agonist treatment causes a sustained elevation of basal LH secretion (Davis et al., 2003). It can be speculated that induced differentiation of small to large luteal cells requires
pharmacological concentrations of LH in a pulsatile manner. In the present study, the increase of basal LH secretion was not adequate to alter the proportion of steroidogenic luteal cells.

The proportion of small to large luteal cells reported in the present study is less than previous estimates obtained from fixed luteal tissue (7.6:1; O'Shea et al., 1989) and substantially less than estimates obtained from dispersed luteal cells (20:1; Hansel et al., 1987) from cattle. The ratios reported in the present study are closely related to the 4.5:1 ratio of small to large luteal cells reported by Rodgers et al. (1984) for the sheep CL. Selective loss of large luteal cells occurs when luteal tissue is dissociated (Rodgers et al., 1984; O'Shea et al., 1989). Fixed tissue was, therefore, used to evaluate cell numbers in the present study. The criteria used to distinguish small and large luteal cells were effective in identifying the conspicuous large luteal cells. However, small luteal cells were not as readily identified and the number of small luteal cells per view might have been underestimated causing a lesser ratio of small luteal cells to large luteal cells, yet the relative numbers of large luteal cells were not altered with GnRH agonist treatment.

Average mean diameter of small and large luteal cells in all treatment groups is similar to those from previous reports (Koos and Hansel, 1981; O’Shea et al., 1989; Lei et al., 1991; Fields et al., 1992). Neither GnRH agonist nor antagonist treatment changed the diameter of small or large luteal cells. Therefore, the increase in area and weight of the CL in the D3 group is not due to hypertrophy of the steroidogenic cells.
The difference in weight of the CL between the control and D3 group was nearly double. It has been shown that ovulation of smaller preovulatory follicles reduces the size and function of the resulting CL (Vasconcelos et al., 2001; Mussard et al., 2002). Ovulation of larger follicles in the D3 group is an unlikely explanation for increased luteal weight. A number of the heifers ovulated prior to initiation of ultrasonography, so size of the preovulatory follicle was not obtained for heifers in the present study. However, the heifers were allowed to spontaneously ovulate and were randomly assigned to treatment groups prior to detection of estrus. It was assumed, therefore, that variation in size of the preovulatory follicle should be equally distributed across all treatment groups.

Following the preovulatory LH surge, theca-derived small luteal cells undergo mitosis (O’Shea et al., 1980), while granulosa-derived large luteal cells cease to proliferate (McClellan et al., 1975; O’Shea et al., 1980). To maintain small to large luteal cell ratio in CL that are nearly double the size, as in the D3 group, other sources of large luteal cells other than granulosa-derived cells must contribute to this cell population. It has been suggested that the CL contains a population of stem cells that are mitotically active and differentiate into small luteal cells (Niswender et al., 1985). It is speculated that these small luteal cells might develop into large luteal cells under the chronic basal LH stimulation that existed in the present study. However, this model would be proposed for the effects observed in the D3 group, not the D12 group, because mitosis of cells has been observed in developing CL early in the estrous cycle (McClellan et al.,...
1975; O'Shea et al., 1980) not in the CL of the latter stages of the estrous cycle (O'Shea et al., 1986) and the CL in the D12 group did not increase in size.

Steady-state amounts of StAR mRNA were greater and tended to be greater in CL collected from the D3 and D12 deslorelin-treated heifers, respectively. The increase of StAR mRNA is likely the result of increased LH secretion induced by the GnRH agonist. Luteinizing hormone is known to be the tropic hormone that stimulates StAR (Clark et al., 1994), the rate-limiting step of steroidogenesis. In contrast, there was no effect on the steady-state amounts of mRNA encoding for P450_{sc}c and 3β-HSD. This was not surprising considering both these enzymes are in abundance and not rate-limiting. Steady state amounts of P450_{sc}c and 3βHSD mRNA increase no more than two-fold, while serum progesterone concentrations can increase as much as 25-fold in the CL during the estrous cycle of ewes (Niswender et al., 2000). Furthermore, with luteinization of cells the presence of P450_{sc}c in the CL is cAMP independent and is maintained in a constitutive manner (Goldring et al., 1987; Hickey et al., 1989; Oonk et al., 1990). Interestingly, the large luteal cells have greater concentrations of mRNA for P450_{sc}c and 3β-HSD as compared with small luteal cells (Wiltbank et al., 1993; Juengel et al., 1994).

Although it was not determined in the present study, effects of the GnRH agonist treatment on the vasculature of the CL might have attributed to the increase in plasma progesterone concentrations. Treatment of ewes with LH increased the cell volume and diameter of capillary endothelial cells (Farin et al., 1988). It has been reported for female rats (Varga et al., 1985), rabbits (Wiltbank
et al., 1989), and sheep (McCracken et al., 1971; Niswender et al., 1976) that administration of either LH or hCG increases blood flow to the ovary containing the CL. Wiltbank et al. (1989) found that luteal blood flow does not increase when hCG is administered to rabbits. The increase of ovarian blood flow is associated with an increase in circulating progesterone concentrations. The increase in basal LH secretion brought about by GnRH agonist treatment may have increased blood flow to the luteal ovary, likely providing more substrate for steroidogenesis or luteotropic support. The increase in progesterone concentrations that occurred when GnRH agonist was administered on Day 12 of the estrous cycle might be the result of increased blood flow to the ovary, considering size of the CL was not altered and StAR mRNA only tended to increase.

Peters et al. (1994) concluded that development and maintenance of the CL of cows is dependent on episodic release of LH between Days 2 and 12 of the estrous cycle. Daily injections of the GnRH antagonist to heifers suppressed LH pulses as well as mean plasma progesterone concentrations in the previous study. The same model was used in the present study, however progesterone concentrations did not differ from those of the control group. Treatment with SB-75 did not change progesterone concentrations and relative abundance of mRNA encoding for StAR, P450scc, and 3β-HSD. Statistical analysis could not be conducted for cell size and cell ratio for the SB-75 group because only two CL remained after tissue processing, therefore, any conclusions were based on observations of the two CL. The small to large luteal cell ratio appeared to be
altered in sections from the two CL collected from SB-75-treated heifers. It was expected that large luteal cell numbers would be less because they produce the majority of the progesterone (Niswender et al., 1985). In contrast, the number of large luteal cells per field seemed to be greater, while the number of small luteal cells appeared to be less in the CL collected from GnRH antagonist-treated heifers as compared with those from the control group. From the observations made from these two CL, we speculate that small luteal cells did not proliferate because SB-75 suppressed LH secretion, but not progesterone production. The reason luteal function was not diminished may have been due to the greater number of large luteal cells in the GnRH antagonist-treated heifers. It is unclear why the increase in the number of large luteal cells occurred with SB-75 treatment.

In summary, the effects of GnRH agonist treatment that increase plasma progesterone concentrations appear to be different depending on the stage of luteal development when the agonist was administered. Gonadotropin-releasing hormone agonist treatment results in a larger and heavier CL when the agonist is chronically administered over several days beginning on Day 3 of the estrous cycle. Enhanced luteal function in the D3 group is likely the result of greater numbers of cells and increased amount of StAR protein as indicated by the greater amount of mRNA for this protein in the present study. In contrast, size of the CL was unaffected when the agonist was administered beginning on Day 12 of the estrous cycle. Agonist treatment only tended to increase steady-state amounts of StAR mRNA in the CL in the D12 group, though this increase likely
attributed to greater progesterone concentrations. The fully functional CL does not respond to the indirect effects of a GnRH agonist in the same manner, as does the developing CL. Peters et al. (1994) previously reported that treatment with SB-75 to heifers suppressed progesterone concentrations, but in the present study progesterone concentrations were not decreased with SB-75 treatment. Although progesterone concentrations did not decrease, the ratio of small to large luteal cells numbers appeared to be altered with SB-75 treatment. To determine if the observed effect of SB-75 treatment on the ratio of small to large luteal cells is statistically different as compared with ratio of cells from CL collected from control heifers, additional animals will need to be treated, CL collected, and ratio of small to large luteal cells analyzed. In conclusion, administration of a GnRH agonist to cattle increases StAR mRNA and circulating progesterone concentrations.
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**Table 2.1** Primer sequences used for quantitative PCR.
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<th>Cell Ratio</th>
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<td>LLC (µm)</td>
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<td>17.09 ± 0.05*</td>
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<td>342.34 ± 60.67*</td>
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**Table 2.2** Size, cell number, and cell ratio of small luteal cells (SLC, < 22 µm) and large luteal cells (LLC, > 22 µm). *Number and size of cells could not be determined to be statistically different from the CONT, because data were only obtained from two CL.
Figure 2.1 Mean plasma progesterone concentrations from Day 3 to Day 16 of the estrous cycle for control heifers (CONT, n = 6), D3 GnRH agonist-treated heifers (D3, n = 6), D12 GnRH agonist-treated heifers (D12, n = 6), and GnRH antagonist-treated heifers (SB-75, n = 3). *Mean progesterone concentrations are significantly different ($P < 0.05$).
Figure 2.2 Area of the CL (cm²) from Day 2 to Day 16 of the estrous cycle for control heifers (CONT, n = 6), D3 GnRH agonist-treated heifers (D3, n = 6), D12 GnRH agonist-treated heifers (D12, n = 6), and GnRH antagonist-treated heifers (SB-75, n = 3). *CL area was significantly different (P < 0.05).
Figure 2.3 Representative luteal sections used to determine cell numbers. Small (arrow) and large luteal cell (arrowhead) numbers were determined from CL collected from CONT (A), D3 agonist (B), D12 agonist (C), or SB-75 treated heifers. Pictures were taken at 400X magnification.
Figure 2.4  Steady-state amounts of StAR, P450\textsubscript{scC}, and 3β-HSD mRNA in luteal tissue collected from control heifers (CONT, n = 6), Day 3 GnRH agonist-treated heifers (D3, n = 6), D12 GnRH agonist-treated heifers (D12, n = 6), and GnRH antagonist-treated heifers (SB-75, n = 3). *Relative abundance of mRNA was different as compared with the control \((P < 0.05)\). **Relative abundance of mRNA tended to be greater as compared to the control group \((P = 0.09)\).
CHAPTER 3

EXPRESSION OF CD83 IN BOVINE LUTEAL TISSUE BUT PRESENCE OF DENDRITIC CELLS REMAINS UNDETERMINED

INTRODUCTION

Major histocompatibility complex (MHC) class II molecules present antigenic peptides to CD4+ T cells to elicit an immune response. The class II MHC molecules have been identified in the corpus luteum (CL; Fairchild and Pate, 1989; Khoury and Marshall, 1990; Benyo et al., 1991; Kenny et al., 1991; Lawler et al., 1999; Penny et al., 1999). However, the cell type that expresses class II MHC is not known. Part of the difficulty in identifying which cell type contains MHC class II molecules is the CL is a heterogenous tissue consisting of multiple cell types. Furthermore, there are different cell types that could possibly contain class II MHC. For instance one cell type in the CL that is known to express MHC class II is the macrophage, which is an antigen-presenting cell. A certain type of endothelial cell has also been recently identified in the CL that expresses MHC class II (Lehmann et al., 2000). Interestingly, Benyo et al. (1991) identified large dense and large less dense cells that express MHC class II that were believed to be the large steroidogenic luteal cells. A population of small luteal cells also expressed MHC class II.
Dendritic cells (DC) are immune cells important in antigen-specific T cell responses as well as induction of tumor immunity. Recently DC have been identified in the uterus at the fetal-maternal interface and are believed to have an immunoregulatory role during gestation (Kammerer et al., 2000; Blois et al., 2004). Dendritic-like cells have been described in the CL of the mouse (Jasper et al., 2000), but exhaustive research has not been done to determine the identity of the cells described as dendritic-like.

Dendritic cells differentiate from lymphoid or myeloid precursor cells. It has been reported in humans that DC differentiate from the CD14+ monocytes that circulate in the blood (Olweus et al., 1997). In vitro, DC can be generated from CD14+ precursor cells if provided the proper stimulation (Romani et al., 1994; Zhou and Tedder, 1996). Dendritic cells are classified as immature cells that function to process antigens or as mature cells that present antigen to T cells (Steinman and Inaba, 1999; Banchereau et al., 2000). Again, the function of DC is determined by the environment in which the cells reside. At the mature stage, there is an increase in the cell surface MHC class II molecule as well as the co-stimulatory molecules, CD80, CD86, and CD40 (Zhou and Tedder, 1995; Zhou et al., 1996; Schuurhuis et al., 2000). These molecules provide the primary and co-stimulatory signal for T cell activation. A cell surface adhesion molecule, CD83, which has been recognized to be specific for DC, is also upregulated on mature DC (Kozlow et al., 1993; Zhou and Tedder, 1995; Twist et al., 1998).

It is possible that DC are located in the CL to elicit a T cell response during luteal regression. As stated previously, CD14+ monocytes are precursor cells for
DC. During luteolysis there is an infiltration of CD14$^+$ monocytes into luteal tissue (Bauer et al., 2001). Maturation of DC as determined by the upregulation of CD83 is induced by tumor necrosis factor $\alpha$ (TNF-$\alpha$; Morse et al., 1997; Berchtold et al., 1999), CD40 ligation (Caux et al., 1994), and prostaglandins (Steinbrink et al., 2000). Prostaglandin F$_{2\alpha}$ has long been accepted to be the luteolytic factor (McCracken et al., 1972), TNF-$\alpha$ mRNA (Petroff et al., 1999) and protein (Shaw and Britt, 1995) are present in luteal tissue, and TNF-$\alpha$ has been implicated to have a role in luteal function (for review; Pate, 1995; Davis and Rueda, 2002).

Because CD14$^+$ precursor cells infiltrate the CL when PGF$_{2\alpha}$ exerts luteolytic effects and TNF-$\alpha$ mRNA is present, both described as maturation stimuli of DC, it was speculated that DC might be present in regressing luteal tissue. The objective of the present study was to determine if DC were present within the CL as determined by CD83 gene expression or if the steroidogenic cells expressed CD83.

**MATERIALS AND METHODS**

**Reagents**

The reagents for reverse transcription (RT) PCR were purchased from the following companies; random hexamer primers were obtained from Amersham Pharmacia Biotech Inc. (Buckinghamshire, England), primers were purchased from QIAGEN Operon (Alameda, CA), and Dynamo SYBR green was purchased from MJ Research (Waltham, MA). TRIzol and Superscript II were both acquired
from Invitrogen Life Technologies (Carlsbad, CA). The T easy vector, dNTPs, and all enzymes were purchased from Promega (Madison, WI). The pBluescript SK+ vector was purchased from Stratagene (La Jolla, CA). The following reagents used for Northern blot analysis and *in situ* hybridization were all purchased from Roche Applied Science (Indianapolis, IN); CDP-StAR, anti-digoxigenin (DIG) antibody conjugated with alkaline phosphatase, DIG RNA labeling mix, blocking reagent, and nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The EZ rTth polymerase was obtained from Applied Biosystems (Foster City, CA). The following reagents and kits were purchased from Vector Laboratories, Inc. (Burlingame, CA); avidin/biotin blocking kit, DAB peroxidase substrate kit, elite mouse IgG Vectastain ABC kit, mouse IgG. The mouse anti-human CD83 antibody was purchased from Serotec Inc. (Raleigh, NC). All other reagents were purchased from either Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich (St. Louis, MO).

**Tissue Collection**

Corpora lutea were collected transvaginally from dairy cows exhibiting normal estrous cycles. Corpora lutea were removed early (Days 4 and 5), mid- (Days 10 –12) and late (Day 18, n = 4 for each stage) stages of the estrous cycle. Other cows were administered 25 mg of PGF$_{2\alpha}$ on Days 10 –12 of the estrous cycle (Lutalyse; Pharmacia & Upjohn Co., Kalamazoo, MI) to induce luteal regression. Corpora lutea were collected 0, 0.5, 1, 2, 4, and 8 h (n = 4 for all time points) after PGF$_{2\alpha}$ administration. Immediately after removal, one-third of the CL was snap frozen in liquid nitrogen, transported to the laboratory and...
stored at –80°C until RNA was isolated. Another portion of the CL was sliced into smaller pieces and embedded in OCT compound over chilled isopentane. Tissues embedded in OCT were kept at –80°C until cryostat sections were made. The remaining portion of the CL was fixed in Prefer fixative and embedded in paraffin. Spleen, lymph node, liver, kidney, and testis were collected at the local abattoir to be used as controls and were processed similar to the luteal tissue.

**Cloning of CD83**

The CD83 cDNA (232 base pairs) was generated from bovine spleen RNA by RT-PCR. Total RNA was isolated from 0.5 g of frozen tissue using TRIzol according to the manufacturer’s recommendations. Briefly, the tissue was homogenized in TRIzol using a polytron tissue homogenizer. The homogenized tissue was allowed to remain at room temperature for approximately 10 min followed by the addition of chloroform for phase separation. The RNA remaining in the aqueous phase was separated from the organic phase and precipitated with isopropyl alcohol, washed with 75% ethanol, reconstituted in sterile H₂O and stored at –80°C until analysis. Integrity of the RNA was determined by gel electrophoresis using a 1.2% agarose denaturing gel stained with ethidium bromide.

First strand cDNA was synthesized from 2 µg of total RNA using SuperScript II reverse transcriptase and random hexamer primers. Primers to amplify were designed based on the human CD83 sequence (GenBank accession no. NM004233). The original CD83 forward and reverse primers were 5’-AACACTACCAGCTGCAACTC-3’ and 5’-GGAAGATACTCTGTAGCCGT-3’,
respectively. Following reverse transcription, PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems). The reaction included 10% of the reverse transcription reaction and Taq polymerase in a final volume of 50 µl. The PCR conditions were an initial denaturation at 95°C for 1 min, 45 sec followed by 35 cycles at 95°C, 30 sec; 58°C, 30 sec; 72°C, 1 min and a final extension of 72°C, 7 min. Amplification of a single product of a 232 bp size was verified by analyzing a portion of the PCR reaction by gel electrophoresis. The purified PCR product was ligated into T-easy vector and transformed into DH5α competent cells. Insertion of the proper cDNA into the vector was determined by restriction digestion of the purified plasmid using the Not1 enzyme to cut out the insert. Colonies with the correct size insert were sequenced at the OARDC Molecular and Cellular Imaging Center to confirm CD83 cDNA with 92% and 87% homologous regions to the human (GenBank accession no. NM0042332) and mouse (GenBank accession no. NM0098561) sequences, respectively. Other clones amplified with these primers would amplify a 269 bp product that contained extra bases of bovine satellite DNA. To circumvent the problem of non-specific amplification that occurred at times with the original primers designed for CD83, nested primers were designed from the bovine CD83 sequence obtained from the clone that was the expected 232 bp. The nested CD83 forward and reverse primers were 5’-CAGAGAAACCTGACTGGCAC-3’ and 5’-CAGGACGATCTCAGCTCTGT-3’, respectively. The resulting CD83 cDNA was 91% homologous to a region of human CD83 (GenBank accession no.
The nested primers and cDNA generated from these primers were used for all experiments.

The CD40 cDNA (626 bp) was generated from bovine spleen and luteal tissue following the same methods as described above for CD83 cDNA. The CD40 primers were designed based on the bovine CD40 sequence (Genbank accession no. BTU57745) and the cDNA sequence was identified with the same accession no. The CD40 forward and reverse primers were 5'-GCCTTAGCTGCGCTTTG-3' and 5'-CGTCCACTCAGAACCACAGCCA-3', respectively. Amplification was the same for CD40, except the annealing temperature at 60°C.

**Northern Blot Analysis**

The CD83 cDNA was subcloned into the pBluescript II SK(+) vector and linearized in both directions to obtain both sense and anti-sense riboprobes. Detection of CD83 mRNA was performed using a digoxigenin-labeled RNA probe for hybridization to a Northern blot. Briefly, approximately 15 µg of total cellular RNA was electrophoretically separated on a 1.2% agarose denaturing gel. The RNA was transferred to a nylon membrane in 10X SSC buffer. Following a complete transfer, the RNA was fixed to the nylon membrane by baking at 80°C for 2 h. Blots were prehybridized for 1 h followed by an overnight hybridization at 65°C. Blots were extensively washed at 65°C in buffers containing 2.5% (w/v) and 0.5% (w/v) SDS. The blots were blocked and incubated for 30 min in blocking buffer containing anti-digoxigenin fragments conjugated with alkaline
phosphatase (1:20,000). A chemiluminescence detection method (CDP-Star) was used and blots were exposed to Kodak Biomax Light film.

**Quantitative RT-PCR**

Relative abundance of CD83 and CD40 were determined by quantitative RT-PCR using the DNA Engine Opticon 2 System (MJ Research). First-strand cDNA was synthesized from 2 µg of RNA in a final volume of 20 µl using Superscript II reverse transcriptase and random hexamer primers. A cycle test and temperature gradient test (50 – 60°C) was performed for each pair of primers. Briefly 10% of the reverse transcription reaction was used for a final 20 µl reaction volume (1X DyNamo SYBR Green Master Mix, 0.4 µM forward and reverse primers). The PCR conditions were: 95°C, 30 sec; 58.5°C (CD83)/59.3°C (CD40 and G3PDH), 30 sec; 72°C, 1 min for 25 cycles (G3PDH) or 30 cycles (CD40 and CD83). Standard curves that were generated from purified PCR product were included within every PCR reaction. The standard curve for each reaction consisted of four serial dilutions (1:10) that were adjusted such that amplification of the unknown samples would lie within the range of values that were used to develop the curve. Each sample was standardized to G3PDH and expressed as relative values. All samples were run as duplicates. Amplification of the correct product size was verified by gel electrophoresis.

**In Situ Hybridization**

*In situ* hybridization was used to detect which luteal cell type expressed CD83 using a modification of a protocol provided by Roche Applied Sciences. Frozen tissue sections (7 µm) were baked for 2 min at 50°C and subsequently air
dried for 30 min. Incubation in chloroform was included to reduce non-specific background from lipid vesicles. Following fixation in 4% paraformaldehyde, an acetylation step was included to prevent non-specific binding of the riboprobe to positively charged amino groups. The sections were washed in 2X SSC to remove the acetic anhydride. Pre-hybridization and hybridization were performed at the same temperature (42° C) in buffer containing 50% formamide, 10% dextran sulfate, 1X Denhardt’s solution, 4X SSC, and 500 µg/ml salmon sperm. The hybridization buffer included 200 ng/ml of either sense or anti-sense digoxigenin-labeled riboprobe. Following an overnight hybridization in a moisture chamber, sections were washed extensively to remove any unbound riboprobe. Sections were incubated with blocking buffer (100 mM Tris-HCl, 150 mM NaCl; saturated with blocking reagent) prior to incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:200 in blocking buffer). Detection of hybridization signal was determined by alkaline phosphatase and NBT/BCIP chromogen containing Levamisol (1 mM) to inactivate endogenous alkaline phosphatase. After optimal color development the slides were rinsed in distilled H₂O and mounted with aqueous medium.

**RT In Situ PCR**

Reverse transcription in situ PCR allows for the detection of mRNAs that are in small abundance. A direct, one step RT in situ PCR protocol was followed with modifications (Nuovo, 1996) to detect CD83 mRNA in luteal tissue. The integrity of frozen tissue sections was destroyed as a result of the high temperatures during the PCR reaction, so paraffin embedded tissues were used. Sections
were deparaffinized in a clearing agent and washed in 100% ethanol. Although the nested CD83 primers were tested and did not amplify genomic DNA, an overnight incubation with 10U RQ1 DNase at 37°C was included to ensure that adequate DNA digestion was achieved. Sections were washed in sterile H2O followed by 100% ethanol and air dried. The EZ rTth DNA polymerase was utilized for the one-step PCR reaction. The rTth enzyme functions as a reverse transcriptase as well as a DNA polymerase in the presence of manganese. Each section was overlaid with 25 µl of the amplification buffer (1.2 mM dNTPs, 0.9 µM forward and reverse primers, 12 µm digoxigenin-UTP, 2.5 mM Mn(OAc)2, 1X EZ buffer, 5 U rTth polymerase, 40 U RNasin, 400 µg/ml BSA) and covered with a coverslip sealed with nail polish to prevent evaporation. After the reverse transcription reaction at 65°C for 30 min and initial denaturation at 94°C, 35 cycles were performed at 58.5°C, 30 sec annealing and 72°C, 1 min extension followed by a final extension of 72°C, 7 min in a PTC-200 DNA Engine equipped with a twin tower slide block (MJ Research). Amplification buffer was removed from the sections with washes of 0.1X SSC and 0.2X SSC. Incorporated digoxigenin was detected as described for in situ hybridization utilizing NBT/BCIP as the chromogen.

To verify that a detected signal was amplification of CD83 cDNA and not nonspecific incorporation of digoxigenin, DNA was extracted from luteal tissue sections after amplification to confirm specificity of amplification. To obtain adequate amounts of DNA to be purified and ligated into a vector approximately 40 tissue sections from one luteal tissue sample were scraped from the slide into
a microcentrifuge tube and incubated overnight with 3 mg/ml proteinase K at 54°C. A phenol/chloroform/isoamyl alcohol (25:24:1) DNA purification protocol was used. The rTth polymerase enzyme leaves 3’ A overhangs on the amplified DNA, so the isolated DNA was ligated into T easy vector and subsequently transformed into DH5α competent cells. Following purification the circular plasmid was digested with Not I enzyme and separated on a 1.2% agarose gel to determine if the insert was the expected size.

**Immunohistochemistry**

An immunoperoxidase system was employed for immunohistochemical detection of CD83 in luteal tissue. Frozen sections (7 µm) were fixed in ice cold 95% ethanol. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol. Nonspecific binding sites were blocked with a 10% (v:v) dilution of normal goat serum in PBS. Following the blocking step, endogenous biotin was blocked prior to addition of the primary antibody. A monoclonal mouse anti-human CD83 antibody (1:2000) was added to the sections and incubated for 1 h in a moisture chamber at room temperature. A negative control was included on the same slide as the positive sections and consisted of a substitution of the primary antibody with mouse IgG (1:2000). After incubation with primary antibody, sections were treated with a biotinylated secondary antibody (1:200) followed by avidin and biotinylated horseradish peroxidase H solution. Localization of peroxidase was determined using diaminobenzidine tetrahydrochloride (DAB) as the peroxidase substrate. Sections were counterstained with hematoxylin and viewed by light microscopy.
Statistical Analysis

Differences in relative abundance of mRNA for CD83 and CD40 throughout the luteal phase and after PGF$_{2\alpha}$ administration were determined using a one-way ANOVA (SigmaStat 2.0; Jandel Corporation; San Rafael, CA). The Tukey test was used to determine differences between means after a significant F-value was determined.

RESULTS

Generation of a Partial CD83 cDNA

The nucleotide sequence encoding bovine CD83 was obtained from cDNA amplified from bovine spleen and CL. A 232 bp fragment was initially sequenced and shared a 92% and 87% region of homology to the human and mouse sequences (Figure 3.1). Because a 269 bp fragment would occasionally be amplified with this set of primers, nested primers were designed from the generated bovine sequence in the present study. A 99 bp fragment was amplified with the nested primers and contained a region of 91% homology to the human CD83 nucleotide sequence (Figure 3.1).

Quantification of CD83 and CD40 mRNA

To determine whether CD83 and CD40 gene expression vary at different stages of luteal life span and regression, Northern blot hybridization was performed using total RNA. Unfortunately, neither CD83 nor CD40 mRNA was detected by Northern analysis. A 2.4 kb transcript has been identified for human and mouse CD83 mRNA and was the predicted size for bovine CD83 mRNA.
single band was present, however, at approximately 5 kb (Figure 3.2A). The band is similar in size to the 28S ribosomal RNA and appeared in liver, testis, and CL with both sense and antisense riboprobes.

A similar pattern of expression was detected when CD40 mRNA was to be identified by Northern analysis (Figure 3.2B). A stronger signal was shown with the sense as compared with the blot hybridized with the antisense probe. Regrettably, CD83 and CD40 mRNA could not be detected with Northern analysis; only what appeared to be nonspecific binding of the probes to the 28S ribosomal RNA was visible.

Abundance of CD83 and CD40 mRNA was expected to be minimal and therefore, might explain why neither could be detected by Northern blot hybridization. Quantitative PCR, therefore, was used to determine gene expression throughout the luteal life span and regression. The greatest amount of CD83 mRNA was in Day 4 CL and this message was decreased in midcycle and Day 18 CL (Figure 3.3A; \( P < 0.05 \)). During luteal regression, CD83 gene expression was greatest at 4 and 8 h after \( \text{PGF}_2\alpha \) administration (Figure 3.3B; \( P < 0.05 \)).

In contrast, relative abundance of CD40 mRNA was greater at midcycle as compared with luteal tissue from Day 4 of the estrous cycle (Figure 3.4A; \( P < 0.05 \)). There were no differences at Day 5 and Day 18 as compared with Day 4 and midcycle CL. Following \( \text{PGF}_2\alpha \) administration, the amount of CD40 mRNA was greater at 2 h, 4 h, and 8 h as compared with 0.5 h (Figure 3.4B; \( P < 0.05 \)), but there were no differences as compared with time 0 h.
Localization of CD83 mRNA in Luteal Tissue

Similar to Northern analysis, CD83 mRNA could not be localized to a cell type by \textit{in situ} hybridization using a digoxigenin labeled riboprobe (Figure 3.5). The background that appeared in the section hybridized with anti-sense probe was similar to that with the sense probe. Amplification of CD83 cDNA in luteal tissue by reverse transcriptase PCR and direct incorporation of digoxigenin-dUTP was attempted in paraffin sections. Once more the results were inconsistent and ambiguous. A section from midcycle CL and kidney are displayed in Figure 3.6. When compared with the negative control when primers were omitted or with a negative control tissue, it appeared that CD83 mRNA was detected in steroidogenic cells, primarily the large luteal cells. This was convincing because the signal is localized to the cytoplasm as would be expected. As depicted in Figures 3.7 and 3.8, however, the results were inconsistent and it could not be concluded which cell type in the CL expresses CD83.

To confirm if CD83 cDNA was being amplified, DNA was extracted from luteal tissues that had undergone RT \textit{in situ} PCR. A low molecular weight fragment was detected, but not at the expected 99 bp size (Figure 3.9A). Digestion with Not1 revealed that the insert of extracted DNA was in fact not the appropriate size (Figure 3.9B; Lanes 7 and 8). However, digestion of plasmid containing cDNA amplified in solution with Taq polymerase (Lanes 2 – 5) or with rTth polymerase (Lane 6) did contain the correct size insert. Therefore, it was concluded that CD83 cDNA was not amplified using RT \textit{in situ} PCR.
Immunohistochemical Detection of CD83

Since no antibody to bovine CD83 was available, immunohistochemistry with anti-human CD83 antibody was attempted. The anti-human CD83 antibody did not recognize bovine CD83 in lymph or CL (Figure 3.10).

DISCUSSION

The presence of CD83, a cell surface adhesion molecule, is undisputed in the literature to be a marker for mature DC. Therefore, the presence of CD83 was used to determine if DC are present in luteal tissue throughout the luteal life span and regression. A partial bovine cDNA was generated from the known human sequence because a bovine CD83 nucleotide sequence has not been previously published. This is also the first report of CD83 gene expression in the CL. Unexpectedly, CD83 gene expression was the greatest in Day 4 CL. The upregulation of CD83 gene expression in Day 4 CL could be the result of cytokines present during ovulation. The ovary produces the inflammatory cytokines interleukin (IL)-6, IL-1, and TNF-α as well as granulocyte-macrophage colony-stimulating factor during ovulation (Brännström et al., 1994; Murdoch et al., 1997; Tamura et al., 1998). Prostaglandins are essential for ovulation and the requirement of prostaglandins for ovulation was demonstrated decades ago (Armstrong and Grinwich, 1972; Tsafiri et al., 1973). Monocytes cultured with these cytokines as well as prostaglandins are induced to differentiate and mature into DC in vitro (Morse et al., 1997; Berchtold et al., 1999; Steinbrink et al., 2000). Moreover, monocytes that are precursors of DC in vivo and in vitro
(Romani et al., 1994; Zhou and Tedder, 1996; Olweus et al., 1997) infiltrate ovarian tissue during ovulation and luteinization (Bauer et al., 2001). The proper environment is provided for maturation of DC during the luteinization process. To obtain mature DC from precursor cells cultured with cytokines in vitro requires a period of 7 days or longer. Randolph et al. (1998) proposed a model that monocytes that migrate across the endothelium and process antigens can become phenotypical and functional mature DC within 48 h. The time proposed in this model would support the increase of CD83 gene expression that could have occurred from ovulation to Day 4 when the CL was collected.

In cattle and sheep, the CL is resistant to the luteolytic effects of PGF$_{2\alpha}$ before Day 5 of the estrous cycle (Braun et al., 1988; Tsai and Wiltbank, 1998; Silva et al., 2000). Even though it was not expected that CD83 mRNA would be as abundant as it was on Day 4, Day 5 was included to determine if acquisition of luteolytic capacity might alter CD83 gene expression. Amounts of CD83 mRNA were not different on Day 5 as compared with Day 4, midcycle, or Day 18, however, there was a difference between Day 4, midcycle, and Day 18 CL. Day 5 is a pivotal stage of the estrous cycle when amounts of CD83 mRNA begin to decline. When the CL is fully functional, CD83 gene expression was the lowest. It was not expected that CD83 would be high during mid- or late stages of the luteal phase, because DC do not normally take up residence in tissues, but are migratory (Steinman and Inaba, 1999; Banchereau et al., 2000). However, DC were recently identified in the mouse kidney, but a role as resident immune cells remains to be elucidated (Kruger et al., 2004). Because DC activate T cells,
resident DC could be disastrous by inducing a T cell response and causing premature regression of the CL. Expression of the CD83 gene did increase during luteal regression. Similar to ovulation, the factors that are known to induce DC maturation are present during luteal regression including PGF$_{2\alpha}$ (McCracken et al., 1972) and TNF-α (Shaw and Britt, 1995; Petroff et al., 1999) as well as infiltration of monocytes (Bauer et al., 2001).

Dendritic cell maturation and activation requires CD40 ligation. The interaction between the CD4$^+$ T cells that contain the CD40 ligand and the DC that have the CD40 receptor provide a terminal differentiation signal (Schuurhuis et al., 2000). This is the first report of CD40 mRNA being present in luteal tissue. The amount of CD40 mRNA increased from early to midcycle and remained elevated throughout the luteal phase. The temporal expression of CD40 mRNA was similar to CD83 mRNA during luteal regression. The expression of CD40 mRNA throughout the luteal phase and during luteal regression is similar to the temporal expression of mRNAs encoding for CD80 and CD86, co-stimulatory molecules that have also been reported to be present in bovine luteal tissue (Cannon and Pate, 2001).

Although CD83 was expressed at all stages in luteal tissue, the cellular source could not be identified by any of the methods employed in the present study. It is speculated that there was not a great enough abundance of the mRNA to detect by in situ hybridization. Many problems were encountered using the RT in situ PCR procedure. This procedure is known to be inconsistent and difficult (Muro-Cacho, 1997). Possible reasons as to why CD83 mRNA was not
detected with this method could include degradation of mRNA, incompatible fixation methods for this technique, or inability of the designed primers to amplify cDNA within a tissue. The technique could on occasion be used to detect the steroidogenic acute regulatory protein, a known gene expressed in relative greater abundance in luteal tissue. Again, this supports how inconsistent this technique was in the hands of this researcher. Finally, the CD83 protein could not be detected in luteal tissue. Most likely the CD83 monoclonal anti-human antibody did not cross-react with bovine CD83. However, human tissue was unavailable to test the antibody.

The primary question that remains is what cell type within the CL is expressing the mature DC cell adhesion molecule, CD83, in luteal tissue. Possible sources of DC would be non-proliferating precursor monocytes or immature DC. During the early stage of the luteal phase and luteal regression when CD83 gene expression was the greatest the environment would have been favorable for maturation of DC. Furthermore, cell surface molecules known to be upregulated on DC have been reported in the bovine CL including MHC class II molecules (Benyo et al., 1991) and CD80 and CD86 co-stimulatory molecules (Cannon and Pate, 2001) as well as CD40 reported in the present study. However, macrophages also express these cell surface molecules. Macrophages and DC have similar functions albeit DC are greater stimulators of T cell responses.

In conclusion, CD83 mRNA is present in luteal tissue and the greatest expression is coincident with the presence of inflammatory cytokines.
Furthermore, CD83 is upregulated at the same time as CD40 during luteal regression. It remains to be determined if CD83 expression is indicative of the presence of DC within the CL, or if this gene is expressed in cells other than DC.
Figure 3.1 Nucleotide sequence of the bovine CD83 cDNA. The 232 bp CD83 cDNA that was originally amplified was denoted as 'Bovine'. The 99 bp CD83 cDNA generated using the nested primers designed from the bovine sequence was referred to as 'Bovine Short'. Differences in nucleotides between the human and bovine sequences are outlined with yellow.
**Figure 3.2** Representative Northern blots for CD83 mRNA hybridized with antisense (A) and sense probe (B) and CD40 mRNA hybridized with antisense (C) and sense probe (D).
Figure 3.3 Relative abundance of CD83 mRNA throughout the luteal phase (A) and during luteal regression (B).
Figure 3.4 Relative abundance of CD40 mRNA during the estrous cycle (A) and luteal regression (B).
Figure 3.5 *In situ* hybridization. Frozen luteal sections were hybridized with antisense (A) or sense (B) probe to detect CD83 (200x).
Figure 3.6 Representative sections from RT \textit{in situ} PCR to detect CD83 in luteal tissue (A, B) and kidney (C, D). Primers were omitted from RT-PCR as a negative control (B, D). Pictures were taken at 400X magnification.
Figure 3.7 Inconsistent results using the RT *in situ* PCR method to detect CD83. Negative controls included omission of primers (B, D, F). Amplification of StAR was used as a control because the cell type expressing the gene is known (A, B; 100X). Amplification of CD83 was attempted in midcycle (C, D; 200X) and 8 h post-PGF$_{2\alpha}$ (E, F; 200X) luteal tissue.
Figure 3.8 Additional samples of RT in situ PCR. Amplification of CD83 was attempted with Day 4 (A, B; 100X), midcycle (C, D; 100X), and 4 h post-PGF$_{2\alpha}$ (E, F; 100X) luteal sections. Primers were omitted as a negative control (B, D, F).
Figure 3.9 Representative gel showing cDNA extracted from luteal tissue after RT in situ PCR (top gel). The proper size product (99 bp) was not detected when the extracted cDNA was inserted into a plasmid (Lanes 7 and 8; bottom gel). The proper insert was detected when cDNA was amplified in solution with Taq polymerase (Lanes 2 – 5) or rTth polymerase (Lane 6).
Figure 3.10 Representative immunohistochemical staining for CD83. CD83 could not be detected in lymph (A; 200X) or luteal frozen tissue sections (C, E; 200X). The CD83 antibody was substituted by nonspecific mouse IgG for the negative control (B, D, F; 200X).
CHAPTER 4

BOVINE LUTEAL CELLS STIMULATE $\gamma^\delta$ T CELL PROLIFERATION AND INDUCE IFN-$\gamma$ AND IL-10 PRODUCTION

INTRODUCTION

The events that surround the demise of the CL may involve a cell-mediated immune response. Luteal cells are capable of stimulating T cell proliferation in vitro, with cells obtained from regressed CL eliciting a greater T cell response as compared with cells obtained from CL collected during the midportion of the estrous cycle (Petroff et al., 1997). The first signal for T cell proliferation is provided by the interaction of the major histocompatibility complex molecules (MHC) on the antigen-presenting cell with the T cell receptor (Mueller et al., 1989). Bovine luteal cells express class I and II molecules (Fairchild and Pate, 1989) and the percentage of luteal cells that express MHC class II molecules increases at the late stage of the luteal phase and after administration of a luteolytic dose of PGF$_2\alpha$ (Benyo et al., 1991). The proinflammatory cytokine, interferon-gamma (IFN-$\gamma$), stimulates an increase of class I and II MHC molecules on cultured bovine luteal cells (Fairchild and Pate, 1989).

Treatment of luteal cells with interferon-$\gamma$ inhibits LH-stimulated progesterone production (Fairchild and Pate, 1991) and decreases luteal cell viability (Benyo
and Pate, 1992; Jo et al., 1995; Petroff et al., 2001). The γδ T cells (Christmas and Meager, 1990; Taguchi et al., 1991), CD4⁺ T helper 1 (TH1) (Mosmann et al., 1986), and CD8⁺ cytolytic T cells (Boehm et al., 1997) produce IFN-γ. Less has been reported about the effects of the CD4⁺ TH2 cytokines in luteal function. Hashii et al. (1998) reported production of IL-10 and IL-4 by peripheral blood mononuclear cells (PBMCs) when cultured with human luteal cells from early pregnancy. The overall objective of the present study was to determine which T cells are stimulated by bovine luteal cells in vitro, if the response is MHC class I or class II restricted, and which cytokines are predominantly present during this response.

**MATERIALS AND METHODS**

**Reagents**

Culture medium RPMI 1640, Ham’s F-12, L-glutamine, gentamicin, streptomycin, penicillin, and heat-inactivated fetal calf serum were purchased from Invitrogen Life Technologies (Carlsbad, CA). Collagenase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). Ficoll-Hypaque Plus was purchased from Amersham Biosciences (Uppsala, Sweden). Antibodies specific for bovine cell surface molecules were purchased from VMRD Inc. (Pullman, WA) or from the Monoclonal Antibody Center at Washington State University (Pullman, WA). Bovine IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Secondary antibodies conjugated with fluorescein (FITC) or Cy5 were purchased from Caltag Laboratories (Burlingame,
CA) and the phycoerythrin (PE) conjugated antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). The antibodies used for ELISA were obtained from Serotec (Oxford, UK). The IL-4 ELISA kit and recombinant human IL-12 were acquired from eBioscience (San Diego, CA). The ELISA specific for bovine IFN-γ was purchased from Biosource International (Camarillo, CA). The ABC kit, avidin-biotin blocking kit, and DAB kit as well as mouse IgG used for immunohistochemistry were purchased from Vector Laboratories, Inc. (Burlingame, CA). The 96 well culture plates were obtained from Corning (Corning, NJ). The [³H]thymidine was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Lutalyse was purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Collection and Dissociation of Luteal Tissue

The procedure to collect CL was approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Cows exhibiting normal estrous cycles were used in the present study. Corpora lutea (n = 5) were collected during the midluteal phase (Days 10 – 12) of the estrous cycle (Day 0 = estrus) 8 h after a 25 mg i.m. injection of PGF₂α. The time after PGF₂α administration was chosen because it has previously been determined in this laboratory to be effective in stimulating T cell proliferation (Petroff et al., 1997). A blood sample was taken prior to and 8 h after PGF₂α administration to determine if plasma progesterone concentrations declined in response to the luteolytic dose.
of PGF$_{2\alpha}$. Progesterone concentrations were measured by the ELISA procedure as previously described (Petroff et al., 1997).

Luteal tissue was dissociated as previously described (Pate and Condon, 1982). Briefly, luteal tissue was finely minced and placed in Ham’s F-12 medium containing 20 ng/ml gentamicin, 0.5% BSA, and collagenase (2000 U/g tissue). After 1 h incubation at 37°C, medium containing the dispersed luteal cells was decanted and placed on ice. The remaining tissue was dissociated for an additional hour with new medium containing collagenase. After the second dissociation, luteal cells were washed 3 times in BSA-free medium and resuspended in RPMI 1640 medium containing 10% fetal calf serum. To prevent proliferation of luteal cells in culture, luteal cells (7 X 10$^6$) were pretreated with mitomycin C (50 µg/ml) for 20 min at 37°C and washed 3 times with RPMI 1640. Luteal cell number was determined by trypan blue dye exclusion. For all experiments, cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU penicillin, and 100 µg/ml streptomycin.

**Isolation of T Cells**

After CL removal, blood was collected from the jugular vein into a sterile bottle containing acid citrate dextrose (ACD)-A and placed on ice to prevent coagulation of the blood. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient centrifugation method as recommended by the manufacturer. Blood was aliquoted into sterile glass tubes and centrifuged at 1000 x g for 10 min at 4°C. Buffy coat layers were aspirated and added to room
temperature Mg\(^{2+}\)/Ca\(^{2+}\) free phosphate-buffered saline (PBS) containing 2 mM ethylenediamine-tetracetic acid (EDTA) to dilute the buffy coats (1:2). The diluted buffy coats (4 ml) were layered onto Ficoll-Hypaque Plus (3 ml), without disturbing the interface. The tubes were centrifuged at 400 x g for 30 min at room temperature. Following centrifugation, the lymphocyte-rich layer was transferred to a new tube and washed 3 times with 4\(^\circ\)C PBS + 2 mM EDTA. Following the final wash, cells were resuspended in PBS + 2 mM EDTA and yield and viability was determined using trypan blue dye exclusion.

T cells were purified from PBMCs using the MACS Cell Separation System. Briefly, 1.5 x 10\(^8\) PBMCs were incubated with 10 \(\mu\)g/ml each of the mouse anti-bovine MHC class II monoclonal antibodies TH14B, TH81A, and H42A (IgG; Table 2.1) for 20 min at 4\(^\circ\)C. Following the incubation the cells were washed and resuspended in 800 \(\mu\)l PBS containing 2 mM EDTA and 0.5% BSA. The anti-MHC class II labeled cells were incubated with paramagnetic MACS rat anti-mouse IgG\(_{2a+b}\) microbeads (200 \(\mu\)l) for 20 min at 4\(^\circ\)C. After the incubation the labeled cells were washed and resuspended in the PBS buffer containing 0.5% BSA and passed through a MACS MS column that was placed in front of a magnetic stand. The anti-MHC class II labeled cells were retained within the column and the CD3\(^+\) lymphocytes were depleted and captured in the eluate. Viability was evaluated by trypan blue dye exclusion and the number of cells collected was determined using a hemacytometer. The purity of the cell population obtained by cell separation was \(\geq 96\%\) for CD3\(^+\) cells as determined by flow cytometry.
Luteal Cell and T cell Co-cultures

**Experiment 1: Effect of blocking MHC class I and II on T cell proliferation.**

To determine if T cell proliferation was MHC class I or class II mediated, T cells (1 x 10^5) and mitomycin C-treated luteal cells (3.2 x 10^4) were cocultured in duplicate in 96 well culture plates in the presence of mouse anti-bovine MHC class I or mouse anti-bovine MHC class II antibodies. The antibodies were dialyzed against 2 changes of sterile PBS for 24 h to remove sodium azide. The treatments included a cocktail of anti-MHC class II antibodies (TH14B, TH81A, and H42A), anti-MHC class I (H58A), and a combination of these. Antibody concentrations of 1, 5, 10, and 20 µg/ml were used. To stabilize the interaction of the MHC molecules with the T cell receptor complex, staphylococcus enterotoxin B (SEB; 1 µg/ml) was included in all cultures with the exception of the control luteal cell-T cell cultures. Other controls for this experiment included proliferation of luteal cells and T cells in separate cultures, both in the presence of SEB, and a non-specific rabbit anti-bovine IgG antibody to ensure specificity of any effect of anti-MHC class I or class II antibodies. Cultures were carried out for 72 h. At 66 h of culture, 0.5 µCi [³H]thymidine was added to the culture wells. At the termination of the culture, the plate was placed at -80°C until the cells were harvested using a cell harvester (Skatron Instruments, Sterling, VA). Incorporation of [³H]thymidine, an indicator of cell proliferation, was measured by liquid scintillation counting.
Experiment 2: Effect of anti-CD4, anti-CD8, and anti-γδ on T cell proliferation.

Luteal cells were treated with mitomycin C (50 mg/ml) prior to culture to prevent proliferation of these cells in culture. Luteal cells (3.2 x 10⁴) and T cells (1.0 x 10⁵) were cultured in the presence of SEB (1 µg/ml) for 72 h in 96 well culture plates. The cells were treated with antibodies developed in the mouse specific for the bovine CD4 co-receptor, the bovine CD8 α-chain, and the bovine γδ T cell receptor δ-chain at 1, 5, 10, and 20 µg/ml final concentrations (Table 4.1). The cells were pulsed with 0.5 μCi [³H]thymidine during the last 6 h of culture. Cells were placed in the -80°C freezer and analyzed for cell proliferation as stated for experiment 1. Controls for this experiment included luteal cell-T cell cultures in the absence of SEB, luteal cells and T cells cultured separately, both in the presence of SEB, and the addition of anti-bovine IgG.

Experiment 3: Determination of T cell types and cytokine production.

Luteal cells (3.2 x 10⁴) and T cells (1.0 x 10⁵) were cultured for 72 h in 96 well plates. As previously described, luteal cells were treated with mitomycin C prior to culture. Cultures containing T cells or T cells with SEB (1 µg/ml) were used as controls. Following the culture the T cells were separated from the luteal cells and analyzed by flow cytometry. The medium containing the non-adherent T cells was collected and the luteal cells remained adhered to the plate. The medium containing the T cells was centrifuged for 10 min at 400 x g at 4°C. The medium was decanted carefully, so not to disturb the T cell pellet, and stored at -
80°C until analyzed for cytokine detection as described later. T cells were resuspended in PBS (4°C) and prepared for flow cytometry.

**Flow Cytometry**

After stimulation, T cells were prepared for 3 color flow cytometric analysis. The T cells were labeled by indirect immunofluorescence. All incubations with the antibodies and washes were performed at 4°C. Cells were incubated with mouse anti-bovine CD3, mouse anti-bovine CD4, and mouse anti-bovine δ or mouse anti-bovine CD3, mouse anti-bovine CD8, and mouse anti-bovine δ (2.5 µg/ml each) for 30 min, the cells were washed with PBS (4°C). To determine the purity of the cell population analyzed by flow cytometry, the cells were labeled with the anti-bovine CD3 antibody that is present on CD4⁺, CD8⁺, and γδ T cells. T cells were incubated for 30 min with the appropriate secondary antibodies. Goat anti-mouse IgG₁ conjugated to phycoerythrin (PE; 0.0625 µg/ml) was used to detect CD3⁺ T cells, goat anti-mouse IgG₂a conjugated to fluorescein isothiocyanate (FITC; 1 µg/ml) to detect CD4⁺ or CD8⁺ cells, or goat anti-mouse IgG₂b conjugated to Cy5 (1 µg/ml) was used to detect γδ T cells. The concentration for each secondary antibody that gave the least background was determined prior to analysis. Following incubation with the secondary antibody, cells were washed twice, fixed in 0.5% paraformaldehyde and analyzed within 3 days. Flow cytometric data were collected using a FACSCalibur and analyzed using the CellQuest program. Prior to each analysis, unstained T cells were used to determine autofluorescence and each secondary antibody was analyzed separately to compensate for spectral overlap. Two separate cell populations
were detected by forward and side scatter properties that were considered to be smaller resting T cells or larger activated T cells. A total of 10,000 cells was analyzed for the region of activated T cells determined by forward and side scatter properties.

Detection of Cytokines

IFN-γ ELISA

Medium was collected from cultures containing T cells plus luteal cells with SEB at 48 h of culture to assess if cytokine production was different from 48 to 72 h. Interferon-γ was analyzed in culture supernatants using a commercially available ELISA specific for bovine IFN-γ according to the procedure supplied by the manufacturer (Biosource Int., Camarillo, CA). Briefly, 100 µl of samples and controls were added to the anti-bovine IFN-γ coated 96 well plate and incubated for 1 h at room temperature. The wells were washed 3 times and 100 µl of anti-bovine IFN-γ horseradish peroxidase conjugate solution was added. After the incubation with conjugate, the wells were washed as before and IFN-γ was detected using 3,3',5,5'-tetramethylbenzidine (TMB) chromagen solution. Values of duplicate samples are given as mean O.D. value (450 nm).

IL-4 ELISA

A recombinant human IL-4 ELISA assay (eBioscience, San Diego, CA) was used because neither an ELISA assay kit nor antibodies were available specific for bovine IL-4. Plates were coated with anti-human IL-4 antibody overnight at 4°C. Standards and culture supernatants (100 µl) were added to the appropriate
wells and incubated for 1 h at room temperature. The standard curve was generated from serial dilutions of rhuIL-4. After washing, 100 µl of biotin-conjugated anti-human IL-4 was added to the wells as the detection antibody. Detection of IL-4 was determined using avidin-horseradish peroxidase. Concentrations of IL-4 are reported as pg/ml. Because it had not been reported whether the rhuIL-4 kit used in the present study would cross-react with bovine IL-4, medium collected from Concanavalin A (ConA) stimulated bovine PBMCs were used as a control. Peripheral blood mononuclear cells were isolated as previously described. The PBMCs (1.0 x 10⁶) were treated with ConA (1 mg/ml) for 48 h when the medium was collected and stored until needed. Serial dilutions of medium collected from ConA stimulated PBMCs and unstimulated PBMCs were included as duplicates to assess for cross-reactivity.

IL-10 ELISA

Interleukin-10 in culture supernatants was analyzed using a pair of monoclonal antibodies that react with recombinant bovine IL-10 in an ELISA (Kwong et al., 2002). Plates were coated with 6 µg/ml of mouse anti-bovine IL-10 capture antibody (CC318; Table 4.1) in coating buffer overnight at room temperature. Plates were washed 4 times with PBS containing 0.01% Tween 20 (v:v) after each incubation and all samples and buffers were added in 100 µl aliquots. Non-specific binding sites were saturated with PBS containing sodium casein (1 mg/ml) for 1 h. Culture supernatants were added to the appropriate wells in duplicate. Because rbOIL-10 is commercially unavailable and rhuIL-10 did not cross-react with the antibodies specific for bovine IL-10, serial dilutions of
medium from ConA stimulated PBMCs were used to generate a standard curve. Biotin-conjugated mouse anti-bovine IL-10 detection antibody (CC320; 2.0 \( \mu \)g/ml) was added and incubated at room temperature for 1 h. The streptavidin-horseradish peroxidase enzyme (1:500) was used for detection of IL-10. Values are reported as mean O.D. (450 nm).

**IL-12 ELISA**

Interleukin-12 in culture supernatants was analyzed using a procedure similar to the IL-10 ELISA described previously. Only single samples were analyzed to detect IL-12, because there was not enough medium remaining to analyze duplicate samples. The standard curve (ng/ml) was generated from serial dilutions using rhuIL-12. The mouse anti-bovine IL-12 capture antibody (CC301; 8.0 \( \mu \)g/ml) and mouse anti-bovine IL-12 biotin-conjugated detection antibody (CC326; 8.0 \( \mu \)g/ml) have been reported to react with bovine IL-12 in an ELISA (Hope et al., 2002). The streptavidin-horseradish peroxidase enzyme (1:500) was used for detection of IL-12.

**Immunohistochemical Localization of \( \gamma\delta \) T Cells in CL**

An immunoperoxidase system was used to detect \( \gamma\delta \) T cells in luteal tissue during the midluteal phase (day 10) and 8 h after PGF\(_{2\alpha}\) administration. Immediately after removal, luteal tissue was sliced into small blocks and frozen over chilled isopentane in OCT compound. Frozen sections (7 \( \mu \)m) were fixed in ice cold 95% ethanol. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol for 10 min on ice. All remaining incubations were performed at room temperature. Nonspecific binding sites were saturated with a
10% dilution of normal goat serum in PBS for 1 h. Endogenous biotin was saturated with avidin solution for 15 minutes followed by incubation with the biotin solution for 15 min, prior to addition of the primary antibody. A monoclonal antibody specific for the δ chain of the T cell receptor (GB21A; 1:500) was used to examine the presence of γδ T cells in luteal tissue. This antibody is specific for two populations of bovine γδ T cells having the phenotype WC1+CD2−CD4−CD8− and WC1−CD2+CD8+/- (Davis et al., 1996; MacHugh et al., 1997). Substitution of the primary antibody with mouse IgG (1:500) was used as a negative control and was included on the same slide as the section stained for γδ T cells. After incubation with the primary antibody (1 h), sections were treated with biotinylated horse anti-mouse IgG (1:200) for 30 min followed by a 30 min incubation with avidin and biotinylated horseradish peroxidase H solution. Localization of peroxidase was determined using diaminobenizidine tetrahydrochloride (DAB) as the peroxidase substrate. Sections were counterstained with hematoxylin and viewed by light microscopy.

**Statistical Analysis**

Concentrations of mean plasma progesterone before and after PGF$_{2\alpha}$ administration were compared using a paired T-test. Cell proliferation and flow cytometry data were analyzed by two-way ANOVA followed by the Tukey test. Cell proliferation data were normalized using the square root transformation function and were presented as the square root of dpm counts ± SEM. Mean O.D. values for IFN-γ and IL-10 were analyzed using a two-way ANOVA with all pairwise comparisons being determined by the Tukey test. Mean O.D. values for
IFN-γ were presented as log₁₀. All statistical analyses were performed using SigmaStat software (Jandel Corporation; San Rafael, CA).

RESULTS

Plasma progesterone concentrations decreased after a luteolytic dose of PGF₂α for 4 of the 5 cows (data not shown). A blood sample from one cow was destroyed prior to analysis. Regressing luteal cells were used because T cell proliferation in coculture is greater following PGF₂α as compared with prior to PGF₂α treatment (Petroff et al., 1997). The results are shown in Figure 4.1. Minimal proliferation occurred when T cells or luteal cells were cultured independently of each other. The lack of T cell proliferation when T cells were cultured with SEB indicated that the removal of antigen-presenting cells was effective using the separation method used in the present study. T cell proliferation in the presence of SEB required that the T cells were cocultured with luteal cells (P < 0.05). Differences in T cell proliferation could be observed by microscopy while cells were still in culture (Figure 4.2). To determine if T cell proliferation was induced via MHC class I or class II, anti-MHC class I and anti-MHC class II antibodies were added to the cocultures in various concentrations. Proliferation of T cells was not attenuated with any concentration of anti-MHC class I antibody (Figure 4.3), anti-MHC class II (Figure 4.4), or a combination of both (Figure 4.5). However, when cells were cultured with anti-MHC class I at 20 µg/ml either alone or in combination with anti-MHC class II, proliferation was not different as compared with T cells treated with SEB.
To determine which T cell population was stimulated to proliferate when cultured with luteal cells, antibodies were used to compete with the CD4 and CD8 T cell co-receptor molecules which interact with the MHC class II and class I molecules, respectively. Anti-CD4 did not inhibit T cell proliferation even at the greatest concentration (Figure 4.6). The proliferation of T cells was suppressed with the greatest concentration of anti-CD8 antibody (P < 0.05; Figure 4.7, 4.8).

The presence of γδ T cells in CL was examined by immunohistochemistry (Figure 4.9). The γδ T cells were identified in bovine CL collected on day 10 of the estrous cycle and 8 h post-PGF$_{2\alpha}$. In the T cell proliferation assay, the anti-δ T cell receptor antibody did not inhibit proliferation of T cells stimulated by luteal cells (Figure 4.10).

The proliferative response of T cells after 72 h of culture was assessed by flow cytometry. Representative scatter diagrams for one CL are shown in Figure 4.11. The number of CD4$^+$ T cells per 10,000 total cells was less in cultures that contained T cells plus luteal cells with SEB as compared with cultures that contained T cells alone (P < 0.05; Figure 4.12). The number of CD4$^+$ cells in luteal cell-T cell cultures in the absence of SEB was not different as compared with cocultures that contained SEB. In contrast, the number of γδ T cells was greater when T cells were cultured with luteal cells as compared with T cells only (P < 0.05; Figure 4.13). The number of CD8$^+$ T cells did not differ among treatments (Figure 4.14). Indirect immunofluorescence imaging was used to visualize CD4$^+$, CD8$^+$, and γδ T cells (Figure 4.15). No co-labeling was detected with antibodies for CD4 and γδ. A few cells were positive for CD8 and γδ as
determined by flow cytometry, but no co-labeling was observed by indirect immunofluorescence imaging. A few cells were larger in size, indicating these cells had been stimulated as compared with the smaller resting T cells.

Cytokine production by activated T cells can be used to determine the nature of the T cell response, either helper or cytolytic. Luteal cells stimulated T cell production of the proinflammatory cytokine, IFN-\(\gamma\) (\(P < 0.05\); Figure 4.16). Culture medium collected at 48 h from separate culture wells with T cells plus luteal cells with SEB was analyzed for cytokines. There was no increase in IFN-\(\gamma\) detected in the medium from 48 h to 72 h of culture (2.386 ± 0.158 vs 2.646 ± 0.158 O.D. value). Concentrations of IL-12 in culture medium for all treatments were at or below detection of the ELISA (2.0 ng/ml; Figure 4.17). Medium collected from ConA-stimulated PBMCs contained concentrations of IL-12 (Figure 4.18) and IL-4 (Figure 4.19) that were detected by the ELISA used in the present study. The TH2 cytokine, IL-4, was not detected in the culture supernatants (2.0 pg/ml sensitivity, data not shown). Interleukin-10, an anti-inflammatory and immunosuppressive cytokine was detected in culture medium. T cells stimulated by luteal cells in the presence of SEB produced the greatest amount of IL-10 (\(P < 0.05\); Figure 4.20) and there was no difference in the amount of IL-10 between 48 h and 72 h of culture in medium collected from T cells cultured with luteal cells in the presence of SEB (0.30 ± 0.04 vs 0.356 ± 0.04 O.D. value). Serial dilutions of medium collected from ConA-stimulated PBMCs were included as a positive control for IL-10 (Figure 4.21).
DISCUSSION

In the present study, luteal cells stimulated T cell proliferation. The findings in the present study support previous reports from our laboratory that luteal cells can function as antigen-presenting cells to induce a MHC-dependent T cell response (Petroff et al., 1997; Cannon et al., 2003). Superantigens, including SEB, are produced by *staphylococcus aureus* and characterized by their ability to stabilize the interaction of MHC class II and the V\(_\beta\) segment of the \(\alpha\beta\) T cell receptor (for review; Li et al., 1999). Superantigens bypass antigen specificity by binding outside the MHC peptide groove and stimulating massive numbers of T cells (Marrack and Kappler, 1990).

To determine if T cell proliferation induced by luteal cells was MHC class I or class II mediated, antibodies specific for these molecules were added to the cultures to compete with the interaction between luteal cells and T cells. Unexpectedly, the addition of anti-MHC class II antibodies did not inhibit T cell proliferation in the present study. These results differ from those of a previous study in which addition of anti-MHC class II antibodies to cultures attenuated luteal cell-induced T cell proliferation (Petroff et al., 1997). Petroff et al. (1997) reported that addition of anti-MHC class II antibodies attenuated T cell proliferation when luteal cells were obtained from regressing CL but not from midcycle. The ability of luteal cells to stimulate T cell proliferation is enhanced, possibly as a result of changes to luteal cells in response to a decline in progesterone as a result of PGF\(_{2\alpha}\) (Cannon et al., 2003). The luteal cells used in culture for the present study might not have been altered in such a manner that
MHC class II mediated T cell proliferation could be suppressed. The discrepancy between the two studies is unclear and warrants further investigation. Proliferation of T cells was not attenuated by the addition of anti-MHC class I antibody, although the extent of proliferation was not different from T cells with SEB. Therefore, some inhibition of T cell proliferation did occur, although not great enough to significantly suppress T cell activation. Based on the results of the present study, T cell proliferation stimulated by luteal cells was not MHC class I or class II dependent.

Antigenic peptides presented via class II MHC molecules interact with the T cell receptor present on CD4+ T cells (Doyle and Strominger, 1987). Interruption of the CD4 co-receptor with the addition of anti-CD4 antibody to the cultures did not suppress T cell proliferation. Oddly, activated CD4+ T cells were less abundant when cultured with luteal cells as compared with the number of these cells when T cells were cultured alone. Although, the number of these cells was altered when cultured with luteal cells, proliferation was not inhibited with the addition of either an anti-MHC class II or an anti-CD4 antibody, which indicates another factor other than the interaction of luteal cells with T cells might alter the response of this T cell population.

The CD8+ T cells are activated by antigenic peptides presented via class I MHC (Swain, 1981; Meuer et al., 1982). Although more research has focused on the effect of SEB to selectively induce the interaction of MHC class II positive antigen-presenting cells and CD4+ T cells, the interaction of CD8+ T cells with MHC class I positive cells is stabilized by SEB (Kawabe and Ochi, 1990; Fuller
and Braciale, 1998). Suppression of T cell proliferation was attenuated when cells were treated with the anti-CD8 antibody. Luteal cell-induced T cell proliferation did not alter the number of activated CD8+ T cells. The possibility exists that CD8+ T cells have a role in luteolysis because the number of these cells increase during the late stage of the luteal phase (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002).

The γδ T cells are a unique population of T cells that do not require antigen processing or presentation via MHC molecules to be activated. Cows have proportionately more γδ T cells than do humans or mice (Hein and Mackay, 1991; Kronenberg, 1994). It has been proposed that these cells recognize stress-induced self-antigens defined as MHC class I-related molecules, MICA/MICB (Groh et al., 1998; 1999; Wu et al., 2002). These molecules have been identified in intestinal epithelium (Groh et al., 1998) and many carcinomas including those found in the lung, breast, ovary, and kidney (Groh et al., 1999). This is the first report that γδ T cells are localized within the CL of the cow. T cell proliferation was not inhibited with the addition of the antibody specific for the δ chain of the γδ T cell receptor. It has previously been reported that the anti-δ antibody used in the present study can inhibit T cell proliferation (Sathiyaseelan et al., 2002). A possible explanation for the difference in the ability of the antibody to inhibit T cell proliferation between the two studies is that in the previous study Fab1 fragments that contain only the antigen binding sites of the antibody were used, whereas in the present study the whole antibody was used. Furthermore, a greater concentration was used in the previous study as compared with the
concentrations used in the present study. Because little is known how this population of T cells is activated, the difference between using the whole antibody versus just the antigen-binding portion might affect the ability of the antibody to compete with the luteal cells to inhibit T cell proliferation.

The number of γδ T cells was greater when T cells were cultured with luteal cells as compared with T cells alone. It is speculated that the γδ T cell-induced proliferation was not MHC-restricted. As stated previously γδ T cells recognize stress-induced self-antigens (Groh et al., 1998; 1999; Wu et al., 2002). Further research will need to be conducted to determine if luteal cells express these self-antigens that would be recognized by the γδ T cells to activate this population of T cells.

As stated previously, a factor other than luteal cell-T cell interaction might suppress the activation of CD4+ T cells observed in the present study. Because the portion of the γδ T cells increased as the portion of CD4+ T cells in the T cell population decreased, the presence or abundance of γδ cells might have a role in CD4+ T cell responses. Chiodini and Davis (1992) have reported CD4+ T cell non-responsiveness mediated by the presence of γδ T cells. In the presence of bacterial antigens, γδ cells suppress CD4+ T cells (Ferens et al., 1998; Rhodes et al., 2001), possibly by selective anergy (Kawabe and Ochi, 1990). The γδ T cells present in the bovine CL, might have a role in the type of immune response that occurs during luteolysis.
To assist in identification of T cells that were activated, cytokine production was characterized in the cocultures. Interleukin-12 is produced by macrophages and dendritic cells (for review; Watford et al., 2003). Interleukin-12 has been shown to stimulate IFN-γ production by macrophages and T cells (Munder et al., 1998; Fukao et al., 2000). Interleukin-12 could not be detected in the culture medium from T cells stimulated with luteal cells or SEB. The lack of detection of IL-12, provided further evidence that the cultures did not contain contaminating antigen-presenting cells that produce IL-12. As anticipated, T cells activated in culture did not secrete IL-4. The IL-4 cytokine produced by the CD4⁺ T⁺₂ cells stimulates B cells (Mosmann et al., 1986). Although B cells have been identified in the CL, Penny et al. (1999) reported that B cells are an insignificant population of immune cells in the bovine CL.

Luteal cell-induced T cell proliferation was correlated with production of both IFN-γ and IL-10. These cytokines have opposing functions, IFN-γ is a pro-inflammatory cytokine whereas IL-10 is anti-inflammatory and immunosuppressive. Activated CD4⁺ T⁺₁ cells (Mosmann et al., 1986), CD8⁺ T cells (Boehm et al., 1997), and γδ T cells (Christmas and Meager, 1990; Taguchi et al., 1991) produce IFN-γ. It could not be concluded from the present study which T cell population was stimulated to produce IFN-γ, but it was speculated based on the data obtained from flow cytometry that the CD8⁺ T cells and γδ T cells were major contributors of IFN-γ production. Fikri et al. (2001) reported that activated γδ T cells express IFN-γ but not IL-10. The production of IL-10 was
probably increased to suppress the proinflammatory effects of IFN-\(\gamma\) and was likely produced by the CD4\(^+\) T helper cells. The majority of CD4\(^+\) T cells only produce either IFN-\(\gamma\) or IL-10 (Cardell et al., 1993; Assenmacher et al., 1994), but cells can co-express these cytokines (Assenmacher et al., 1994). Further studies will need to be designed to determine which T cells were stimulated to produce cytokines.

In conclusion, luteal cell-induced T cell activation was primarily through a non-MHC restricted response. The \(\gamma\delta\) T cells that recognize antigens not required to be presented by MHC molecules, showed the greatest stimulation by luteal cells, whereas the CD4\(^+\) T cells seemed to be nonresponsive to luteal cell stimulation. This was the first report of \(\gamma\delta\) cells within the CL as well as these cells being stimulated by bovine luteal cells. Interestingly, IFN-\(\gamma\) and IL-10 were produced in the cultures. The detection of IFN-\(\gamma\) was significant, because this cytokine has been described to have a potential role in luteolysis. It was speculated that IL-10 was upregulated not because of the stimulation of the T cells by the luteal cells, but to suppress the proinflammatory response that would result from IFN-\(\gamma\) production. Further studies will be required to determine how luteal cells stimulate \(\gamma\delta\) cells and to determine which T cell is stimulated to produce cytokines.
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<td>Davis et al., 1987</td>
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<td>Petroff et al., 1997</td>
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<td>Cannon et al., 2003</td>
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<tr>
<td>TH81A5</td>
<td>Bovine MHC class II</td>
<td>IgG$_{2a}$</td>
<td>Davis et al., 1987</td>
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<td>Cannon et al., 2003</td>
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<tr>
<td>CC301</td>
<td>Bovine IL-12</td>
<td>IgG$_{2a}$</td>
<td>Hope et al., 2002</td>
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<tr>
<td>CC326</td>
<td>Bovine IL-12:biotin</td>
<td>IgG$_{2b}$</td>
<td>Hope et al., 2002</td>
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<tr>
<td>CC318</td>
<td>Bovine IL-10</td>
<td>IgG$_{2b}$</td>
<td>Kwong et al., 2002</td>
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<tr>
<td>CC320</td>
<td>Bovine IL-10:biotin</td>
<td>IgG$_{1}$</td>
<td>Kwong et al., 2002</td>
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**Table 4.1** List of monoclonal antibodies
Figure 4.1 T cell proliferation determined by $[^3]$Hthymidine incorporation. T cell proliferation was determined in cultures containing luteal cells with SEB (LC + SEB), T cells with SEB (TC + SEB), luteal cells plus T cells (LC + TC), or T cells and luteal cells in the presence of SEB (TC + LC + SEB). Data are representative of the mean for five animals. Data are presented as a square root transformation of dpm. Different letters denote significant differences (P < 0.05).
Figure 4.2 Microscopic images of T cells in culture. T cells showed little clumping when cultured alone (A). Small clusters of T cells were observed when cocultured with luteal cells (B). Interaction of luteal cells and T cells was evident by the large clusters of cells observed when cultures contained SEB (C). 200x magnification.
Figure 4.3 Effects of anti-MHC class I antibody on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of $[^3]$Hthymidine incorporation (dpm). Different letters denote significant differences ($P < 0.05; n = 5$) among means.
Figure 4.4 Effects of anti-MHC class II antibody on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of $[^3H]$thymidine incorporation (dpm). Different letters denote significant differences ($P < 0.05; n = 5$) among means.
Figure 4.5 Effects of anti-MHC class I and II antibodies on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of [$^3$H]thymidine incorporation (dpm). Different letters denote significant differences ($P < 0.05$; $n = 5$) among means.
Figure 4.6 Effects of anti-CD4 antibody on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of $[^3]$Hthymidine incorporation (dpm). Different letters denote significant differences ($P < 0.05$; $n = 5$) among means.
Figure 4.7 Effects of anti-CD8 antibody on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of $[^3]$Hthymidine incorporation (dpm). Different letters denote significant differences ($P < 0.05$; $n = 5$) among means.
Figure 4.8 Effects of anti-CD8 antibody in culture. Cultures that contained 20 µg/ml anti-CD8 (B) showed a less number of cells and less clumping of cells as compared to cultures that contained 1 µg/ml antibody (A).
Figure 4.9 Immunohistochemical detection of γδ T cells in CL. The γδ T cells were detected in CL collected midcycle (A) and 8 h post-PGF$_{2α}$ (B). Bovine spleen was used as a positive control (C) with a great proportion of the positive cells in the periarterial region (PR). The negative control (D) was substitution of the primary antibody with nonspecific mouse IgG. 200X magnification.
Figure 4.10 Effects of anti-δ T cell receptor antibody on T cell proliferation. Luteal cell (LC) and T cells (TC) were cultured separately in the presence of SEB, cocultured without SEB, or cocultured with SEB and various concentrations of antibody. T cell proliferation is presented as square root transformation of \([^3H]\)thymidine incorporation (dpm). Different letters denote significant differences (P < 0.05; n = 5) among means.
Figure 4.11 Representative scatter diagram of T cell activation. Percentage of T cells labeled with anti-bovine CD4 and anti-bovine CD3 antibodies is shown in the first column. Cells were labeled with anti-bovine CD3 antibody to determine purity of the isolated cell population analyzed by flow cytometry. Percentage of T cells labeled with anti-bovine CD8 and anti-bovine δ antibodies are shown in the second column. The percentage of unstained cells is shown in the lower left quadrant. The upper left quadrant depicts the percentage of cells labeled with either the anti-CD3 antibody (first column) or anti-δ antibody (second column). The percentage of either CD4+ (first column) or CD8+ cells (second column) is shown in the lower right quadrants. The percentage of cells that were CD3+/CD4+ (first column) or δ+/CD8+ (second column) is shown in the upper right quadrant. The numbers within each quadrant represent the percentage of cells for the specific cell type as described above.
Figure 4.12 Number of activated CD4$^+$ T cells determined by flow cytometry. Different letters denote differences (P < 0.05) in number of CD4$^+$ T cells per 10,000 cells counted. Data represent five replicates with different animals.
Figure 4.13 Number of activated $\gamma\delta$ T cells determined by flow cytometry. Different letters denote differences (P < 0.05) in number of $\gamma\delta$ T cells within the 10,000 counts collected. Data represent means obtained from five animals.
Figure 4.14 Number of activated CD8\(^+\) T cells determined by flow cytometry. Different letters denote differences (P < 0.05) in number of CD8\(^+\) T cells within the 10,000 counts collected. Data represent means obtained from five animals.
Figure 4.15 Examination of indirect immunofluorescence with confocal microscopy. The indirect immunofluorescent staining procedure was verified using microscopy. The $\gamma\delta$ T cells were detected with the red filter (A and C) and CD4$^+$ T cells (A) and CD8$^+$ T cells (C) were detected with the green filter. Cells labeled as $\gamma\delta$ cells appeared to be larger as compared with CD4$^+$ cells (A and B, arrow).
Figure 4.16 IFN-γ production in T cell cultures. Culture medium was collected after 72 h of culture. Data are presented as log(10) transformed values. Different letters denote differences (P < 0.05; n = 5) among means.
Figure 4.17 IL-12 in T cell cultures. Interleukin-12 was at or below the detection limit (2.0 ng/ml) of the ELISA for cultures that contained T cells alone, T cells treated with SEB or if luteal cell-T cell cocultures were untreated or treated with SEB. Culture medium collected from PBMCs (n = 3) that were cultured in the presence or absence of ConA was included as a positive control for IL-12. *Data were extrapolated because IL-12 concentrations in the samples (n = 2) were greater than the standard curve.
Figure 4.18 Positive control for IL-12 ELISA. Serial dilutions of medium collected from PBMCs that were stimulated with ConA for 48 h served as positive controls for IL-12. Medium collected from PBMCs that were cultured without ConA treatment were also included. Data represent means of three experiments. *Data were extrapolated because concentration of IL-12 was greater than the standard curve.
Figure 4.19 Controls for the IL-4 ELISA. Interleukin-4 could not be detected in the medium collected from cultures containing T cells alone or cocultured with luteal cells and treated with SEB. Samples of medium collected from PBMCs stimulated with ConA were included in the ELISA. Interleukin-4 could be detected in medium collected from PBMCs stimulated with ConA (n = 3).
Figure 4.20 IL-10 production in T cell cultures. Data represent replicate experiments from five animals. Different letters indicate differences (P < 0.05) of mean O.D. values.
Figure 4.21 Positive control for IL-10 ELISA. Serial dilutions of medium from PBMCs that were stimulated with ConA for 48 h served as positive control for IL-10. Medium collected from PBMCs cultured for 48 h without stimulation were also included in the ELISA. Data represent O.D. value means from three experiments.
CHAPTER 5

CONCLUSION

The CL is a transient tissue that is dependent on tropic hormone stimulation. Development of a functional CL and regression of the CL involves the endocrine and immune systems. The neuroendocrine system is a primary regulator of reproduction. Release of GnRH from the hypothalamus stimulates the release of LH from the anterior pituitary. Luteinizing hormone is the primary luteotropin in ruminants. Ovulation of the ovarian follicle, early development of the CL, and regression of the CL involve the participation of immune cells and the cytokines produced by these cells. The objective of this dissertation was not to address a major question about any one aspect of luteal function, but was focused on how these different systems, neuroendocrine and immune, interact with the CL to ensure that luteal development and regression occur, in a cyclical manner if pregnancy does not occur.

Gonadotropin-releasing hormone is widely used in assisted reproductive programs, such as estrous synchronization. The use of GnRH is to induce a LH surge and subsequent ovulation of an ovarian follicle. The use of a GnRH agonist to alter luteal function has not been applied in beef production. Depending on the stage of the estrous cycle and route of administration, GnRH can either enhance
or diminish circulating progesterone concentrations. We have previously shown that chronic administration of a GnRH agonist to cattle when the CL was developing increased the size of the CL and plasma progesterone concentrations.

The study presented in this dissertation was a follow-up study to determine what was altered within the CL as a result of treatment with a GnRH agonist to increase plasma progesterone concentrations. The amount of mRNA encoding for StAR was greater in CL collected from GnRH-agonist treated heifers as compared with control heifers. The rate-limiting step of progesterone synthesis is regulated by StAR. Furthermore, LH regulates StAR, so it was not a surprising finding that StAR mRNA was more abundant in CL collected from heifers treated with the agonist beginning on Day 3 of the estrous cycle as compared with the control group. A primary question that still remains is what contributes to the increase in size and weight of the CL. The increase in the size of the CL that was brought about by GnRH agonist administration was not attributed to an increase in size of the steroidogenic cells. Furthermore, the ratio of small luteal cells to large luteal cells was not altered so treatment did not alter differentiation of small to large luteal cells as hypothesized. The increase of progesterone production by the CL when cattle are chronically administered a GnRH agonist was likely attributed to an increase in StAR.

Another objective of this dissertation was to determine if dendritic cells were present in bovine luteal tissue. Dendritic cells are antigen-presenting cells that induce cell-mediated immunity as well as contributing to tumor immunity.
Dendritic-like cells have been reported in the ovary of the mouse (Jasper et al., 2000) as well as the cell surface molecules that are present on dendritic cells, such as MHC class II molecules (Fairchild and Pate, 1989; Khoury and Marshall, 1990; Kenny et al., 1991) and the CD80/86 co-stimulatory molecules (Cannon and Pate, 2001). Dendritic cell activation requires CD40 ligation, provided by CD4⁺ T cells. The mRNA encoding for CD40 was identified in luteal tissue at all stages of the luteal phase and during regression. This was the first report of CD40 gene expression in luteal tissue. These molecules are also located on macrophages. Therefore, to determine if dendritic cells were present in luteal tissue, evaluation of CD83, a cell adhesion molecule considered specific for dendritic cells was conducted.

The CD83 mRNA was detected at all stages of the luteal phase and regression. The mRNA encoding for CD83 was in the greatest abundance when proinflammatory cytokines are present during ovulation and luteal regression. It could not be concluded from the present study if dendritic cells were present in bovine luteal tissue or if the CD83 gene is expressed in non-dendritic cells.

One objective of the third study was to determine if an immune cell not yet identified in luteal tissue was present. An antibody specific for the δ-chain for the γδ T cell receptor was available and the γδ T cells were identified by immunohistochemistry in bovine luteal tissue. The γδ T cells are more abundant in circulation of cows as compared with humans and mice. The αβ CD4⁺ and CD8⁺ T cells have been identified in luteal tissue and the function of these cells in immune responses are known as well as their interaction with MHC
class II and MHC class I antigen-presenting cells, respectively. A study previously conducted in this laboratory reported that bovine luteal cells are capable of stimulating MHC class II-dependent T cell proliferation (Petroff et al., 1997).

The present study addressed which T cell was stimulated and what cytokines were produced by luteal cell-induced T cell activation. The \( \gamma\delta \) T cells were the prominent cells that were stimulated by luteal cells. These cells do not require MHC presentation of antigenic peptides to be activated. The lack of T cell inhibition when MHC class I or II antibodies were added to the culture support the hypothesis that luteal cells stimulate \( \gamma\delta \) cells in a MHC-independent manner. The percentage of CD4\(^+\) cells in the T cell population decreased when stimulated with luteal cells. Stimulation of CD8\(^+\) T cell proliferation was not altered when T cells were cultured with luteal cells, however anti-CD8 antibody inhibited T cell proliferation. It could be concluded that activation of CD4\(^+\) T cells is more tightly regulated as compared to the \( \gamma\delta \) T cells or that these cells contribute less to luteal regression as compared with the \( \gamma\delta \) or CD8\(^+\) T cells.

Neither IL-12 nor IL-4 were produced in luteal cell-T cell cocultures. Interferon-\( \gamma \) and IL-10 were produced in luteal cell-T cell cocultures. Interestingly, IFN-\( \gamma \) and IL-10 are antagonistic in their roles to mediate an immune response. Interferon-\( \gamma \) is proinflammatory, whereas IL-10 is anti-inflammatory and immunosuppressive. It was speculated that IL-10 was produced as a protective mechanism because of the abundant concentration of IFN-\( \gamma \) in the cultures.
The present study provided more information to how the immune system might have a role in luteolysis. Luteal cells stimulate $\gamma\delta$ T cells to a greater extent than either CD4$^+$ or CD8$^+$ T cells. It is speculated that the luteal cells have self-antigens that are recognized by $\gamma\delta$ T cells. The role of $\gamma\delta$ T cells during luteolysis needs further investigation. It was determined in the present study which T cell was stimulated by luteal cells, but not which T cell secreted the cytokines. It is speculated that different T cell populations secreted IFN-$\gamma$ and IL-10.
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