The Role of the Immune System in Regression of the Bovine Corpus Luteum.

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Declaration

I hereby declare that this thesis is entirely my own composition and that the research described therein is my own work, except where specifically stated within the text.

Lesley Anderson

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Abstract

In recent years there has been increasing interest in the role of the immune system in reproductive physiology. The aim of this study was to quantify immune cell populations within the cow corpus luteum (CL) throughout the oestrous cycle in order to investigate whether these cells could be involved in controlling luteal function, particularly around the time of luteolysis. Although prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the uterus is known to be the luteolytic substance in the cow, events occurring at the level of the CL at this time are less clearly defined. Immune cells and their cytokine products have significant potential to influence the cells of the CL at luteolysis.

Six CL were collected from each of four stages of the oestrous cycle, identified on the basis of their gross appearance, for preliminary immunohistochemical studies. Immune cell populations and MHC II expression varied throughout the oestrous cycle. In particular, the number of macrophages, T-lymphocytes (CD5+, CD4+) and MHC II expression was significantly higher in late stage CL (after luteolysis) compared to all other stages. To study in more detail the cellular events associated with luteolysis, the oestrous cycles of 19 cows were synchronised. CL were collected between days 16 and 20 of the following oestrous cycle. A significant increase in the number of T-lymphocytes (CD5+, CD8+) was detected in CL collected from day 16 onwards, compared to days 13-14. This increase occurred prior to functional luteolysis. Artificially-induced luteolysis was then assessed as a potential model for further studies around luteolysis. CL collected 6, 12 and 24 hours after luteolysis, induced using a single injection of 25mg $PGF_{2\alpha}$, had undergone dramatic structural regression which bore little resemblance to events during normal luteolysis and so this model was rejected. The role of endogenous $PGF_{2\alpha}$ in inducing the influx of T-lymphocytes was then investigated. Production of $PGF_{2\alpha}$ was inhibited in 12 cows between days 15 and 18 of the oestrous cycle and artificially replaced in six cows for 24 hours before collection of the CL on day 18. The number of macrophages was significantly lower in

all animals in which $PGF_{2\alpha}$ was inhibited compared to control animals but T-lymphocyte numbers were not significantly altered.

Cytokine production within the CL was also studied using the reverse trancriptase polymerase chain reaction (RT-PCR). Tumour necrosis factor-α, (TNF-α), interleukin-1β (Il-1β) and interferon-γ (IFN-γ) were detectable in similar amounts in CL at all stages of the oestrous cycle and after induced luteolysis and $PGF_{2\alpha}$ inhibition. Monocyte chemoattractant protein-1 (MCP-1) was higher in CL from animals after functional luteolysis compared to CL collected prior to luteolysis but the increase occurred before macrophage numbers in the CL increased. MCP-1 may be involved in chemotaxis of monocytes into the CL during luteal regression.

In conclusion, these results provide further evidence of a role for immune cells, particularly T-lymphocytes, in controlling CL function, outwith their involvement in structural luteolysis. Increasing concentrations of $PGF_{2\alpha}$ may be one of the major factors influencing the presence of macrophages, and possibly other immune cells, in luteal tissue. The presence of mRNA for TNF-α, IL-1β, IFN-γ and MCP-1 at all stages studied indicates a potential role for these substances in the CL although more detailed investigations are required. MCP-1 appears to have a specific involvement in the chemotaxis of monocytes to the CL in preparation for structural regression.

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Chapter 1

Literature Review

1.1. Introduction

In the last thirty years there have been significant advances in our understanding of the reproductive cycle in the cow and how it is controlled. This has led to the development of improved methods of oestrous synchronisation and artificial insemination as well as embryo transfer. All of these techniques are now used successfully in a wide range of management systems. However there are still many reproductive processes that are not fully understood and new areas of research relating to reproduction are emerging. In particular, the role of the immune system, which would previously have been regarded as being somewhat irrelevant to reproduction, has recently come under investigation. This interest has been stimulated as advances in the fields of immunology and reproductive endocrinology have revealed an overlap in function between the two systems (Mori, 1990; Stern and Coulam, 1992).

The cells of the immune system and their products have the capacity to influence reproduction from the level of the hypothalamus to the uterus (Tabibzadeh, 1994). In particular, immune cell populations in the ovary may affect the lifespan and function of ovarian structures especially around key ovarian events such as ovulation and luteolysis (Brannstrom and Norman, 1993; Pate, 1995; Terranova and Montgomery Rice, 1997). However, the mechanisms by which these cells may exert their effects have yet to be fully established, partly because of the vast number of potential cell interactions involved.

In this review the basic physiology of the oestrous cycle in the cow will be discussed followed by the structure and function of the bovine corpus luteum and luteolysis. The immune system and its role in reproduction will then be discussed in some detail. For the purposes of this review I will concentrate on the cow with reference to

other species where relevant. The luteal phase of the oestrous cycle will be emphasised, with limited reference to the control of follicular development except in relation to the immune system.

1.2. Basic physiology of the bovine oestrous cycle

The hormonal events and ovarian dynamics associated with the oestrous cycle in the cow are well known (Hansel and Convey, 1983; Greenwald and Roy, 1994) and may be summarised briefly as follows; the normal oestrous cycle in the cow lasts around 21 days. During this time the CL is the dominant ovarian structure between days 2 and 17 (Hansel *et al.*, 1973). The CL forms after ovulation from the granulosa and theca interna cells of the collapsed ovulatory follicle and its primary function is to produce progesterone which will prepare the reproductive tract for pregnancy (see Smith *et al.*, 1994 for a review). Luteal development is rapid and maximum size is reached around days 6-9 of the oestrous cycle (Hafs and Armstrong, 1968; Ireland *et al.*, 1980). During luteal development progesterone production by the CL increases, resulting in peak systemic concentrations around day 8. The high systemic progesterone concentrations are then maintained until luteolysis begins around day 17-18 (Hafs and Armstrong, 1968; Wiltbank, 1994).

Regression of the CL at the end of its lifespan in the non-pregnant cycle occurs in response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which is released from the uterus. (Luteolysis is discussed in detail in this review in Section 1.4). There is a rapid decline in the concentrations of follicle-stimulating hormone (FSH) and progesterone during luteolysis. The decline in progesterone concentrations removes the negative feedback effect on both gonadotrophin-releasing hormone (GnRH) from the hypothalamus and luteinising hormone (LH) from the anterior pituitary. These hormones control the growth of the ovulatory follicle and therefore progesterone inhibits maturation of a dominant follicle until after luteolysis, although waves of follicular growth, controlled by LH pulses, continue to occur throughout the lifespan of the CL (Fortune, 1994; Greenwald and Roy, 1994). Factors affecting follicular development and selection of the dominant follicle in the cow are reviewed by Webb *et al.* (1992)

and follicular steroidogenesis is described by Gore-Langton and Armstrong (1994). Ovulation in the cow is caused by the preovulatory LH surge which is itself induced by the high concentrations of oestradiol produced by the pre-ovulatory follicle. Ovulation takes place around 24 hours after the LH surge and then the next cycle of luteal development begins.

1.3. The Corpus Luteum

1.3.1 Gross structure

The gross appearance of the CL changes markedly throughout the oestrous cycle and these changes can be used to determine the approximate age of luteal tissue collected from animals with unknown reproductive histories (Ireland *et al.*, 1980; Fields and Fields, 1996). The early CL developing after ovulation grows rapidly between days 1 and 4 to around 1-1.5cm in diameter. At this stage the point of follicular rupture is still visible and the tissue is soft and creamy red in colour with a blood-filled centre. Between days 5 and 12 the CL matures to its maximum size of 2-2.5cm and the tissue becomes more firm in texture and orange in colour, although the apex is still red. A fluid filled lacuna can sometimes be found. The presence or absence of a lacuna during the CL lifespan does not appear to alter luteal function (Okuda *et al.*, 1988). After this stage the CL does not alter in size but becomes firmer in texture, has a vivid homogenous orange colour and distinct blood vessels develop over the capsule. After the onset of luteolysis, the CL decreases rapidly in size until it is eventually absorbed into the ovarian stroma. The regressing CL is much more fibrous and somewhere between bright yellow and white in colour.

During pregnancy the CL is maintained at its maximum size throughout gestation (see Gibori, 1993 for a review article). The CL is required functionally until the last 70 days of pregnancy (see Niswender and Nett, 1994, for a review). The CL of pregnancy is similar in structure to a mid-cycle CL (Sawyer, 1995) but regresses much more slowly after parturition than the CL from a non-pregnant cycle and small,

white areas of luteal tissue (corpus albicans) can often be seen in ovaries which are remnants of CL from previous pregnancies (Ireland *et al.*, 1980).

1.3.2. Ultrastructure

The CL of the cow, as in other ruminants, is made up of a variety of cell types (see O'Shea, 1987 and Fields and Fields, 1996 for review articles). Luteal cells make up a significant proportion of these cells but there are also large numbers of fibroblasts and endothelial cells as well as cells of the immune system, particularly macrophages (O'Shea *et al.*, 1989; Lei *et al.*, 1991). The bovine CL also contains a large amount of collagen (Luck and Zhao, 1993; Luck *et al.*, 1995). Many of the non-luteal cells present are associated with the significant vascular network in luteal tissue as the CL receives the highest blood flow, relative to its size, of any organ (Smith *et al.*, 1994).

The luteal cells have been classified on the basis of cell diameter into two distinct populations: small luteal cells and large luteal cells (Ursely and Leymarie, 1979; Koos and Hansel, 1981). Small luteal cells are around 10-22μm in diameter but there is some variation in the size specified for large luteal cells: >25μm from enzymatically dispersed luteal cell studies (Hansel *et al.*, 1987) and >35μm from morphometric studies (O'Shea *et al.*, 1989). Although the two cell populations are distinct there is likely to be some overlap in size and it is more difficult to clearly differentiate the two luteal cell populations by light microscopy in the sheep than the cow (O'Shea *et al.*, 1990; Parkinson *et al.*, 1994). In recent years there has been some suggestion that subpopulations of large and small luteal cells may also exist (Fields and Fields, 1996; Schmitt *et al.*, 1996)

1.3.3. Luteal cells - ultrastructural and functional differences

In addition to difference in size, large and small cells also have different ultrastructures and functions (Niswender *et al.*, 1985; Fields and Fields, 1996).

1.3.3.1. Ultrastructural differences

The ultrastructures of large and small luteal cells in the cow CL have been studied in detail (Koos and Hansel, 1981; O'Shea *et al.*, 1990). Both cell types contain intracellular structures associated with steroidogenesis including numerous mitochondria and abundant smooth endoplasmic reticulum. The small cells have a relatively smooth surface with a cup-shaped nucleus which stains intensely for chromatin. Large cells have a central round nucleus with less dense chromatin than small cells and a more distinct nucleolus. The surface membrane of the large cell is highly convoluted, with numerous microvilli which hold large and small cells tightly together. This tight cell adhesion has to be overcome when attempting to disperse luteal tissue to obtain populations of luteal cells for *in vitro* studies. The cytoplasm of large cells also contains Golgi complexes and rough endoplasmic reticulum, both structures being required for protein production.

One of the distinguishing features of large cells is the presence of small electron dense granules within their cytoplasm which are released by exocytosis (Parry *et al.*, 1980; O'Shea *et al.*, 1990). These granules were initially thought to contain progesterone because of a correlation between progesterone concentrations and granule release during the oestrous cycle of the sheep (Gemmell *et al.*, 1974; Stacy *et al.*, 1976). However, immunocytochemical studies showed that these granules contained oxytocin and its carrier protein neurophysin (Fields and Fields, 1986; Sawyer *et al.*, 1986) as well as vasopressin (Wathes *et al.*, 1984) and relaxin in pregnant cows (Fields *et al.*, 1980). A later study showed that these secretory granules were present in large luteal cells at all stages of the luteal lifespan and that the number of cells containing them varied between 3% on day 3 of the oestrous cycle and 85% on day 19 (Fields *et al.*, 1992). The substances contained in these secretory granules have a variety of functions (Smith, 1986; Schams, 1987). The role of oxytocin is discussed in detail in Section 1.4.3.1.

1.3.3.2. Functional differences

Although large and small cells both contain the cell organelles required for steroid synthesis they differ greatly in basal progesterone production as well as in their response to a variety of hormones including LH and $PGF_{2\alpha}$ (see Hansel *et al.*, 1991 for a review).

Basal production of progesterone by large luteal cells is around 20 times higher than that of the small cells (Koos and Hansel, 1981) and these high basal levels account for at least 80% of the progesterone produced by the CL *in vivo* (Niswender *et al.*, 1985). However small luteal cells are far more responsive to stimulation by LH in the cow and the sheep (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz *et al.*, 1982). Initially this was thought to be due to the presence of greater numbers of LH receptors on small cells compared to large cells (Fitz *et al.*, 1982). However, more recent studies have found equal numbers of LH receptors on large and small luteal cells, or greater numbers of LH receptors on large luteal cells compared to small luteal cells (Harrison *et al.*, 1987; Chegini *et al.*, 1991). The numbers of LH receptors present on luteal cells is regulated by progesterone, particularly in early CL (Jones *et al.*, 1992).

Small and large luteal cells also differ in their responsiveness to $PGF_{2\alpha}$, the main luteolytic hormone in the cow. Studies *in vitro* have demonstrated that $PGF_{2\alpha}$ stimulates progesterone production by small cells but inhibits progesterone production by large cells (Alila *et al.*, 1988; Davis *et al.*, 1989). Specific $PGF_{2\alpha}$ receptors are found on both types of luteal cells (Powell *et al.*, 1975) but the majority are present on large cells (Braun *et al.*, 1988). It appears therefore that large cells are the main target for the luteolytic effect of $PGF_{2\alpha}$ in late stage CL. Binding affinity for $PGF_{2\alpha}$ is higher in late stage CL compared to early or mid-cycle (Rao *et al.*, 1979). Receptors for prostaglandin E_2 (PGE_2), which is a luteotrophic substance in the cow (Fitz *et al.*, 1993), are also more abundant on large cells than small cells (Fitz *et al.*, 1982).

Two morphologically distinct cell types, identifiable as large and small luteal cells, are also present in the CL of pregnancy (Sawyer, 1995). In the pregnant cow CL both cell types produce similar amounts of progesterone when unstimulated and respond in the same way to gonadotrophins (Chegini *et al.*, 1984). Another study found that bothsmall and large luteal cells from pregnant cows contained equal amounts of progesterone but neither cell type responded to LH-stimulation *in vitro* (Weber *et al.*, 1987).

1.3.4. The Development of the CL

1.3.4.1. CL formation after ovulation

The CL develops rapidly from the remnants of the dominant follicle after ovulation (see Smith *et al.*, 1994, for a review). After follicular rupture there is folding of the follicular wall and fibroblasts, endothelial cells and theca interna cells migrate into the early CL (O'Shea *et al.*, 1980). The theca interna and granulosa cells luteinise and large and small luteal cells increase in size and develop their characteristic features. Increasing amounts of progesterone are produced as the luteal cells, particularly large luteal cells, enlarge (Farin *et al.*, 1986). Increased production of progesterone is also associated with the rapid development of the blood supply in the CL (see Section 1.3.4.3.). Blood vessels gradually invade the collapsed follicle to establish a major network of capillaries which will ensure that the majority of luteal cells are in close contact with a blood supply (Niswender *et al.*, 1976). After the blood supply is established and luteinisation has taken place the changes within the CL are associated with rapid growth of the tissue due to hypertrophy of the luteal cells as well as proliferation of both luteal and non-luteal cells (Jablonka-Shariff *et al.*, 1993).

Development of the CL is under the control of a large number of local and systemic factors (Smith, 1994). In recent years there has been particular interest in the role of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinase (TIMP) in CL formation. Metalloproteinases, such as gelatinase and collagenase, are locally produced proteins extensively involved in tissue remodelling and angiogenesis, both

significant processes in the CL. MMP activity has been observed in day 4 CL when angiogenesis and cell proliferation will be occurring rapidly (Tsang *et al.*, 1995). However, MMP-2, MMP-9 as well as TIMP-1 and TIMP-2 were also present in CL collected between days 6 and 16 of the oestrous cycle (Goldberg *et al.*, 1996). It appears that metalloproteinases have a role within the CL, not only during the period of maximum growth, but also throughout its functional lifespan.

1.3.4.2. The origin of luteal cells

The cellular origin of the individual luteal cell types has been a subject of great debate. Donaldson and Hansel (1965) stated, from histological studies, that luteal cells in the cow CL developed from both the theca and granulosa layers of the follicle and a similar finding was described by Lobel and Levey (1968). Later studies in the sheep indicated that the theca interna was the source of the small luteal cell and the granulosa cell the source of the large luteal cell (O'Shea *et al.*, 1980). Similar findings were then described in the cow where bovine granulosa and thecal cells, when luteinised in culture, differentiated into large luteal type cells and small luteal like cells respectively (Meidan *et al.*, 1990).

In the cow CL, the novel use of monoclonal antibodies to antigens expressed on theca and granulosa cell surfaces allowed clearer identification of the origins of the two luteal cell types (Alila and Hansel, 1984). Indirect immunofluorescence was used to look at binding of these antibodies at different stages of the CL lifespan. Large cells were seen to primarily express granulosa cell antigen although this expression declined with age of CL. The majority of small cells expressed thecal cell antigen throughout the lifespan of the CL. However, around days 10-12 of the oestrous cycle up to 46% of the large cells also expressed thecal cell antigen compared to 10% at days 4-6.

These results provided evidence of the cellular origin of luteal cells but also indicated that some small luteal cells develop into large luteal cells during the lifespan of the CL, a process first suggested by workers studying basic histology of the bovine CL

(Donaldson and Hansel, 1965). There is however some suggestion that the use of enzymatically-dispersed cells in the monoclonal antibody study might have artificially altered the luteal cell populations and hence, skewed the results (O'Shea *et al.*, 1990).

An ultrastructural study in the cow CL revealed no evidence of a separate population of large cells that might have been formed from small luteal cells (O'Shea *et al.*, 1990), although a later morphometric study did find evidence that a proportion of small luteal cells differentiated into large luteal cells late in the oestrous cycle (Lei *et al.*, 1991). In the sheep no change in large luteal cell numbers, indicative of differentiation of small luteal cells into large cells, was detectable by morphometric analysis of luteal tissue from different stages of the oestrous cycle (O'Shea *et al.*, 1986; Farin *et al.*, 1986). However, a later study in the sheep showed that significantly more small luteal cells differentiated into large luteal cells in ewes treated with human chorionic gonadotrophin (hCG) and LH *in vivo* compared to sheep CL from ewes treated with saline prior to collection of the CL (Farin *et al.*, 1988). The evidence for the differentiation of small luteal cells into large luteal cells as part of luteal development remains somewhat inconclusive.

1.3.4.3. Angiogenesis

During development of the CL there is formation of a vascular network under the influence of a range of angiogenic factors (Smith *et al.*, 1994; Redmer and Reynolds, 1996). In addition angiogenesis is an important process during luteal regression when rapid vascular restructuring takes place (O'Shea et al., 1977; Redmer *et al.*, 1988). The vascular component of luteal tissue is extensive and the mature CL receives one of the greatest rates of blood flow to any organ, reaching 10-15ml/min per gram of tissue (Ford and Chenault, 1981). The greatest flow rates are found during the luteal phase and a rapid decline in flow is associated with luteolysis (Ford and Chenault, 1981; Wise *et al.*, 1982). Adequate blood flow is vital for the support of luteal cells, as well as the transport of luteal cell products, and is important for regulation of CL

function (Niswender *et al.*, 1976). The mechanisms involved in controlling the blood supply of the CL vary throughout the lifespan of the CL (Keyes and Wiltbank, 1988).

The multiple factors involved in controlling angiogenesis in the ovary have recently been reviewed (Redmer and Reynolds, 1996). Heparin binding growth factor proteins, in particular fibroblast growth factors and vascular endothelial growth factors appear to have a specific role in affecting angiogenesis within the corpus luteum throughout its lifespan. However other, as yet undefined, factors are also likely to be involved.

The bovine CL produces fibroblast growth factors throughout the oestrous cycle although there is some variation in the results of different studies, probably related to the different methods of detection used. Messenger RNA for fibroblast growth factor 2 (FGF-2) was present in CL at all stages of the oestrous cycle but was highest late in the luteal phase (Stirling *et al.*, 1991). Using immunohistochemical staining, Zheng *et al.* (1993) reported that FGF-2 was more abundant than FGF-1 and varied in concentration with the greatest amount present in the middle stage (Zheng *et al.*, 1993). Vascular endothelial growth factor (VEGF) has been described in most detail within the ovine CL. VEGF mRNA is present in ovine luteal tissue and the amount of mRNA encoding VEGF in the ovine CL is 2-3 fold greater between days 2-4 compared to days 8-15 (Redmer and Reynolds, 1996).

The expression of different angiogenic factors is thought to be controlled by the various luteotrophic and luteolytic factors present within the CL at different stages of the cycle. Expression of mRNA for FGF-2 is increased by LH and inhibited by $PGF_{2\alpha}$ in bovine luteal cells *in vitro* (Redmer *et al.*, 1988, Stirling *et al.*, 1991). In addition, angiogenesis, particularly during luteal regression, may be regulated by the presence of FGF receptors. In particular the receptor for FGF-2 (FGFR-2) is only present in the vasculature late in the oestrous cycle (Redmer and Reynolds, 1996). It is unclear why angiogenic growth factors are present throughout the lifespan of the CL as most rapid vascular development occurs early in luteal development. However, it appears that angiogenic factors are also involved in maintenance of vascular

supply in the mature CL. Angiogenic factors could also have other roles within the CL, possibly affecting CL differentiation and function (Redmer and Reynolds, 1996).

1.3.5. The Cellular Composition of the CL

Various techniques have been used to count cells from the CL, particularly to calculate the relative proportions of large:small luteal cells. Unit gravity sedimentation of dispersed luteal tissue separates different cell types, but large cell populations are contaminated with small cells using this technique (Ursely and Leymarie, 1979). Fluorescence-activated cell sorting reduces this contamination and is also less damaging to the luteal cells than unity gravity sedimentation (Alila *et al.*, 1988; Brannian *et al.*, 1993). However, both techniques involve dispersion of luteal tissue and the dispersion process, particularly treatment with collagenase, is thought to result in selective loss of large luteal cells (Rodgers *et al.*, 1984; O'Shea *et al.*, 1989).

This artificial reduction in large cell numbers may explain the variations in large:small luteal cell ratios from different studies. It is generally accepted that there are far more small cells than large cells present in the cow CL at all stages of the oestrous cycle, but estimates of actual figures for small luteal cells:large luteal cells from dispersed luteal cell studies have varied from 5:1 (Weber *et al.*, 1987) to between 20:1 and 40:1 (Hansel *et al.*, 1987).

Morphometric analysis of intact slices of luteal tissue has been used to calculate the numbers of different cell types present in luteal tissue. A detailed morphometric study by O'Shea *et al.* (1989) showed that the ratio of large:small luteal cells was in the region of 1:7.6 in mid-cycle cow CL. Luteal cells accounted for only 30% of the total number of cells present and around 70% of the total cell volume. However this study was based on CL collected after synchronisation of oestrus using two injections different cell populations from CL formed naturally (Hansen *et al.*, 1987). Another study where oestrus was synchronised using a combination of progesterone treatment

and a single injection of $PGF_{2\alpha}$ found that 40% of the total cell population from mid-cycle CL was made up of luteal cells with 44% large luteal cells and 56% small luteal cells, a considerably lower ratio of large:small cells than in any other study (Lei *et al.*, 1991).

Despite differences in luteal cell numbers, both of these morphometric studies found that non-luteal cells made up the majority of cells present in mid-cycle CL (60-70%). Endothelial cells were the most abundant non-luteal cell type (O'Shea *et al.*, 1989). The presence of such large numbers of non-luteal cells is, as discussed earlier, associated with the large capillary network in the CL but it is also possible that these cells may play a role in altering CL function. In particular, endothelial cells have been shown to interact with small and large luteal cells in luteotrophic and luteolytic events *in vitro* (Girsch *et al.*, 1995).

The cellular composition of the mid-cycle CL (around day 12 of the oestrous cycle) has been studied in the most detail. However, the proportions of different cell types within the CL are not static but vary at different stages of its lifespan in both the sheep and the cow (Farin *et al.*, 1989; Lei *et al.*, 1991). Once again the actual numbers vary between studies. Parry *et al.* (1980) found that luteal cells made up 51% of the total number of cells in the cow CL on day 6 of the oestrous cycle, 72% on day 15 and 60% on day 20 whereas Lei *et al.* (1991) found that luteal cells made up only 25% of the total cell population in both early luteal phase CL (day 6) and late luteal phase CL (day18) and was highest (40%) on day 12.

1.3.6. Corpus luteum function and control

The CL is influenced by a wide range of factors of both local and systemic origin which influence its functional activity in different ways at different stages of the oestrous cycle (see Rothchild, 1981; Behrman *et al.*, 1991; Niswender and Nett, 1994 and Milvae *et al.*, 1996 for review articles). The cellular composition of the CL, both luteal cells and non-luteal cells, influences CL responsiveness to different hormonal stimuli as well as cellular events within luteal tissue itself (Stouffer and Brannian,

1993). In particular, the relative numbers of large and small luteal cells will influence the CL because of their different functional properties (Section 1.3.3.2.). Although large and small cells differ functionally when examined in separate populations *in vitro*, the two cell types do interact (Harrison *et al.*, 1987; Del Vecchio *et al.*, 1994; 1995) and this is likely to be significant in terms of overall luteal cell function *in vivo* (Redmer *et al.*, 1991).

Progesterone production is not only influenced by the cellular composition of the luteal tissue. Release of progesterone during the luteal phase of the oestrous cycle and its abrupt decline after luteolysis are under the control of a wide variety of luteotrophic and luteolytic factors (Alila and Dowd, 1991; Niswender *et al.*, 1994). The factors associated with luteolysis are discussed in Section 1.4.

1.3.6.1. Luteotrophic support of the CL

The main luteotrophic hormone in the ruminant is LH which is released from the anterior pituitary in response to GnRH from the hypothalamus (Hansel, 1966; Niswender and Nett, 1994). The studies which provided evidence for the role of LH are reviewed by Alila and Dowd (1991). There is some debate in the literature about the exact role of LH at different stages of luteal lifespan. In the sheep LH is required for normal luteal function throughout the CL lifespan but the early CL is most resistant to withdrawal of LH (see Baird, 1992 for review). In the cow however, it appears that LH is required for normal CL development in heifers between days 2-7 of the oestrous cycle and is not important in maintaining progesterone production between days 12 and 17 (Peters *et al.*, 1994).

Although LH is the most significant luteotropic hormone its effect is not acute as progesterone production continues in the absence of LH. In the bovine this is most likely related to high baseline production of progesterone by large luteal cells (Wiltbank, 1994). However, development and overall function of the CL is reduced when LH support is withdrawn. LH production and activity itself is under the control of a variety of processes. Progesterone has a negative feedback effect on LH release (Goodman and Karsch, 1980) which results in the less frequent release of LH pulses during the luteal phase than during the follicular phase (Rahe *et al.*, 1980; Walters *et*

al., 1984), although the pulse height is higher. In addition, LH receptor numbers vary according to the stage of cycle (Diekman *et al.*, 1978; Chegini *et al.*, 1991) and increased production of progesterone as the CL develops is associated with an increase in the numbers of LH receptors (Jones *et al.*, 1992). The binding of LH to these specific receptors results in activation of cyclic AMP and Ca^{2+}-polyphosphoinositol-protein kinase C second messenger systems. The products of these pathways lead to stimulation of progesterone biosynthesis (Alila and Dowd, 1991; Niswender and Nett, 1994).

Prostaglandin E2 (PGE2) may also have a luteotrophic role in the CL, particularly during the maintenance of pregnancy (Fitz *et al.*, 1993; Shelton *et al.*, 1990). Uterine PGE2 concentrations in cows were higher during the luteal phase and decreased during the follicular phase (Cerbito *et al.*, 1994). Another product of the arachidonic acid cascade; prostacyclin (PGI-2) also has a luteotrophic effect (Milvae *et al.*, 1986; Hansel and Dowd, 1986)

Whether or not oxytocin acts as a luteotrophic hormone is unclear as results of studies by various groups are contradictory. Different studies have found that oxytocin either stimulates (Tan *et al.*, 1982), inhibits (Miyamoto and Schams, 1991) or has no effect (Milvae and Hansel, 1983) on progesterone production by luteal cells *in vitro*. The differences between studies are likely to result from variation in dose rates of oxytocin used as well as the use of luteal tissue collected at different stages of the oestrous cycle and pregnancy. In sheep, loss of luteal oxytocin did not alter progesterone production and so it seems unlikely that oxytocin is of importance in providing luteotrophic support (Sheldrick and Flint, 1983). Oxytocin has a more significant role in the luteolytic events in the CL (Flint *et al.*, 1986) and this is discussed in detail in Section 1.4.3.1.

A number of local factors also affect CL function and they may have luteotrophic or luteolytic effects. These can be substances produced by luteal cells, by endothelial cells and fibroblasts or cells of the immune system, and may act on the cell that produces them (autocrine effect) or affect surrounding cells (paracrine effect). There are very many substances which can affect luteal function and the list is continually

expanding but they include a range of growth factors and cytokines (see Behrman *et al.*, 1993; Tsafiri and Adashi, 1994 and Pate, 1996 for review articles). The effect of cytokines in the CL will be discussed in detail in Section 1.6.

1.4. Luteolysis

Luteolysis takes place at the end of the functional lifespan of the CL of the non-pregnant cycle and also at the end of pregnancy. Two separate processes are involved beginning with functional regression, which is characterised by a rapid decline in progesterone release, followed by structural regression when the luteal tissue itself is destroyed (Knickerbocker *et al.*, 1988; Niswender *et al.*, 1994). The actual mechanisms involved in the process of luteolysis are complicated and there is some variation between species. For this part of the review I will concentrate on cattle and sheep with reference to other species where relevant.

1.4.1. The luteolytic signal; uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

It is now accepted that $PGF_{2\alpha}$ released from the uterus is the primary hormone which causes luteolysis in the cow and the sheep (Hansel *et al.*, 1973; McCracken *et al.*, 1981; Silvia *et al.*, 1991). This conclusion is based on a large number of studies over a period of many years.

A uterine source for the luteolytic substance was first suggested when it was discovered that removal of the uterus lengthened the lifespan of the CL in both cattle and sheep (Wiltbank and Casida, 1956; Moor *et al.*, 1970). A role for $PGF_{2\alpha}$ in the initiation of luteolysis was indicated when later studies showed that luteal regression was delayed by inhibitors of prostaglandin synthesis infused directly in to the uterus (Lewis and Warren, 1977; Milvae *et al.*, 1986) and that the oestrous cycle was prolonged in animals immunised with PGF antibodies (Fairclough *et al.*, 1981). Less direct evidence of a role for uterine $PGF_{2\alpha}$ in luteolysis is the increase in systemic concentrations of the major metabolite of $PGF_{2\alpha}$ around this time (Nancarrow *et al.*,

1973) and the fact that luteolysis can be successfully induced using exogenous $PGF_{2\alpha}$ (Hansel and Convey, 1983).

After endometrial $PGF_{2\alpha}$ had been established as the luteolysin, the transport route of $PGF_{2\alpha}$ to the CL was investigated. In the sheep metabolism of $PGF_{2\alpha}$ by the lungs is rapid, although in the cow it is somewhat slower, and this meant that delivery via the systemic blood flow was not possible. A local veno-arterial countercurrent system, allowing transfer of $PGF_{2\alpha}$ from the uterine vein directly into the ovarian artery, was discovered (Hixon and Hansel, 1974; Hansel and Blair 1996).

1.4.2. Secretion of $PGF_{2\alpha}$

$PGF_{2\alpha}$ is metabolised by the lungs to 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM), a more stable metabolite of $PGF_{2\alpha}$, with a half-life of around eight minutes (Kindahl *et al.*, 1981). Concentrations of $PGF_{2\alpha}$ metabolites in peripheral plasma have been demonstrated to be a reliable indicator of uterine synthesis and release of $PGF_{2\alpha}$ (Guilbault *et al.*, 1984) and are widely used to determine patterns of secretion of $PGF_{2\alpha}$ release *in vivo* (Kindahl *et al.*, 1989).

In the cow PGFM concentrations in peripheral plasma and uterus are minimal until around day 14-15 of the oestrous cycle when increased amounts of $PGF_{2\alpha}$ are detectable in the endometrium and small pulses of PGFM can be detected in plasma (Shemesh and Hansel, 1975; Parkinson and Lamming, 1990). This is followed by the release of larger pulses of $PGF_{2\alpha}$ which will actually induce luteolysis. These pulses last 1-5 hours (Nancarrow *et al.*, 1973; Kindahl *et al.*, 1976a, 1976b) and they are completely inhibited by pregnancy (Basu and Kindahl, 1987). The initial high amplitude pulses induce luteolysis and an associated rapid decline in progesterone secretion. However, several pulses of $PGF_{2\alpha}$ continue after the decrease in progesterone concentration and it is thought that they are required for complete structural regression.

1.4.3. Control of the pulsatile pattern of $PGF_{2\alpha}$ release

Although uterine $PGF_{2\alpha}$ is now established as the luteolytic hormone and the pulsatile secretion pattern of prostaglandin release is well documented, exactly how this pattern of release is controlled *in vivo* is still a matter of debate. There are multiple systemic and local factors involved in this process (reviewed by Silvia *et al.*, 1991).

1.4.3.1. The role of oxytocin

Oxytocin stimulates release of $PGF_{2\alpha}$ from the uterus (Newcomb *et al.*, 1977; Lafrance and Goff, 1990) and is released, primarily from the large cells of the CL, in a similar pulsatile pattern to that of $PGF_{2\alpha}$ from the uterus at the time of luteolysis (Vighio and Liptrap, 1986; Walters *et al.*, 1984). From these results it seems likely that this substance is involved in some way in influencing hormonal events at this time (Flint and Sheldrick, 1983; Flint *et al.*, 1986; Schams, 1989). It has been suggested that there is a significant degree of positive feedback between the two hormones such that uterine $PGF_{2\alpha}$ stimulates luteal oxytocin release and oxytocin then stimulates $PGF_{2\alpha}$ release from the uterus (Flint and Sheldrick, 1983).

However, other studies have been unable to show a direct link between the two hormones. Luteal cells *in vitro* do not release oxytocin in response to $PGF_{2\alpha}$ stimulation although it has been suggested that this is due to an indirect action of $PGF_{2\alpha}$ on luteal cells which is blocked in some way when cells are cultured (McCann and Flint, 1990). There is also evidence that $PGF_{2\alpha}$ functions independently of oxytocin *in vivo*. In the sheep recent work has shown that $PGF_{2\alpha}$ pulses are released at normal frequency, although reduced magnitude, in the absence of the luteal source of oxytocin (Mann and Lamming 1995b) and depletion of stored oxytocin from the CL prior to luteolysis in normally cycling cattle did not affect luteolysis or the duration of the oestrous cycle (Kotwica and Skarzynski, 1993). However it is possible that in these studies oxytocin derived from the posterior pituitary played a more significant role in the absence of an ovarian source.

Oxytocin has been measured in CL at different stages of the oestrous cycle. Maximal concentrations of oxytocin have been observed in cow luteal tissue at mid-luteal phase and then gradually decline and maximum expression of the oxytocin gene is associated with luteinisation (see Wathes and Denning-Kendall, 1992 for a review). These temporal relationships are difficult to fit with the theory of positive feedback between uterine $PGF_{2\alpha}$ and luteal oxytocin at the time of luteal regression (Abdelgadir *et al.*, 1987; Wathes *et al.*, 1984; Parkinson *et al.*, 1992) as the oxytocin concentration falls prior to a decrease in progesterone concentration and luteolysis.

The relationship between release of $PGF_{2\alpha}$ and oxytocin during the oestrous cycle remains somewhat unclear. The timing of the development of oxytocin receptors within the uterus would seem to indicate that the two substances do interact at luteolysis. Increased endometrial responsiveness due to an increase in the concentration of oxytocin receptors occurs from around day 13 of the oestrous cycle onwards but is most marked between days 16 and 19 (Meyer *et al.*, 1988; Mirando *et al.*, 1993). The development of oxytocin receptors would appear therefore to be an important process in the initiation of luteolysis (McCracken *et al.*, 1981; Flint *et al.*, 1990) and is thought to be controlled by the concentrations of steroid hormones, in particular progesterone and oestrogen (Sheldrick and Flint, 1985; Jenner *et al.*, 1991).

It is also unclear what substance initiates the pulsatile release pattern of $PGF_{2\alpha}$ and oxytocin in the first place. In the sheep there is some evidence that $PGF_{2\alpha}$ first initiates the release of oxytocin (Moore *et al.*, 1986) but other workers suggest that oxytocin derived from the posterior pituitary is involved (McCracken *et al.*, 1984). Maintenance of the pulsatile release pattern of both substances is under the control of various factors including the presence of oxytocin receptors in the uterus (Flint *et al.*, 1990) and changes in the steroid environment, in particular the concentrations of progesterone and oestradiol (Silvia and Taylor, 1989).

The duration of each pulse of $PGF_{2\alpha}$ and oxytocin was thought to be limited by depletion in oxytocin concentrations which would also explain the 5-12 hour delay

between pulses as stores are replenished (Flint and Sheldrick, 1983). However, it now appears more likely these effects are due to the uterus becoming refractory to further stimulus for a limited period of time (Sheldrick and Flint, 1986), a theory supported by studies that have shown that prolonged treatment with oxytocin extends luteal lifespan in cattle (Howard *et al.*, 1990; Lutz *et al.*, 1991) and inhibits oxytocin-induced $PGF_{2\alpha}$ secretion (Lutz *et al.*, 1991).

In addition to oxytocin receptors in the uterus there are also oxytocin receptors on luteal cells (Okuda *et al.*, 1992) and treatment with $PGF_{2\alpha}$ increases the numbers of these receptors on luteal cells *in vitro* (Okuda *et al.*, 1995). It appears therefore that $PGF_{2\alpha}$ and oxytocin also act in some autocrine/paracrine manner at the level of the CL as well as between the uterus and CL.

1.4.3.2. The role of oestradiol

Oestradiol concentrations, as mentioned above, do appear to be involved in affecting the timing of events around luteolysis. Reduced concentrations of oestradiol have been shown to affect the ability of the uterus to release $PGF_{2\alpha}$ in response to oxytocin, weakening the luteolytic signal (Mann and Lamming, 1995a). Oestradiol also increases uterine responsiveness to oxytocin possibly through controlling the development of oxytocin receptors and increasing endometrial production of $PGF_{2\alpha}$ (Parkinson *et al.*, 1990; Beard and Lamming, 1994). It may also be involved in regulating synthesis and release of oxytocin from the posterior pituitary gland as well as having a synergistic effect with progesterone in enhancing oxytocin-induced release of $PGF_{2\alpha}$ (Lafrance and Goff, 1988; Lamming and Mann, 1995b). In the sheep the progesterone:oestradiol ratio has been shown to influence the release of $PGF_{2\alpha}$ in response to oxytocin (Beard *et al.*, 1994).

1.4.3.3. The role of progesterone

Likewise progesterone may also have a role in controlling $PGF_{2\alpha}$ release. The uterus requires progesterone stimulation for around 10-14 days in order for natural $PGF_{2\alpha}$

release to take place in the sheep (Louis *et al.*, 1977). The spontaneously-formed cow CL does not respond to artificially induced luteolysis until after day 5 of the oestrous cycle (Hafs *et al.*, 1974; Henricks *et al.*, 1974) whereas luteolysis can be artificially induced by day 2 after ovulation in a CL induced by treatment with hCG (Howard and Britt, 1990). Elevated progesterone concentrations during the development of the hCG-induced CL may result in this early response to $PGF_{2\alpha}$. However, artificially increasing progesterone concentrations during days 1-3 of the oestrous cycle did not result in a more rapid response to $PGF_{2\alpha}$ by spontaneously formed CL (Battista *et al.*, 1984). Therefore it appears that other factors are involved in this process (Howard and Britt, 1990).

The exact actions of progesterone are still being investigated but this hormone could have a role in stimulating the accumulation of substances required for synthesis of $PGF_{2\alpha}$ (Silvia *et al.*, 1991) or may affect the ability of oxytocin to stimulate release of uterine $PGF_{2\alpha}$ (Lafrance and Goff, 1988). Early withdrawal of progesterone also causes premature release of $PGF_{2\alpha}$ and so it may act as a prostaglandin inhibitor for much of the oestrous cycle with loss of this inhibitory effect occurring after prolonged exposure of the uterus to progesterone (Kindahl *et al.*, 1981). It has also been suggested that progesterone blocks formation of endometrial oxytocin receptors in ruminants (Mirando *et al.*, 1993; Lau *et al.*, 1992, 1993). However, a more recent study showed that ovariectomised cows, treated with progesterone, released $PGF_{2\alpha}$ in response to an oxytocin challenge, whereas untreated cows and those treated with oestradiol did not, although oxytocin receptors were present in the uteri of all animals (Lamming and Mann, 1995a). This suggests that progesterone is involved in controlling oxytocin receptor function. In addition, progesterone is involved in inhibiting the luteolytic signal in the cow (Mann and Lamming, 1995c).

1.4.4. $PGF_{2\alpha}$ sites of action

Specific receptors for $PGF_{2\alpha}$ are found on all luteal cells (Powell *et al.*, 1975) but the majority are located on large luteal cells which are presumably the main site of action

in vivo (Braun *et al.*, 1988). The $PGF_{2\alpha}$ receptor is induced rapidly after luteinisation (Tsai and Wiltbank, 1995; Wiltbank *et al.*, 1995). The number and affinity of binding sites for $PGF_{2\alpha}$, as well as mRNA encoding the $PGF_{2\alpha}$ receptor, increases between days 3 and 20 of the oestrous cycle in the cow CL before being reduced after luteal regression (Rao *et al.*, 1979; Sakamoto *et al.*, 1995). mRNA encoding the $PGF_{2\alpha}$ receptor in sheep is also found primarily on large luteal cells (Niswender *et al.*, 1995) and is abundant between days 2 and 12 of the oestrous cycle but, like the cow, is reduced after luteolysis (Eldering *et al.*, 1995).

The direct effects of $PGF_{2\alpha}$ on luteal cells *in vitro* have been studied in some detail with varying results. In contrast to the known luteolytic role of $PGF_{2\alpha}$ *in vivo*, progesterone secretion by cow luteal cells treated with $PGF_{2\alpha}$ is increased (Hixon and Hansel 1979; Davis *et al.,* 1988; Meidan *et al.*, 1992). Pate and Condon (1984) found that $PGF_{2\alpha}$ had no effect on basal production of progesterone but did inhibit agonist-stimulated progesterone production after luteal cells had been cultured for 24 hours. Conversely, in the sheep $PGF_{2\alpha}$ either had no effect on, or inhibited, progesterone production *in vitro* (Fletcher and Niswender, 1982; Fitz *et al.*, 1993). A detailed study using microdialysed cow CL concluded that the variation in results and apparent contradiction in effects of $PGF_{2\alpha}$ *in vitro* compared to its luteolytic effects *in vivo* may be due to lack of cell-cell contact in culture conditions (Miyamoto *et al.*, 1993).

1.4.5. $PGF_{2\alpha}$ mechanisms of action

Although the luteolytic substance was identified many years ago, it is still not clear exactly how $PGF_{2\alpha}$ exerts its effects at the cellular level. A range of possible mechanisms of $PGF_{2\alpha}$ action on luteal cells has been described (see Knickerbocker *et al.*, 1988; Wiltbank and Niswender, 1992 and Michael *et al.*, 1994 for review articles).

There are direct cytotoxic effects of $PGF_{2\alpha}$ on luteal cells (Braden *et al.*, 1988) as well as changes in cell membrane structure related to the loss in cellular function (Carlson *et al.*, 1982). Decreased utilisation of cholesterol for steroidogenesis by luteal cells treated with $PGF_{2\alpha}$ has been noted in sheep and cattle (Pate and Condon, 1989; Wiltbank *et al.*, 1990; Grusenmeyer and Pate, 1992) as well as reduced steroidogenic enzyme activity and reduced numbers of LH receptors (Fletcher and Niswender, 1982). There is also evidence of a disruption of LH- stimulation of progesterone production (Thomas *et al.*, 1978; Wakeling and Green, 1981; Fletcher and Niswender, 1982).

Altered luteal blood flow may be involved but it is not clear if this is a cause or a consequence of luteolysis although the reduction in blood flow is unlikely to be significant enough to cause hypoxia (Pharriss *et al.*, 1970; Niswender *et al.*, 1976; Nett *et al.*, 1976). The induction of high levels of intracellular calcium have also been suggested as a mechanism of action for $PGF_{2\alpha}$ during luteolysis (Alila *et al.*, 1990a,1990b; Duncan and Davis, 1991; Wegner *et al.*, 1990).

1.4.6. Inhibition of luteolysis in the pregnant animal

In the event of pregnancy, the CL does not undergo luteolysis but remains functional. This requires 'maternal recognition of pregnancy', a process influenced by many different factors and leading to a variety of hormonal events which inhibit luteolysis (Bazer *et al.*, 1994; Niswender *et al.*, 1994; Hansen, 1995). Luteolytic peaks of $PGF_{2\alpha}$ are completely inhibited by pregnancy (Basu and Kindahl, 1987) although there is some evidence that the uterine and peripheral concentrations of $PGF_{2\alpha}$ are slightly higher in pregnant than in non-pregnant cattle and sheep around day 17 of the cycle (Inskeep and Murdoch 1980; Williams *et al.*, 1983; Parkinson *et al.*, 1990). This phenomenon may be due to prostaglandin synthesis by the embryo (Shemesh *et al.*, 1979). However the bovine blastocyst has also been shown to be capable of synthesis of progesterone which could be significant in protecting the CL from luteolysis (Shemesh *et al.*, 1979).

The CL of early pregnancy has been shown to be more resistant to the luteolytic effects of $PGF_{2\alpha}$ than CL from non-pregnant cycles in the sheep between days 10 and 13 after oestrus (Silvia and Niswender, 1984). However, this effect is lost between days 16 and 26 after oestrus (Silvia and Niswender, 1986). One explanation for this development of resistance to $PGF_{2\alpha}$ is the production of a protein by the developing embryo which is known as bovine trophoblast protein-1 (bTP-1). bTP-1 and ovine TP-1 (o-TP-1) are members of the alpha-interferon family (Imakawa *et al.*, 1987; Chard, 1991). More recently they have been identified as a unique subclass of interferons now named as interferon-tau (IFN-τ) and associated specifically with maintenance of pregnancy (Roberts *et al.*, 1992). Expression of mRNA for both ovine and bovine IFN-τ occurs when the conceptus elongates and increased expression takes place before the time of luteolysis (Roberts *et al.*, 1992).

Various studies have investigated the effects of bTP-1 *in vivo* and *in vitro*, using either the actual protein or recombinant interferons (see Thatcher *et al.*, 1995 for a review). bTP-1 extends the lifespan of the CL when infused into the uterus of normally cycling cows (Knickerbocker *et al.*, 1986; Helmer *et al.*, 1989b). Progesterone secretion by granulosa and luteal cells in culture is increased by low concentrations of recombinant bovine interferon-α (Luck *et al.*, 1992). Injection of recombinant bovine interferon-$\alpha_I 1$ (rBoIFN-α) or recombinant IFN-τ prolongs CL lifespan and increases the inter-oestrous interval (Plante *et al.*, 1989; Meyer *et al.*, 1995). In the sheep intrauterine recombinant IFN-τ also prolongs CL lifespan (L'Haridon *et al.*, 1995). The results of these and many other studies demonstrate a clear anti-luteolytic effect for IFN-τ *in vivo* although the exact mechanisms through which this happens are still being investigated.

IFN-τ is thought to act through inhibition of prostaglandin synthesis at the level of the endometrium (Helmer *et al.*, 1989b; Thatcher *et al.*, 1989). Treatment of cows with IFN-τ resulted in reduced secretion of uterine $PGF_{2\alpha}$ (Meyer *et al.*, 1995). This effect on prostaglandin synthesis appears to be restricted to $PGF_{2\alpha}$ as synthesis of PGE, which is luteotrophic in the cow, is increased (Helmer *et al.*, 1989a). This may

be due to a direct effect on epithelial cells which are the major source of $PGF_{2\alpha}$ rather than stromal cells which produce mainly PGE (Bazer *et al.*, 1991). It has also been suggested that IFN-τ might act through the inhibition of development of endometrial oxytocin receptors (Bazer *et al.*, 1991). rBoIFN-α also reduces oxytocin-induced release of $PGF_{2\alpha}$ from the uterus *in vivo* (Plante *et al.*, 1991). Recent studies have demonstrated that, in the sheep, IFN-τ may exert an anti-luteolytic effect by blocking the action of $PGF_{2\alpha}$ on large luteal cells *in vitro* (Wiltbank *et al.*, 1992a).

Although the anti-luteolytic and luteotrophic effects of IFN-τ are well documented in the sheep and the cow some workers suggest that there may also be another substance(s) involved in luteal preservation during early pregnancy (Wiltbank *et al.*, 1992b). Granulocyte-macrophage colony-stimulating factor (GM-CSF), which is present in the endometrium, has been shown to increase the expression and secretion of IFN-τ from ovine conceptus tissues (Imakawa *et al.*, 1993). It is likely that various growth factors, colony stimulating factors and other cytokines have a role in the complicated process of CL maintenance for pregnancy.

1.4.7. Local $PGF_{2\alpha}$ production within the CL

The CL itself is also a potent source of prostaglandins, including $PGF_{2\alpha}$, which could be involved in cell-cell communication within luteal tissue (Rodgers *et al.*, 1988, Rodgers, 1990). Luteal $PGF_{2\alpha}$ does not appear to play a significant role around luteolysis in the cow as secretion is greater in cells from CL collected early in the oestrous cycle compared to mid- and late-stage CL (Milvae and Hansel, 1983; Rodgers *et al.*, 1988). However, some studies have shown that the cytokine products of immune cells can stimulate production of $PGF_{2\alpha}$ by luteal cells in culture (Nothnick and Pate, 1990; Fairchild Benyo and Pate, 1992) and it is possible that this may be significant in events occurring at the cellular level around luteolysis. This topic is discussed in detail in Section 1.6. There is evidence of a luteotrophic role for luteal-derived PGI_2 (Milvae, 1986).

1.4.8. Structural luteolysis

Following functional luteolysis and the decline in progesterone production, the luteal tissue itself is destroyed. Within a few days of functional luteolysis the CL has reduced in size considerably, being present as a corpus albicans on the ovary. Before the end of the next oestrous cycle, the regressing CL will have become almost invisible. Such rapid regression of a relatively large structure involves dramatic ultrastructural and morphological changes (Lobel and Levey, 1968; Gemmell *et al.*, 1976).

One of the earliest ultrastructural changes to occur is alteration to the cell membranes which leads to increased membrane permeability and loss of cellular function (Carlson *et al.*, 1982). There is some evidence that superoxide radicals which are by-products of metabolic activity and have various roles in reproduction (see Riley and Behrman, 1991, for a review), may be associated with the membrane changes that take place at luteolysis (Sawada and Carlson, 1991). At the structural level, changes in blood vessels are the first visible alteration, with loss of endothelial cell wall structure and degenerating cellular fragments in blood vessels (Lobel and Levey, 1968; O'Shea *et al.*, 1977; Archbald *et al.*, 1981). Luteal cell changes then become obvious, lipid accumulates in the cytoplasm and the cells become vacuolated and shrunken, gradually losing their distinctive appearance. At this stage macrophages, involved in the destruction and phagocytosis of dead and dying luteal cells, become the predominant cell type (Lobel and Levey, 1968; Gemmell *et al.*, 1976). Increased numbers of lipid droplets in the cytoplasm of the macrophages indicates active phagocytic activity (Katabuchi *et al.*, 1989).

The vascular changes associated with the destruction of the CL of pregnancy are similar to those seen in the CL from a non-pregnant cycle. However, the whole process is slower and in the CL of pregnancy, large luteal cells appeared intact within 24 hours of parturition whilst there were signs of cellular damage associated with the small luteal cells (Archbald *et al.*, 1981; O'Shea and Wright, 1985).

1.4.9. The Role of Apoptosis in luteal destruction

Apoptosis is a form of physiological cell death which appears to be genetically controlled and occurs as part of the cycle of cell growth and cell death throughout the body tissues including the ovary. The role of apoptosis in ovarian function has recently been reviewed (Tilly, 1996). Apoptosis occurs in the CL and, unlike the cellular events associated with structural regression which initiate an influx of macrophages and destruction of the luteal cell wall, apoptosis is a more subtle process of cell death. It involves shrinkage of the whole cell and fragmentation of DNA in the nucleus as well as formation of characteristic apoptotic bodies (pieces of membrane containing DNA fragments which then bud off from the cell: see Cohen, 1993, for a review). Macrophages are then involved in phagocytosis of the dead cells but without involvement of other leukocytes as would be expected in an inflammatory response.

The enzyme involved in cleaving of the DNA during apoptosis, a Ca^{2+}/Mg^{2+} - dependent endogenous endonuclease has been located in both granulosa and luteal cells in the rat (Zeleznik *et al.*, 1989). Apoptosis has been associated with follicular atresia of small follicles in the chicken (Tilly *et al.*, 1991), rat (Hughes and Gorospe, 1991) and sheep (Murdoch, 1995a) whilst luteal cell apoptosis in the mouse CL has recently been connected with expression of mRNA for the $PGF_{2\alpha}$ receptor (Hasumoto *et al.*, 1997). Changes to vascular endothelial cells during luteal regression were suggestive of apoptosis (O'Shea *et al.*, 1977), but later studies in the sheep have shown that apoptosis occurs not only in endothelial cells but also in the luteal cells and fibroblasts of the CL (Sawyer *et al.*, 1990).

DNA fragmentation has been described in CL collected after functional luteolysis from the rat (Matsuyama and Takahashi, 1993), rabbit (Dharmarajan *et al.*, 1994), and sheep (Rueda *et al.*, 1995; Murdoch, 1995b). Similarly, DNA fragmentation is seen in cells from the bovine CL after induced and natural luteolysis (Juengel *et al.*, 1993) but not before the onset of luteolysis (Zheng *et al.*, 1994). There is some

evidence that tissue metalloproteinase inhibitors produced within the CL may also be involved in the cellular changes associated with apoptosis at the time of luteal regression (Juengel *et al.*, 1994). Concentrations of TIMP-1 and TIMP-2 mRNA increased in luteal tissue after induced luteolysis (Juengel *et al.*, 1994) prior to oligonucleosome formation which is associated with apoptosis (Juengel *et al.*, 1993).

Apoptosis is controlled by a variety of factors which can either suppress or induce cell death. Some of these factors have been detected within ovarian tissue from a range of species (see Tilly, 1996 for a review article). A proto-oncogene *bcl*-2 is involved in suppression of apoptosis along with antioxidants such as glutathione peroxidase and superoxide dismutase. $PGF_{2\alpha}$ acts within the ovary to increase generation of reactive oxygen species (Sawada and Carlson, 1991). This process has been linked with the induction of apoptosis therefore these substances may have a role within luteal tissue in protecting cells from cell death.

Another member of the BCL-2 gene family, *bax*, induces cell death and there is some evidence that this bax gene may have a role in follicular degeneration. In recent years there has also been increasing interest in another group of proteases that may be involved in regulation, particularly induction, of apoptosis (Tilly, 1996). This includes interleukin-1β-converting enzyme (ICE), cysteine protease P32 (CPP32) and ICE-and-*ced*-3-homologue 1(ICH-1).

Expression of ICE, CPP32 and ICH-1 has been reported in the rat ovary. In addition there is some evidence that expression of CPP32 and ICH-1 is gonadotrophin dependent although this is not the case with ICE (Flaws *et al.*, 1995). There are no reports of these substances within the cow ovary and further studies will be required, both *in vitro* and *in vivo*, to assess their role in normal reproductive physiology.

Results from all species studied indicate that at any specific time point the number of cells demonstrating signs of apoptosis within the CL is limited. This does not mean

that apoptosis can be assumed to be unimportant in luteal regression as the process takes place extremely rapidly. However more detailed studies will be necessary to look at the significance of apoptosis *in vivo* as well as which cell types are involved and the timing of events (Hansel and Blair, 1996). Studies using luteal cell cultures *in vitro* may be of limited use because the techniques involved, such as collagenase dissociation, can themselves induce apoptosis (Juengel *et al.*, 1993).

1.5. The Immune System and the Ovary

The CL is made up of a large number of non-luteal cells (section 1.3.5.). These include cells associated with the immune system such as lymphocytes, macrophages and lymphocytes. An overlap in function between ovarian function and the immune system has been recognised for some time (Bukovsky and Presl, 1979). In particular, there has been increasing interest in the role of specific immune cell types in affecting luteal function as well as other reproductive processes (see Adashi, 1990; Pate, 1995, for review articles).

In this review the established roles of immune cells will be summarised to give some background to this project and the main processes in which these cells are involved (namely inflammation and the immune response, concentrating on cell-mediated immunity) will also be summarised. Immune cell populations and their cytokine products in the ovary will be discussed.

1.5.1. A brief overview of the cells of the immune system.

Immune cells (white blood cells or leukocytes) are vital in the body for the recognition and destruction of potentially harmful foreign antigens. These cells act

both non-specifically in the process of inflammation, and specifically in the processes of humoral and cell-mediated immunity (Kuby, 1994). A variety of immune cell types, and the surface protein molecules they bear (known as major histocompatibility complexes: MHC), are involved in all these processes. They have distinct roles, although there is a significant degree of interaction and overlap in function between them.

1.5.1.1. Lymphocytes

The lymphocyte is central to both the cell-mediated and humoral immune response. Lymphocytes make up 20-40% of white blood cells in the body and possess the attributes of diversity, specificity, memory and self/ non-self recognition required for the operation of successful immuno-defense mechanisms (Kuby, 1994). All other cells involved, although vital, play a more accessory role in terms of the immune response. Lymphocytes can be divided into 3 groups; (1) B-lymphocytes which are derived from the bone marrow and are involved in the antibody response to antigen within the body (humoral response), (2) T-lymphocytes which are involved in the cell-mediated response to antigen and which can be divided into several sub-groups, and (3) null cells which do not express the membrane molecules distinguishing T- and B- lymphocytes and which include the cell population known as natural killer cells which are thought to be involved in host defence against tumour cells.

T-lymphocytes are the type of lymphocyte with the greatest functional potential in the ovary. This is mainly due to their capacity to release a variety of cytokine products which can have effects on cells outwith the immune system. There are two subpopulations of T-lymphocytes: T-helper (T_h) cells and T-cytotoxic (T_c) cells which can be distinguished by their membrane glycoproteins. Both have different roles in the cell-mediated immune response, as discussed in Section 1.5.3. However this division of T-cells into two subsets with different functions is not absolute and there is a degree of overlap between them.

1.5.1.2. Monocytes and Macrophages - the mononuclear phagocytic system.

Monocytes, derived from the bone marrow, circulate within the blood stream and migrate into body tissues where they differentiate into macrophages. These have a phagocytic role in the body tissues. Some macrophages are fixed in specific areas, whereas others (known as 'wandering' macrophages) migrate within the body. Macrophages are involved in phagocytosis of exogenous antigens and there is a significant degree of interaction between macrophages and other leukocytes, particularly T-lymphocytes. The phagocytic response is greatly enhanced by stimulation of the cells with interferon-γ (IFN-γ), a T-helper cell-derived cytokine. Macrophages can themselves be a potent source of cytokines, in particular tumour necrosis factor-α (TNF-α).

1.5.1.3. Neutrophils

Neutrophils (polymorphonuclear leukocytes) are involved in the process of inflammation and make up 50-70% of circulating white blood cells. They are attracted to sites of infection and tissue damage by various chemotactic factors, including the cytokine products of activated T-cells. Increased numbers of neutrophils are released from the bone marrow in response to infection. Neutrophils, like macrophages, are phagocytic cells which contain lytic enzymes and bactericidal substances in granules within the cytoplasm.

1.5.1.4. Eosinophils/basophils

Eosinophils and basophils are included with neutrophils in the family of granulocytes because of the granular appearance of the cytoplasm, although they are much fewer in number. Eosinophils, although phagocytic, are not associated with inflammation but with defence against parasite infestations in the body. Degranulation of eosinophils is seen in response to histamine and so eosinophils also have a role in allergic responses. Basophils are non-phagocytic and, in conjunction with mast cells within body tissues, are also involved in the development of allergic responses. They

release histamine and other vasoactive substances from intracytoplasmic granules during allergic responses.

1.5.1.5. Major histocompatability complexes (MHC)

The MHC is a surface glycoprotein molecule which is important in enabling the immune system to recognise self from non-self. Class I MHC molecules are found on all nucleated cells whereas class II molecules are generally associated with cells that function as antigen presenting cells such as macrophages. In the immune response T-helper cells respond to antigen in combination with MHC class II molecules (MHC II) by releasing cytokines, whereas T-cytotoxic cells are activated and exhibit cytotoxic activity only on recognition of antigen in combination with MHC class I molecules (MHC I). MHC II expression is classically found only on cells of the immune system. However, recently it has been hypothesised that non-immune cells may also express MHC II, allowing these cells also to present antigen and enhance the immune response (Bottazzo *et al.*, 1986). Expression of MHC II by non-immune cells in the CL during luteolysis could be significant (Fairchild Benyo *et al.*, 1991).

1.5.1.6. Clusters of differentiation

Different sub-groups of T-cells and other immune cells express different membrane molecules which can be recognised by specific monoclonal antibodies. The monoclonal antibodies that reacted with a particular membrane molecule were first named by the individual researcher concerned. However, this led to much confusion and multiple monoclonal antibodies directed against the same membrane molecules. In order to clarify the situation, the antibodies which recognise a particular membrane molecule are grouped together as a cluster of differentiation (CD) for ease of identification. Different subsets of lymphocytes therefore are recognised by different CD numbers For example T helper cells are CD4 positive (CD4+) and T cytotoxic cells are CD8 positive (CD8+).

1.5.2. The Inflammatory Response

The inflammatory response comprises three main events; vasodilation, increased capillary permeability and influx of phagocytic cells (Roitt, 1991). These events are initiated by infection or tissue injury but the exact processes occurring at the cellular level have not been fully established. Complex interactions between chemical mediators released from either invading micro-organisms, damaged cells, various enzyme systems and also leukocytes are involved. Chemical mediators, particularly histamine released by mast cells and basophils in response to tissue injury, lead to vasoconstriction. This causes the tissue capillaries to become engorged and, in combination with increased vascular permeability, forces fluid and cells into the tissues. In particular, cells of the immune system, including neutrophils, macrophages, eosinophils, basophils and mast cells, move out of the capillaries and migrate by a process of chemotaxis to the site of inflammation. Once present in the site of inflammation activated macrophages and lymphocytes then produce cytokines such as TNF-α, Il-1 and interleukin-6 (Il-6) which are also involved in controlling the inflammatory response.

1.5.3. The Immune Response - Cell-mediated immunity

Cell-mediated immunity is a form of specific or acquired immunity where the immune system can recognise and eliminate foreign molecules. Unlike the inflammatory response, cell-mediated immunity, along with humoral (antibody-based) immunity, is specific, has memory and the ability to diversify and differentiate self from non-self (Roitt, 1991, Kuby, 1994). Although humoral immunity is involved mainly in elimination of bacteria, it sometimes plays a role in cell-mediated immunity. However, its role is secondary as B-cells do not appear to be localised in significant numbers in the ovary. This review will concentrate on cell-mediated immunity.

The cell-mediated immune response is dependent on recognition of antigen by T-cells which initiates a chain of responses involving various effector immune cells. Briefly, T_h cells are activated in response to antigen presented in combination with

MHC II molecules on specific antigen-presenting cells (APCs) such as macrophages. However in addition to this, activation of T_h cells is also dependent on a second signal which induces RNA and protein synthesis by the T_h cells. This is thought to be interleukin-1 (Il-1) released from the antigen-presenting cell.

After activation the T_h cells secrete cytokines, particularly interleukin 2 (Il-2) which stimulates proliferation of T_h cells as well as activation of T_c cells. Release of Il-2 results in expression of Il-2 receptors on T_c cells. These receptors are not present on resting cells. The induction of Il-2 receptors occurs in response to activation of T_h cells by antigen and IL-1 as described above. For this reason the Il-2 receptor may be used as an indicator of activation of T-lymphocytes.

The T_c cells, activated by Il-2, are involved in the cytotoxic response through membrane damage and cell lysis of cells which are presented in combination with MHC I molecules. In addition to activating the antigen-specific T_c cells, various cytokines activate non-specific cytotoxic cells such as macrophages (enhancing their phagocytic activity) as well as natural killer cells. Activated T_c cells produce cytokines which may be involved in activation of other cell types, but to a lesser degree than T_h cells.

This is obviously an oversimplification of a complicated process which is not yet fully understood. New information is continually being produced as investigative techniques advance. However, even a basic understanding of the cellular events which occur during inflammation and cell-mediated immunity have led workers to consider a role for these processes in reproduction.

1.5.4. Immune cells and the ovary

The huge range of functions and interactions of immune cells within the inflammatory and immune responses has become increasingly evident as research has advanced. In particular, increasing information about the dramatic effects of various cytokine products of the immune cells on surrounding cells has prompted researchers

to consider a role for these cells outwith the immune system. The ovary, undergoing a continual cycle of hormonal and structural changes throughout reproductive life, is a potential site of action for these cells (Norman and Brannstrom, 1994).

1.5.5. Immune cells and the follicle

A specific role for the immune system within the follicle has been indicated for some time. The cellular events seen during ovulation are very similar to those seen during the inflammatory process (Section 1.5.2.). Therefore ovulation in the rat has been considered to be a form of inflammatory reaction (Parr, 1974; Espey, 1980; Espey, 1994). Morphological studies in the sheep ovary have also revealed that vasodilation, increased vascular permeability, tissue oedema as well as accumulation of neutrophils and eosinophils are all features of the ovulatory process (Cavender and Murdoch, 1988).

A number of earlier studies provided indirect evidence of a role for immune cells in the follicle, particularly around ovulation. High dose anti-inflammatory drugs, such as aspirin, suppressed ovulation in the rat (Parr, 1974). Ovulation was also delayed in mature rats treated with anti-thymocyte serum to suppress maturation of T-lymphocytes (Bukovsky *et al.*, 1977) whereas leukocyte supplementation of perfused rat ovaries *in vitro* caused an increase in the ovulation rate (Hellberg *et al.*, 1991). However, a later study failed to show any effect on ovulation in rats whose leukocyte populations had been severely depleted (Chun *et al.*, 1993). In the sheep another anti-inflammatory drug, indomethacin, delayed ovulation. This did not appear to be caused by inhibition of prostaglandin synthesis in the follicle but was related to suppression of a specific leukocyte chemoattractant agent involved in attraction of various types of immune cell to the follicle around ovulation (Murdoch and McCormick, 1989, 1991, 1993).

Other studies have shown direct effects of immune cells on granulosa or thecal cells *in vitro*. Peritoneal macrophages increased progesterone secretion by murine granulosa cells *in vitro* (Kirsch *et al.*, 1981). Conversely, gonadotrophin-induced

progesterone production was markedly inhibited by macrophages, particularly activated macrophages, in cultures of rat granulosa cells, although macrophages did appear to increase the number of viable granulosa cells present in culture (Shakil and Whitehead, 1994). T- and B- lymphocytes stimulated progesterone production by granulosa cells from the rat (Hughes *et al.*, 1991) and human (Emi *et al.*, 1991) whereas in the pig, granulosa cell progesterone production was stimulated by lymphocytes only in the presence of monocytes (Maier *et al.*, 1990).

A variety of immune cell types, particularly macrophages, have been recorded in the follicle from a range of species. In the human, macrophages were found associated with the perifollicular capillaries and increased in number in the follicle after ovulation (Katabuchi *et al.*, 1989). In the mouse there was a rapid entry of macrophages into the follicle at ovulation (Simon *et al.*, 1994) and macrophages have also been associated with the transformation of preantral to antral follicles in the human ovary (Bukovsky *et al.*, 1995b). After ovulation the number of macrophages in the collapsed pig follicle increased almost fourfold (Standaert *et al.*, 1991).

Lymphocyte numbers were insignificant in follicles from women and mice. However, they were present in significant numbers in pig follicles and increased dramatically in number after ovulation (Standaert *et al.*, 1991). Detailed studies in the rat (Brannstrom *et al.*, 1993a) and women (Brannstrom *et al.*, 1994c) showed that neutrophils, as well as macrophages, were the most significant cell types present in the medullary and thecal areas of the preovulatory follicle and increased in number at ovulation. The increase in numbers of neutrophils after ovulation may be related to hormonal events at this time because inducing ovulation with hCG in the rat results in increased numbers of neutrophils in the rat ovary (Chun *et al.*, 1993).

Eosinophils are present in the sheep ovulatory follicle (Cavender and Murdoch, 1988). However, these cells appear to be of limited importance, as artificially reducing the number of eosinophils using prednisilone did not affect ovulation (Murdoch and Steadman, 1991). Eosinophils were also the most prominent blood cell

type present in the preovulatory follicle within the pig ovary (Standaert *et al.*, 1991). In the cow an increased number of mast cells was observed in the growing dominant follicle from day 19 onwards (Nakamura *et al.*, 1987) and increased numbers of mast cells were seen in the rat ovarian bursa at pro-oestrus (Gaytan *et al.*, 1991). The exact significance of these cell types is not clear but mast cell products, including histamine and heparin, are associated with inflammation and so they may be involved in events around ovulation (see Krishna *et al.*, 1989 for a review). In the hamster, degranulation of mast cells in the ovary was associated with the LH surge prior to ovulation (Krishna and Terranova, 1985): however, antihistamine treatment did not inhibit ovulation in the sheep (Halterman and Murdoch, 1986).

1.5.6. Immune cells and the corpus luteum

The role, if any, of immune cells in the CL, apart from their involvement in structural luteolysis, is not clear. Suppression of lymphocyte production in the cow using anti-lymphocyte serum resulted in a reduction in both the concentration of circulating progesterone and LH in treated cows (Alila and Hansel, 1984). Suppression of macrophages and T-lymphocytes in the rabbit using high dose prednisilone during the luteal phase did not alter progesterone profiles and luteolysis proceeded as normal (Seiner *et al.*, 1992) whereas in the rat immunosuppression with dexamethasone prevented luteal regression (Wang *et al.*, 1993). Macrophages increased progesterone secretion by mouse luteal cells *in vitro* (Kirsch *et al.*, 1981) and activated neutrophils inhibited LH-stimulated progesterone production in cultured rat luteal cells (Pepperell *et al.*, 1992). These results demonstrate that a specific, non-phagocytic role for the immune cell in the CL is possible but that this varies between species.

One type of immune cell has an established role in the CL. The macrophage is involved in the destruction of luteal tissue after luteolysis (Lobel and Levey, 1968; Paavola 1979; Bagavandoss *et al.*, 1991). Macrophages are relatively easy to identify in tissue on the basis of their cell morphology and were recorded in CL prior to the development of specific monoclonal antibodies which could be used for immunohistochemistry. Macrophages were described in guinea pig CL and their

presence in large numbers in regressing luteal tissue suggested that they might be involved in luteal destruction (Paavola 1979). In the cow, macrophages were also described in regressing luteal tissue (Lobel and Levey, 1968) and increased numbers of macrophages were found in regressing rabbit CL in comparison to young CL (Bagavandoss *et al.*, 1988; 1990).

Roby and Terranova (1989) used immunohistochemistry to look at bovine and rat ovaries and found macrophages to be present in the CL of both species. In the cow, they were found in thecal cords which extend from the capsule into the centre of the CL whereas in the rat, macrophages were localised in the centre of the CL. In the pig, macrophages were the most prominent leukocyte in the CL and greatest numbers of macrophages were recorded in the early developing CL and during luteal regression (Standaert *et al.*, 1991; Hehnke-Vagnoni *et al.*, 1995). Macrophages infiltrated the pig CL in response to implantation of luteal tissue with a $PGF_{2\alpha}$-impregnated implant (Hehnke *et al.*, 1994).

Macrophages are also present in CL after ovulation. Kirsch *et al.* (1981) identified macrophages in mouse CL after induction of ovulation using gonadotrophins. In the human CL, the presence of macrophages in the early CL suggested a role for these cells in the proliferation and luteinisation of granulosa cells as well as in influencing progesterone production (Katabuchi *et al.*, 1989). A more recent study in the human CL found macrophages to be the most significant immune cell type at all stages of the luteal lifespan (Nouza *et al.*, 1995).

Although macrophages have been described in the greatest detail in the CL, there are other immune cell types present which, in addition to any other effect, are likely to be involved in attraction and activation of macrophages after luteolysis. Lymphocytes were first described in CL from day 14 of the oestrous cycle in the cow (Lobel and Levey, 1968). Bagavandoss *et al.* (1990) found T-lymphocytes to be present in similar numbers at different stages of pseudopregnancy and pregnancy in the rabbit including after luteal regression. In the rat, T-lymphocytes were consistently sparse in

the CL throughout pregnancy and pseudopregnancy (Brannstrom *et al.*, 1994a) whereas in the pig the highest numbers of T-lymphocytes were found in the corpus haemorrhagicum, immediately after ovulation (Standaert *et al.*, 1991).

The situation in the human CL is not clear as different studies have yielded conflicting results. Using an image analysing system, few T-lymphocytes (CD3+, CD4+ or CD8+) were recorded at any stage of CL lifespan (Petrovska *et al.*, 1992), whereas Wang *et al.* (1992c) recorded CD4+ and CD8+ T-lymphocytes in the human CL close to blood vessels. Bukovsky *et al.* (1992) did not find any T-lymphocytes in developing and mature human CL initially but in a later study they did observe T-lymphocytes (CD3+, CD4+ or CD8+) between degenerating granulosa luteal cells in regressing CL. There was also some evidence of invasion of CD4+ cells into older CL after degenerative changes were already present (Bukovsky *et al.*, 1995a). Brannstrom *et al.* (1994c) found no variation in populations of CD8+ or CD3+ T-lymphocytes in the human CL throughout the menstrual cycle. However, the ratio of CD8+:CD4+ T-lymphocytes was higher than that seen in the blood, suggesting that there might be a preferential attraction of the CD8+ cells into the CL.

B-lymphocytes do not appear to be an important component of the CL leukocyte population in any species studied. Wang *et al.* (1992c) found no B-cells in human CL at any stage of the menstrual cycle. Many other studies have not investigated B lymphocytes at all, presumably because these cells are involved in antibody production and are considered unlikely to affect luteal cell function.

Neutrophils have been described in luteal tissue. In the pig CL, neutrophils were present in similar numbers throughout the oestrous cycle (Standaert *et al.*, 1991). One study in women did not find any neutrophils in CL at any stage (Bukovsky *et al.*, 1992), although a later study did describe significant numbers of neutrophils in human CL. Highest numbers of neutrophils were present after ovulation, but were present in constant numbers for the rest of the CL lifespan, with no increase in regressing CL (Brannstrom *et al.*, 1994c). In the rat, neutrophils were present in large

numbers in CL of pregnancy and pseudopregnancy although their numbers declined as the CL aged (Brannstrom *et al.*, 1994a). Neutrophils are phagocytic cells and may be involved in structural luteolysis. However, luteal regression does not appear to be associated with an increase in neutrophil numbers, and macrophages seem to play a far more important role in destroying luteal tissue after luteolysis.

Eosinophils have been described in greatest detail in the CL of the sheep and the pig. As their distinctive granular cytoplasm makes identification of these cell types relatively easy, the limited detail in the literature suggests that these cells are not present in large numbers in mature and regressing CL from other species. Eosinophils were observed in regressing sheep CL after luteolysis was induced with $PGF_{2\alpha}$, with highest numbers present between 2-4 hours after $PGF_{2\alpha}$ injection (Nett *et al.*, 1976; Murdoch, 1987). In the pig, eosinophils were the most prominent cell type in the naturally regressing CL and were also present in high numbers in the corpus haemorrhagicum (Standaert *et al.*, 1991). Eosinophils were found in very low numbers in rat CL at all stages (Brannstrom *et al.*, 1994a).

It can be seen from these studies that immune cell populations in CL from different species vary considerably. There are also variations between studies in the same species which are difficult to explain but are likely to reflect different techniques of tissue collection, preparation and cell counting. It is likely that the most recent studies using specific monoclonal antibodies and morphometric analysis systems are most representative of the situation *in vivo*, but a considerable volume of work is still required, particularly to investigate cell populations at different stages of the CL lifespan.

1.5.7. MHC expression in the CL

MHC class I is expressed by all cell types and therefore is found on cells of the CL. However, MHC II is expressed by a more limited number of cell types and increased expression of MHC II enhances the immune response (Section 1.5.1.5.). It is possible

therefore that altered MHC II expression in the CL could be involved in the immune processes which occur around the time of luteal regression.

MHC II molecules were present on human and rhesus monkey granulosa-lutein cells, but not on theca-lutein cells or large antral follicles (Khoury and Marshall, 1990). The same study showed no MHC II expression in any cell type in the rat CL. Another study, which looked in detail at MHC II expression in the human CL at different stages of the oestrous cycle (Petrovska *et al.*, 1992), found that MHC II expression was associated with both granulosa-lutein and thecal-lutein cells and was highest during the time of peak endocrine activity (days 7-9) and fell during luteal regression. However, Bukovsky *et al.* (1995a) found that both MHC I and MHC II expression in human granulosa-lutein cells was enhanced in late-luteal and regressing CL. It is not clear why the results from these two studies conflict, but may be due to the different techniques employed.

In the rabbit, MHC II expression associated with activated T-lymphocytes did not vary throughout the CL lifespan or after luteal regression. MHC II expression on other cell types was not investigated (Bagavandoss *et al.*, 1990). However, MHC II expression increased in the rat CL during the later stages of both pseudopregnancy and pregnancy (Brannstrom *et al.*, 1994a). The cells expressing MHC II appeared to be macrophages but this was not confirmed.

Some of the most detailed studies on MHC II expression have been performed using the cow CL. Expression of both MHC I and MHC II on bovine luteal cells was first recorded in a study looking at the effects of the cytokine interferon-gamma (IFN-γ) on MHC expression (Section 1.6.3; Fairchild and Pate, 1989). A later study by the same group looked at MHC expression in the CL during the oestrous cycle and after induced luteolysis. MHC I expression did not vary significantly between any of the groups studied, whereas MHC II expression increased with age of CL and after induced luteolysis (Fairchild Benyo *et al.*, 1991). In mid-cycle CL (days 10-12) greatest MHC II expression was associated with a subgroup of large cells described

as "large less-dense cells" and expression in this cell population increased by day 18, along with increased expression in the small cell population. In the sheep, MHC II expression was present on both large and small cell populations on days 10 and 14 of the oestrous cycle and day 14 of pregnancy, although there was reduced MHC II expression on large cells from CL collected during pregnancy (Kenny *et al.*, 1991).

Results from both the cow and the sheep demonstrate that MHC II expression occurs on luteal cells *in vivo*. This in turn suggests that altered MHC II expression may be involved in the immune mechanisms which occur around luteolysis. However, these ruminant studies are based on collagenase dispersion of luteal tissue followed by analysis of MHC II expression on dispersed cells by flow cytometry. It is possible that the process of tissue preparation could alter MHC II expression and identifying the specific cell types that are expressing MHC II by flow cytometry presents some difficulties. For these reasons, further analysis of intact luteal tissue is needed to confirm the results from flow cytometric analysis and to identify more accurately the cell types involved.

1.6. Cytokines and the ovary

One of the main reasons for the increased interest in immune cells within the CL is the volume of information which is becoming available about the cytokine products of these cells. Cytokines were originally associated with regulation of the amplitude and duration of immune-inflammatory responses and were named according to the presumed cell source (for example, lymphokines from lymphocytes and monokines from monocytes). However, in recent years it has become apparent that other cell types, including epithelial and endothelial cells as well as cells of the reproductive tract, also have the potential to produce cytokines and that the term cytokine now includes a much wider range of cell products than originally described (Blackwill and Burke, 1989).

The wide range of effects and increasing overlap between the function of some cytokines and the role of hormones makes the definition of a cytokine somewhat

difficult (Rothwell, 1991). However the majority of cytokines are low molecular weight proteins which act as intercellular messengers between the same or different cell systems, although a few may have more widespread effects within the body. They act locally in an autocrine or paracrine fashion, exerting their effects by combining with high affinity cell surface receptors to alter RNA and protein synthesis. They are highly potent, acting at minute concentrations and are released from cells for very short periods of time. The transient nature of their release and the huge number of possible interactions that may occur between different cytokines and cytokine-secreting cells makes it difficult to define specific roles for individual cytokines. In any case, it is likely that the effects of individual cytokines are of limited significance *in vivo*, and that any response seen will be the result of a vast network of cytokines acting in combination (Tabibzadeh, 1994). However, in order to limit this complexity, the majority of investigations have involved detailed studies of individual cytokines, or at most two or three cytokines in combination.

The cytokine family is large and constantly increasing in number. It includes the interleukins, tumour-necrosis factors, colony-stimulating factors, interferons, growth factors, activin, inhibin and chemotactic factors. Recently there has been particular interest in the role of various growth factors on CL function (Lobb and Dorrington, 1993; Liebermann *et al.*, 1996) and the insulin-like growth factors have been studied in some detail (Adashi, 1992b; Amselgruber *et al.*, 1994; Spicer and Echternkamp, 1995). Such a wide range of factors obviously has an enormous potential range of effects which is outwith the scope of this review. However, in summary, these cell products act as a communication system within a cell network, acting to connect and influence various body systems, including the reproductive system (Kennedy and Jones, 1991).

1.6.1. Cytokines and their effects within the reproductive system.

There is now a large amount of literature available describing the effects of cytokines at all levels of the female reproductive tract, including the hypothalamus and pituitary gland (Tabibzadeh, 1994; Spangelo and Gorospe, 1995), uterus (Tabibzadeh and

Sun, 1992; Philippeaux and Piguet, 1993) and ovary (Adashi, 1992a; Vinatier *et al.*, 1995).

Within the ovary a range of cytokines has been studied. In particular, there has been much interest in the potential roles of tumour necrosis factor-α (TNF-α), interleukin-1β (Il-1β) and interferon-γ (IFN-γ) on ovarian follicular and luteal development (Kokia and Adashi, 1993; Terranova *et al.*, 1993; Pate, 1996). These three cytokines are of particular interest for a variety of reasons and, because they have already been studied in detail in the field of immunology, assessment of their roles in the ovary is somewhat easier.

1.6.2. The effect of cytokines on follicular function and ovulation.

As discussed earlier, there are populations of immune cells within ovarian structures and tissues throughout the oestrous cycle, all with the potential to affect surrounding cells (Section 1.5.). Within developing and ovulatory follicles, lymphocytes, eosinophils, mast cells and particularly macrophages have been recorded, although there is some inter-species variation. More recent research has focused on the cytokine products of these cells and their specific effects on follicular cells *in vitro* and *in vivo* using a variety of approaches. Several possible mechanisms of action for these cytokines have been recorded.

1.6.2.1. Effects of cytokines on follicular cells *in vitro*

Effects on steroid synthesis have been observed in some species. Gorospe and Kasson (1988) observed an increase in basal and FSH-induced progesterone production by rat granulosa cells cultured with lymphocyte supernates. Production of oestrogen and progesterone by pig granulosa cells in culture was altered by stimulated and unstimulated splenocytes, potent sources of various cytokines, although the effect varied between cells from follicles of different sizes (Hughes *et al.*, 1990). Other workers described more specific effects of individual cytokines. TNF-α increased production of progesterone and $PGF_{2\alpha}$ by human granulosa cells *in*

vitro (Zolti *et al.*, 1990). However in the pig, TNF-α inhibited insulin- and FSH-stimulated (but not basal) progesterone biosynthesis by granulosa cells and stimulated production of PGE_2 and $PGF_{2\alpha}$ (Veldhuis *et al.*, 1991).

In the preovulatory rat follicle both TNF-α and Il-1β stimulated progesterone secretion and production of $PGF_{2\alpha}$ and PGE *in vitro* (Brannstrom *et al.*, 1993c). This effect was seen in cultures of thecal cells alone: granulosa cells were unaffected, a finding that confirms a previous study showing that Il-1β was expressed only in the rat theca-interstitial cell *in vivo* (Hurwitz *et al.*, 1991b). However another study found that IL-1β stimulated release of various prostaglandins only from mixed cultures of rat thecal and granulosa cells, with no effect on the individual cell populations (Kokia *et al.*, 1992). Pig granulosa cells also responded to Il-1β with inhibition of basal and LH-stimulated progesterone production by porcine granulosa cells *in vitro*, an effect that was dependent on the dose of Il-1β. LH-induced luteinisation was inhibited (Fukuoka *et al.*, 1988, 1989). hCG-stimulated androgen production by rat thecal cells in culture was inhibited by both Il-1α and Il-1β, although Il-1β had a more dramatic effect (Hurwitz *et al.*, 1991a)

FSH-stimulated oestradiol production in the rat was inhibited by TNF-α (Adashi *et al.*, 1989). Similar findings were described when bovine granulosa cells were treated with a range of cytokines *in vitro*. All cytokines tested, including TNF-α, IL-1β and IFN-γ, had a potent inhibitory effect on FSH-induced oestradiol production from cells collected from small follicles, but had no effect on cells collected from large follicles (Spicer and Alpizar, 1994).

There is less information on the effects of interferons on follicular function. IFN-γ inhibited FSH-stimulated oestradiol and progestin secretion and LH-receptor formation in immature rat granulosa cells, whilst IFN-α had no effect (Gorospe *et al.*, 1988). Similar findings were described in the rat, where both recombinant IFN-γ and a mixture of rat IFN-α / IFN-γ inhibited FSH-induced inhibin and progesterone secretion from cultured granulosa cells (Xiao and Findlay, 1992).

Cytokines are not only capable of affecting steroid biosynthesis by cells, they can also directly affect the replication and development of follicular cells. In the pig, Il-1α has been shown to stimulate proliferation of granulosa cells in culture in addition to inhibiting progesterone secretion (Fukuoka *et al.*, 1989). Il-1β inhibited LH/hCG receptor induction by FSH in rat granulosa cells (Kasson and Gorospe, 1989), and Il-1α inhibited luteinisation of porcine granulosa cells in culture (Fukuoka *et al.*, 1988). Il-1β increased LH-induced ovulation in the rat ovary 3-fold (Brannstrom *et al.*, 1993d) and TNF-α induced ovulation in unstimulated, or more effectively in LH-stimulated ovaries (Brannstrom *et al.*, 1995). In the absence of gonadotrophin, Il-1β induced ovulation in the rabbit ovary (Takehara *et al.*, 1994). Further evidence of a specific role for Il-1β in ovulation was provided by a study which demonstrated that an Il-1 receptor antagonist inhibited ovulation in the perfused rat ovary (Peterson *et al.*, 1993).

Although leukocytes are relatively scarce within ovarian follicular structures (Section 1.5.5.), it appears that cytokines may well have a role within the developing follicles. It is difficult to generalise because of differences between studies as a result of different techniques and cell culture conditions as well as inter-species variation. However, it appears that their main role may be to inhibit differentiation of the developing theca and granulosa cells *in vivo* so allowing follicular growth (Fukumatsu *et al.*, 1992).

1.6.2.2. Cytokines in the follicle *in vivo*.

The above studies give some indication of the huge potential range of effects of cytokines *in vitro*. However, these do not necessarily indicate a role for these substances *in vivo*. As in the case of the immune cells themselves, if cytokines do play a significant role within the ovary, their expression may vary at different stages of follicular development. The minute concentrations involved and the transient nature of their production makes detection of cytokines difficult in tissue. However, some workers have demonstrated the presence of these substances within follicular structures. In general, the focus of interest has been IL-1β and TNF-α because of

their role in inflammation, a process which has many similarities to ovulation (Espey, 1980).

Roby and Terranova (1989) used immunohistochemistry to study TNF-α in the rat ovary and found that the most intense staining was associated with the granulosa layer of atretic follicles and with granulosa cells surrounding the antral cavity in pre-ovulatory follicles. Cell blot analysis confirmed that granulosa cells contained and produced TNF-α. A later study found TNF-α mRNA in the rat ovary. The results of this study indicated that this mRNA did not come from white blood cells but that ovarian cells and/or macrophages were the source (Sancho-Tello *et al.*, 1992). In the human follicle, granulosa cells were also found to secrete TNF-α, and TNF-α immunoactivity has been found in human follicular fluid (Roby *et al.*, 1990; Wang *et al.*, 1992b). In the mouse, TNF-α mRNA and protein were observed only in oocytes of follicles after the formation of the second layer of germ cells and the most intense expression was associated with atresia (Chen *et al.*, 1993). A detailed study in the cow demonstrated that production of TNF-α varied between follicular structures. TNF-α bioactivity was significantly higher in the preovulatory follicle (Zolti *et al.*, 1990). TNF-α was present in detectable amounts in human follicular fluid collected from normal and polycystic ovaries (Jasper and Norman, 1995).

A recent study in the sheep showed that TNF-α was present in the thecal endothelial cells of the preovulatory follicle (Murdoch *et al.*, 1997). A role for TNF-α was suggested in follicular rupture as ovulation was inhibited by intrafollicular injection with TNF-α antiserum. In addition, TNF-α was shown to induce ovarian cell apoptosis *in vitro* (Murdoch *et al.*, 1997).

In the rat, IL-1β is expressed only by theca-interstitial cells. Expression appears to be gonadotrophin-dependent, with a 4-5 fold increase in Il-1β expression after exposure to hCG. This might suggest a role for this substance around the time of ovulation (Hurwitz *et al.*, 1991b). TNF-α, Il-1 and Il-6 bioactivity have been detected in the ovulating rat ovary (Brannstrom *et al.*, 1994b). In the mouse Il-1α and Il-1β were

found in the thecal layer at ovulation and were also present in granulosa cells after ovulation (Simon *et al.*, 1994). Human follicular fluid cells also contained a small amount of mRNA encoding Il-1β (Polan *et al.*, 1991).

1.6.3. Cytokines and the Corpus Luteum

The presence of immune cells within luteal tissue, as well as in follicular structures, has led workers to consider a possible role for cytokine products within the CL. Although the main luteotrophic and luteolytic factors have been established in most species, there is still little information concerning events occurring at the cellular level within the corpus luteum. Immune cell populations acting through their cytokine products have the potential to affect luteal function profoundly. As with follicular tissue, there is an increasing volume of published work relating to cytokines in the CL. In particular there is considerable interest in the role of TNF-α within the CL (Terranova, 1997).

1.6.3.1. The effect of cytokines on luteal cells *in vitro*

Various cytokines, alone or in combination, have been shown to have a range of effects on luteal cells in culture. Results vary, even within species, and this is likely to reflect the different techniques used.

In the rat, TNF-α inhibited hCG-supported progesterone production by luteinised granulosa cells (Adashi *et al.*, 1990). In contrast, progesterone production by human granulosa-lutein cells in culture was unaffected by TNF-α, although PGE_2 and $PGF_{2\alpha}$ accumulation increased (Wang *et al.*, 1992b). TNF-α has been shown to stimulate proliferation of human granulosa-lutein cells in culture (Wang *et al.*, 1992b).

In human luteal cell cultures, IFN-γ alone (but not IFN-α) inhibited basal and hCG-induced progesterone formation. This effect was increased when TNF-α and IFN-γ were added together to cell cultures (Wang *et al.*, 1992a). Slightly different results were described by Fukuoka *et al.* (1992) who observed inhibition of progesterone

production only by IFN-γ and not TNF-α. This study also demonstrated that IFN-γ, as well as TNF-α and Il-1α, inhibited FSH-stimulated oestradiol production by human luteinised granulosa cells in culture. Another study also found that Il-1β inhibited hCG-stimulated production of oestradiol but that Il-1β did not affect unstimulated production of progesterone or oestradiol (Barak *et al.*, 1992). No alteration in progesterone production was observed when primate luteal cells from mid- and late-stage CL were treated with IFN-γ, TNF-α or IL-1β. However, a significant increase in production of $PGF_{2\alpha}$ by luteal cells treated with IL-1β was observed (Young *et al.*, 1994).

There are several, sometimes contradictory, studies looking at cytokines and pig luteal cells. Pitzel *et al.* (1993) found that TNF-α inhibited basal and hCG-stimulated production of progesterone and oestradiol (which is luteotrophic in the pig), whereas Tekpetey *et al.* (1993) failed to show any effect of TNF-α on progesterone production in porcine large or small luteal cells in culture. In a study looking more specifically at the effects of TNF-α within the ovary around the time of luteolysis in the pig, Wuttke *et al.* (1995) showed that TNF-α inhibited progesterone production by inhibiting both luteal oestradiol production and preventing the stimulatory effect of $PGF_{2\alpha}$ on steroid secretion.

There has been a considerable amount of research in recent years looking at the effects of cytokines on bovine luteal cells in culture. Il-1β had no significant effect on progesterone production by mid-cycle luteal cells (days 9-12) *in vitro* but did cause a dose-dependent increase in prostaglandin ($PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$) release (Nothnick and Pate, 1990). A later study described a decrease in progesterone production when luteal cells were chronically exposed to IL-1β, an effect that did not appear to be mediated by prostaglandins (Townson and Pate, 1994).

IFN-γ caused an initial 50% decrease followed by a dramatic increase (400%) in production of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ by luteal cells (days 9-12) *in vitro*. LH-stimulated progesterone synthesis was also inhibited by treatment with IFN-γ, but

this did not appear to be related to the increase in prostaglandin production as the effect was not reversed by indomethacin, an inhibitor of prostaglandin synthesis (Fairchild and Pate, 1991).

Another study looking at the effects of TNF-α alone, or in combination with other cytokines, found that TNF-α caused a dose-dependent increase in both $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production by cultured bovine luteal cells but had no effect on basal progesterone production (Fairchild Benyo and Pate, 1992). In combination with either IL-1β or IFN-γ, both of which have been shown to stimulate luteal prostaglandin production, TNF-α had synergistic effects on $PGF_{2\alpha}$ production. This study also showed that IFN-γ significantly reduced luteal cell numbers in culture although TNF-α alone did not. However, these two cytokines in combination were extremely cytotoxic, with only 20% of cells maintained compared to controls (Fairchild Benyo and Pate, 1992). A more recent study looked at the effects of cytokines on luteal cells collected at different stages of the oestrous cycle. $PGF_{2\alpha}$ production was stimulated and progesterone production inhibited by TNF-α, IL-1β and IFN-γ alone or in combination, but there was no cytotoxic effect of TNF-α and IL-1β on early (day 6) luteal cells unlike other stages (Greene and Pate, 1995).

Cytokines may also exert their effects by inducing apoptosis in luteal cells. In cultured mouse luteal cells IFN-γ and TNF-α, alone and in combination, decreased the number of viable cells in culture (depending on dose and age of culture) and also increased the amount of fragmented DNA, associated with apoptosis, in luteal cells (Jo *et al.*, 1995).

1.6.3.2. Cytokine production in the CL

The ability of luteal cells to produce cytokines *in vitro* has been investigated as well as the presence of various cytokines in luteal tissue *in vivo*. In the CL there has been particular interest in the presence of TNF-α as this cytokine is produced by macrophages which are present in CL from most species, particularly after luteolysis.

Cells from regressing rabbit CL produced TNF-α *in vitro* in response to lipopolysaccharide whereas this effect was significantly reduced in young CL (Bagavandoss *et al.*, 1988). However no detectable TNF-α bioactivity was present in unstimulated luteal cells, suggesting a limited role for this substance *in vivo*. Although no attempt was made to localise the cell origin of TNF-α production, the presence of large numbers of macrophages in regressing CL led the authors to suggest that these cells were the most likely source (Bagavandoss *et al.*, 1988). A later study found similar results in CL from pregnancy and pseudopregnancy in rabbits where TNF-α was detected in low amounts by bioassay at all stages but was significantly increased in regressing CL (Bagavandoss *et al.*, 1990).

TNF-α was detected by immunohistochemistry in regressing luteal tissue from rat ovaries collected at pro-oestrus. TNF-α was localised in the centre of the CL, and was associated with cells that also stained positively with antibodies to macrophage markers (Roby and Terranova, 1989). Immunohistochemistry was also used to detect TNF-α in unstaged human CL. Activity was observed in both large granulosa-lutein cells and small paraluteal cells (Roby *et al.*, 1990).

There is also evidence of TNF-α production in ruminant CL. A study using immunohistochemistry found immunoreactive TNF-α in bovine CL although no attempt was made to stage the tissue or compare CL of different ages. The staining was most intense in the thecal cords which extend into the CL from the capsule, and smaller numbers of TNF-α-positive cells were seen between luteal cells. As in the rat and the rabbit, these positively stained cells had the morphological appearance of macrophages (Roby and Terranova, 1989). In sheep CL, collected following $PGF_{2\alpha}$-induced luteolysis, TNF-α bioactivity increased only after the decrease in progesterone had occurred but before there was evidence of luteal destruction (Ji *et al.*, 1991). Northern blots and *in situ* hybridisation were also used to look for changes in mRNA for TNF-α within luteal tissue, but none were seen during induced luteolysis, leading the authors to conclude that TNF-α arose from an extraovarian

source, such as leukocytes (Ji *et al.*, 1991). However, limited conclusions can be drawn from this study because artificial induction of luteolysis may not reflect events occurring during natural luteolysis.

Another study in the cow attempted to overcome this problem by using a microdialysis system placed within the ovary to collect samples during both natural and induced luteolysis (Shaw and Britt, 1995). They also found that any increase in TNF-α detected by radioimmunoassay (sensitivity 35pg/ml) occurred after a decline in progesterone in all animals that underwent luteolysis (natural or induced). Cows that did not undergo luteolysis by day 22 and 24 of the oestrous cycle showed no change in TNF-α concentrations. This study is the most detailed so far in regard to investigating events during natural luteolysis, and the collection of samples direct from the ovary is a major breakthrough, allowing more accurate assessment of intraovarian and particularly intraluteal events. However, as discussed by the authors, cytokines can have dramatic effects at such small concentrations that it is possible that this technique, using radioimmunoassay, is not sensitive enough to detect changes in TNF-α concentrations that could have significant effects in the CL.

TNF-α protein was detected by Western blot in all CL collected from pigs during the oestrous cycle (days 8 and 12-15) and during pregnancy (days 12-112). There was no obvious variation in immuno-intensity between groups (Hehnke-Vagnoni *et al.*, 1995). In the same study, immunohistochemistry showed that TNF-α was associated with blood vessels, connective tissue and the capsule of the CL. Further analysis revealed that many of the cells that stained positively for TNF-α also stained positively for an endothelial cell marker. This finding corresponds with the identification of TNF-α receptors on the small cell population (*i.e.* small luteal cells or endothelial cells) of the pig CL (Richards and Almond, 1994). In the pig, unlike other species described above, TNF-α was only found in functional CL and there was no positive staining for TNF-α in CL where progesterone concentrations had fallen after functional luteolysis. The authors indicate that this is probably related to the decrease in endothelial cells which occurs at the time of luteolysis (Hehnke-Vagnoni

et al., 1995). TNF-α has previously been shown to inhibit proliferation of endothelial cells in the rabbit CL (Bagavandoss and Wilkes, 1991). Significantly, in the pig CL, macrophages were not associated with TNF-α immunostaining at any stage (Hehnke-Vagnoni *et al.*, 1995).

1.6.4. Monocyte Chemoattractant Protein-1 (MCP-1) and the CL.

In recent years there has been interest in the role of MCP-1 in the CL. MCP-1 is a member of the intercrine β family of cytokines which are involved in inflammation and tissue repair (Oppenheim *et al.*, 1991; Leonard and Yoshimura, 1990). MCP-1 acts as a specific chemoattractant for monocytes when injected into the ears of rats (Zachariae *et al.*, 1990) and also acts as a chemoattractant for monocytes *in vitro* (Yoshimura *et al.*, 1989).

These specific chemoattractant effects of this cytokine led workers to consider a role for MCP-1 in the CL, particularly around the time of macrophage infiltration during structural regression. MCP-1 has been found in pig luteal cells (Hosang *et al.*, 1994a) and a recent study showed increased MCP-1 expression in late stage rat CL (Townson *et al.*, 1996). An increase in MCP-1 was also observed in rat CL after luteolysis induced using prolactin (Bowen *et al.*, 1996). It appears that MCP-1 may be the substance which induces the influx of macrophages into the CL (Pate, 1996).

MCP-1 is a member of the chemokine superfamily which includes a large number of cytokines involved in adhesion and chemotaxis of a variety of leukocytes (Taub, 1996). At present the majority of studies focus on the potential role of MCP-1 within the CL but other chemokines may also be significant. Macrophage inflammatory protein-1 (MIP-1α and MIP-1β), T-cell activation gene 3 (TCA-3) and regulated-upon-activation, normally T-cell expressed and secreted (RANTES) are all involved in monocyte chemoattraction and some of these substances are also involved in T-cell migration. The chemokine superfamily is still expanding and the specific role of many of these substances in inflammation *in vivo* has yet to be clearly defined. It is likely however that in future some of these chemokines will be found to have a role in the changing leukocyte populations within the CL.

1.7. Conclusion

In recent years there have been considerable advances in our understanding of how the immune system functions and, in particular, the potential actions of their cytokine products are being revealed. The exact role of the immune system in reproduction is unclear at present because of the vast number of potential interactions that may be involved. However, it is rapidly becoming clear that the reproductive system and immune system are not independent entities, as was once thought, but interact in the control of all reproductive processes. Future studies will involve establishing the exact roles of individual immune cell types and cytokines within ovarian structures, where possible. This is likely to be complicated by the number of cell and cytokine interactions involved. Only after establishing the roles of key cells and substances will it be possible to establish the order of events involved in the immunological control of reproductive function.

The basic physiological processes which occur during the reproductive cycle of the cow have been understood for many years. This has allowed the development of various hormone treatments which may be used to improve fertility rates and treat reproductive conditions in commercial herds. However, the success of such fertility programmes and treatments is limited. It appears that other factors, which have yet to be clearly defined, must be involved in controlling the oestrous cycle in the cow. There is a limited amount of published research investigating the potential role of the immune system in affecting reproduction in the cow. In particular, the role of the immune system in controlling luteal function *in vivo* has not been studied.

The aim of the research described in the following chapters was to investigate firstly, the populations of immune cells within the cow CL throughout the oestrous cycle and particularly around the time of luteolysis, in order to assess the potential role of these cells in CL function. Secondly, to investigate the immune cell populations within CL where luteolysis was artificially induced and artificially inhibited and finally, to look for further evidence of a role for the immune system within luteal tissue by studying cytokine production within CL at different stages of the oestrous cycle.

Chapter 2

Materials and Methods

The techniques which have been used repeatedly in the course of this work are described in this chapter. Any other techniques which were used in single experiments are described in the appropriate chapter. Detailed protocols for the molecular biology techniques can be found in Appendix 1.

2.1. Materials

The sources of the individual chemicals are detailed within the protocols. Frequently-used materials and chemicals including solvents, chemicals for buffers and plastic or glass ware were obtained from the Sigma Chemical Co., Poole, Dorset, UK or BDH Chemicals Ltd., Poole, Dorset, UK.

2.2. Methods

2.2.1. Blood sampling

Blood samples (7-10ml) were collected from either the coccygeal or jugular veins. Blood samples collected at slaughter were taken from the heart within 15 min of slaughter. All blood samples were collected into lithium heparin using either glass Vacutainer tubes (143U heparin/tube, Becton Dickinson, U.K.) or glass tubes (10ml) containing 200U heparin/tube (Sigma Chemical Co., Poole, Dorset, U.K.).

After collection, blood samples were mixed gently and then centrifuged (30 min, 1000 *g*, 4°C or room temperature). Plasma was then stored at -20°C prior to progesterone and/or PGFM assay.

2.2.2. Classification of CL

Where ovaries were collected from animals of unknown reproductive history, the CL were classified using the method described by Ireland *et al.* (1980). Luteal tissue was classified as Stage I (days 1-4 of the oestrous cycle), Stage II (days 5-10), Stage III (days 11-17) and Stage IV (days 18-20) according to a variety of characteristics which are summarised in Table 2.1. Any CL which did not fit clearly into any of the four stages were disregarded.

Table 2.1

Method for classification of CL from animals of unknown reproductive history[1].

Characteristics	Stage I (days 1-4)	Stage II (days 5-10)	Stage III (days 11-17)	Stage IV (days 18-20)
External appearance	red, recently ovulated, point of rupture not covered by epithelium	point of rupture covered by epithelium, apex of CL red or brown	tan or orange	light yellow to white
Internal appearance	red, occasionally filled with blood, cells loosely organised	red or brown at apex only, remainder of CL is orange.	orange	orange to yellow
Diameter	0.5-1.5cm	1.6-2.0cm	1.6-2.0cm	<1cm
Vasculature on the surface of CL	not visible	generally limited to periphery	same as in II, but it will cover apex of CL late in this stage.	not visible
Follicles >10mm diameter	absent	present	may be absent or present	present

[1] Taken from Ireland *et al.* (1980)

2.3. Hormone Assay techniques

2.3.1. LH Binding assay

2.3.1.1. Reagents

^{125}I-hLH (100Ci/g) was purchased from Keith Ferguson, Dept. of Chemical Pathology, Hammersmith Hospital, London, UK. Human chorionic gonadotrophin (hCG, Chorulon:1500 IU hCG/ampoule) was purchased from Intervet Laboratories, Cambridge, UK.

1) SET (homogenizing) buffer

   0.3M sucrose, 1mM EDTA, 10mM Tris-HCl, pH 7.4.

2) Assay buffer

   40mM Tris-HCl, pH 6.5

3) BSA

   5% BSA in 40mM Tris-HCl, pH 7.4

4) ^{125}I-hLH tracer

   Diluted in assay buffer to give 100,000cpm/100µl.

5) hCG for non-specific binding tubes

   Dilute 1 vial Chorulon (1500U/vial) in 3ml assay buffer to give a stock solution of 500IU hCG/ml.

6) Polyethylene glycol (PEG) [MW, 8,000]

   25% PEG in 40mM Tris-HCl, pH 7.4.

7) IgG

   0.5% IgG in 40mM Tris-HCl, pH 7.4

2.3.1.2. Preparation of luteal homogenate

Luteal cell pellets were homogenised on full power in a Polytron homogeniser (Kinematica, Lucerne, Switzerland) for 10 sec in ice-cold SET buffer (approx 10ml/g) and filtered through four layers of cheese cloth. Homogenates were then stored on ice if used immediately, or at -20°C for longer term storage. Portions of

frozen luteal tissue were weighed and homogenised in ice-cold SET buffer (10ml/g). The average weight of luteal tissue used was 250mg.

2.3.1.3. Measurement of specific binding of ^{125}I-LH

The samples were assayed using the method described by Bramley *et al.* (1987). All samples were set up in triplicate. Non-specific binding (NSB) was set up in duplicate and assessed by inclusion of 50 IU Chorulon in the incubation. A preparation of sheep CL homogenate with a binding capacity of 5.1fmol ^{125}I-hLH/μg DNA was included as an internal standard in each assay (interassay coefficient of variation 6%). A set volume (50μl) of each luteal homogenate was used initially but this volume was adjusted (between 20-100μl) where initial binding was outwith the assay range.

Each tube contained 1ml total volume;

50μl of luteal homogenate
750μl of 0.5% BSA in assay buffer
100μl of ^{125}I-hLH tracer
NSB 100μl of Chorulon (50IU)
Bo 100μl of assay buffer

The tubes were vortexed and incubated at 20^{0}C for 16-20 hours.

After incubation, the tubes were chilled on ice and bound and free hormone were separated by polyethylene glycol precipitation (PEG) as follows;
0.5ml of 0.5% IgG was added to all tubes except total counts followed immediately by 1ml of ice-cold 25% PEG. The samples were then vortexed and centrifuged at 2000 *g* for 30 min. The supernatants were carefully aspirated and the ^{125}I associated with the pellet was counted in a multiwell Packard "Crystal" gamma counter at an efficiency of 75%.

Specific binding was calculated as the difference between total binding and binding in the presence of excess unlabelled hormone. The results for each sample were expressed per microgram of DNA.

2.3.2. Progesterone Assay

Plasma progesterone concentrations were analysed by radioimmunoassay as described by Corrie *et al.*, (1981) and modified to be a non-extraction assay by Law *et al.* (1992).

2.3.2.1. Reagents

Assay buffer (Phosgel);

18g NaCl
3g gelatin (swine skin)
200mg Thimerosal
200ml 0.5M PO_4 (pH 7.4)

Made up to 2000mls using double distilled water and stored at 4°C.

2.3.2.2. Preparation of standards

Standard solutions were prepared from P-0130 (Sigma Chemical Co., Poole, Dorset) diluted in assay buffer to give 8 standards ranging from 7.8-1000pg/tube. Two standard curves were prepared where more than 100 samples were being assayed.

2.3.2.3. Assay protocol

All samples and standards were assayed in glass tubes (12 x 75mm). Two quality controls (1 high progesterone, 1 low progesterone) were assayed with the plasma samples.

1. 50µl of ovariectomised bovine plasma was added to each standard (100µl) which was assayed in triplicate. The standards were made up to 500µl using assay buffer.

2. Plasma samples (100µl) were dispensed in duplicate and made up to 500µl with assay buffer.
3. 100µl ^{125}I-labelled progesterone (I-140, [Amersham International plc, Amersham, UK], made up in assay buffer and 1mg/ml 8-anilino-1-naphthalene sulfonic acid (ANS) to give between 13,000-15,000 counts per minute in 100µl) was added to each tube.
4. 200µl antiserum R31/8 (1:16,000) was added to all tubes except totals and blanks (NSB). Blanks received 200µl of assay buffer.
5. All tubes were vortexed and then left to incubate for 3 hours at room temperature.
6. In order to separate free and bound antibody 100µl each of donkey-anti-rabbit serum 1:35 (10% 0.1M EDTA) and normal rabbit serum 1:300 were added to all tubes except totals.
7. The tubes were vortexed and left to incubate at 4°C overnight.
8. All tubes except totals were prewashed by adding 1ml of cold assay buffer and centrifugation at 2000 *g* for 30 min at 4°C.
9. The supernatant was then carefully decanted and the tubes allowed to dry briefly.
10. The activity in the precipitates was then counted.

2.3.2.4. Assay calculations

Progesterone assays were analysed using Assayzap (Zaristow software, Haddington, East Lothian, Scotland). Assay sensitivities and coefficients of variation are detailed in each chapter.

2.3.3. 13,14-Dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) Assay

Plasma samples were assayed for the stable metabolite of $PGF_{2\alpha}$, 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM), using the assay developed by Dr R. Kelly, MRC Centre for Reproductive Biology, Edinburgh (Kelly *et al.*, 1986).

2.3.3.1. Buffers

Assay Buffer;

1.8l PBS (0.05M) 1:10

18g sodium chloride

2g sodium azide

2g gelatin (dissolve first in 200ml ddH_2O)

MOX;

164g anhydrous sodium acetate

20g methoxylamine hydrochloride

1200ml dH_2O

200ml ethanol

pH 5.6 and make up to 2l with dH_2O.

2.3.3.2. Preparation of standards

A standard curve was prepared by diluting a top standard of PGFM (5120pg/ml) by 50% in assay buffer and continuing this serial dilution to give 10 standard dilutions ranging from 1pg/100µl -512pg/100µl. 20% MOX was added to each standard used in the assay.

2.3.3.3. Extraction method for prostaglandins from plasma

1. 0.5ml 20% ethanol and 10µl acetic acid were added to 0.5ml of each plasma sample in a 1.5ml polypropylene tube.
2. The samples were mixed gently, left at room temperature for 5 minutes and centrifuged at maximum speed in a microfuge.
3. The supernatants were applied to Bond-Elut C18 columns that had first been primed with 2 column volumes (0.85ml) of 100% ethanol and then 1 column volume of 10% ethanol.
4. The columns were washed with 1 column volume of distilled water and then 1 column volume of hexane.

5. The samples were eluted into 20ml glass tubes with 2 volumes (0.75ml) of ethyl acetate and then evaporated to dryness under nitrogen at 60°C (30-45 min).
6. 100µl of phosphate-buffered gelatin-saline pH 7.8 and 100µl of MOX B solution was used to reconstitute the precipitate.
7. Finally the tubes were incubated at 60°C for 1 hour to allow methyloximation to take place and then diluted to a final volume of 500µl with PBS.

2.3.3.4. Assay protocol

All standards (100µl) were assayed in triplicate. Two standard curves were prepared for the assay. Samples (25µl and 100µl) were analysed in duplicate and made up to 100µl with assay buffer. Two different volumes of samples were used because it was likely that the concentrations of PGFM would vary considerably between the different dose rates.

Method

1. 100µl of samples and standards were dispensed into polypropylene tubes.
2. 100µl of tracer (radiolabelled 13, 14 dihydro-15-keto $PGF_{2\alpha}$ coupled to proline-tyrosine, supplied by Dr R. Kelly), 20,000cpm/100µl, was added to all tubes.
3. 100µl of R-144 (1:40,000) was added to all tubes except blanks (NSB) which received 100µl of assay buffer.
4. The tubes were vortexed and then covered and incubated for 3 hours at room temperature.
5. 100µl of normal rabbit serum (NRS,SAPU, 1:200) and 100µl of donkey-anti-rabbit serum (DARS, SAPU, 1:20,) and 10% 0.1M EDTA were added to all tubes except total counts, vortexed and incubated overnight at 4°C.
6. 1ml of cold assay buffer was added to each tube except totals and the tubes were then centrifuged at 2000 *g* for 30 min (4°C).
7. The supernatants were carefully decanted, tubes allowed to drain and then the precipitates were counted in a gamma counter.

2.4. DNA Assay

DNA in each sample was assayed fluorometrically using the method described by West *et al.* (1985).

2.4.1. Reagents

1) EDTA

10mM EDTA in ddH_20, pH 12.3.

2) KH_2PO_4

2M KH_2PO_4

3) NaCl-Tris buffer

100mM NaCl in 10mM Tris, pH 7.

4) Bisbenzimide

Stock 200mg/ml stored at 4°C in dark. Diluted 1/1000 in NaCl-Tris buffer immediately prior to use.

2.4.2. Preparation of standard curve

Calf thymus DNA (Sigma, Poole, Dorset) was used as the DNA standard. A stock solution was prepared (2mg/ml) and stored at 4°C.

To prepare a standard curve the stock solution was diluted with ddH_2O to give a range of 9 dilutions from1µg/ml-200µg/ml, aliquoted and stored at -20°C prior to use. A quality control containing 50µg DNA/ml was also prepared and stored at -20°C.

2.4.3. Assay method

All standards and samples were prepared in triplicate. Samples of luteal homogenates were first diluted 1/5 in NaCl-Tris buffer to bring the DNA concentrations within the range of the standard curve. Duplicate quality controls and tubes containing no DNA

were included in each assay. Two sets of standards were prepared and put through the fluorimeter before and after the samples.

Protocol;

Glass test tubes were used throughout.

1) 1.4ml cold EDTA (10mM, pH 12.3) was added to 10µl of each sample or standard.
2) All tubes were incubated at 37°C for 20 min and then cooled on ice for 5 min.
3) 2M KH_2PO_4 was added to each tube in sufficient volume to adjust pH to 7.0 (calculate volume by adding 2M KH_2PO_4 in small volumes to 14ml EDTA and divide amount by 10 to calculate amount required per tube.)
5) 1.5ml bisbenzimide (200ng/ml diluted in NaCl Tris buffer) was added and the tubes were vortexed briefly.
6) Fluorescence was then measured using a fluorimeter [Excitation =350nm (340nm nearest filter) Emission=455nm].
7) The standard curves were then plotted on graph paper and concentrations of DNA calculated from the relative position of the samples on the curve.

2.5. Basic Immunohistochemistry protocol

The technique was based on an avidin-biotin complex (ABC) method described by Hsu *et al.* (1981).

1. Sections of tissue (6µm) were cut on a cryostat (Shandon AS650M, Shandon, UK), then mounted on to gelatin-coated slides and air-dried for 2 hours.
2. The sections were then fixed in cold acetone for 5 min and washed in PBS (pH 7.3).
3. Endogenous peroxidase staining was blocked by treating the sections with 5 units of glucose oxidase/ml^{-1} in 10mM β–D glucose, 1mM sodium azide and 0.1M phosphate buffered saline (pH 7.3) for 50 min at 37°C. The slides were washed for 10 min in PBS.

4. Sections were then treated with 1.5% normal horse serum for 15 min at room temperature, primary antibody was added and the sections were incubated overnight at 4°C.
5. The following day, slides were again washed for 10 min with PBS and then incubated for 30 min at room temperature with biotinylated second antibody.
6. After washing, the slides were incubated with the avidin-biotin reagent for 30 min at room temperature and washed again.
7. The reaction product was visualised by incubating with 3-amino-9-ethyl carbazole (AEC) for 10-15 min.
8. The slides were then washed in tap water, counterstained in Meyer's haematoxylin (2 min) and mounted using Immu-mount (Shandon, Pittsburgh PA 15275), an aqueous mountant.

For each batch of slides, one slide was included in which normal horse serum was substituted for primary antibody (negative control) and sections of bovine lymph node were also included as positive controls.

2.6. Carbol chromotrope stain for eosinophils

Eosinophils were detected on sections from frozen tissue using the carbol chromotrope method described by Lendrum (1944). Sections of gut were used as positive controls.

Method;

1. 6μm sections were cut, fixed in cold acetone for 10 min and air dried before staining in haematoxylin for 1 min (stains nuclei blue).
2. The sections were washed in water, differentiated in acid alcohol and then the blue stain was developed briefly in Scott's tap water substitute.
3. The sections were then stained for 10 min at room temperature in carbol chromotrope solution (5g phenol, 2.5g carbol chromotrope, 500ml distilled water).
4. All slides were rinsed briefly in alcohol and then held in xylene before mounting in DePeX mounting medium (BDH, UK).

2.7. Non-specific esterase staining of macrophages.

Macrophages were stained using the method described by Hudson and Hay (1989) as follows;

1. 6μm sections of tissue were fixed for 30 sec at 4°C in 30ml of 0.1M phosphate buffer, pH 6.6 mixed with 45ml acetone and 25ml formaldehyde solution.
2. The slides were then washed 3 times in distilled water and allowed to air dry.
3. The pararosaniline dye was prepared immediately before use; 6ml pararosaniline stock solution (1g pararosaniline, 5ml 10M HCl, 20ml distilled water. heated to 70°C, cooled to room temperature and filtered.) was added to 6ml 4% sodium nitrite and diluted to 200ml with 0.067M phosphate buffer pH 5.
4. The pH of the activated dye solution was adjusted to pH 5.8 with 0.1M sodium hydroxide.
5. 50mg α-napthyl acetate was dissolved in 2ml acetone and added to the dye solution.
6. The sections were incubated at 37°C for 45 min and then rinsed in distilled water.
7. 0.4% methyl green was used as a counterstain (1-2min) and the sections were finally washed in distilled water, air dried and mounted in DPX.

2.8. Haematoxylin and eosin (H&E) stain

H&E was used to stain sections of CL for morphological studies.

1. Sections of luteal tissue (4μm) were cut onto glass slides.
2. The slides were placed in an automatic processor (Linnistain).
3. The sections were stained in Harris haematoxylin (Surgipath, U.K.) for 1 min and then washed in water for 30 sec.
4. The sections were stained in Scott's tap water substitute for 30 sec and then counterstained in Putt's eosin for 30 sec.
5. Finally all sections were washed in water (2 min), dehydrated, cleared and mounted.

Chapter 3

DISSOCIATION OF THE BOVINE CORPUS LUTEUM

3.1. INTRODUCTION

A variety of techniques may be used to study the corpus luteum *in vitro*. It is possible to study cell populations within the CL using individual sections of tissue by techniques such as immunohistochemistry or electron microscopy. In addition luteal tissue can be dispersed to produce mixed populations of luteal and non-luteal cells that may be used in techniques such as cell culture and also flow cytometry.

However, successful dissociation of luteal tissue for use in these techniques is not an easy process. Dispersion of luteal cells from the CL requires breakdown of the significant collagen component of the CL (Luck and Zhao, 1993) and the separation of closely-interlocking large and small cells (Chegini *et al.*, 1984), whilst minimising damage to the luteal cells themselves. In particular, large cells, with their extensive cytoplasm, are very susceptible to damage during dissociation. Large and small luteal cells differ in many ways, including their responsiveness to LH and basal progesterone production (see review by Hansel *et al.*, 1991) and therefore a high proportion of damaged large cells within a dispersed population of mixed luteal cells may alter significantly the response and properties of mixed luteal cells during *in vitro* studies.

It is not clear at present how large and small luteal cells interact (Del Vecchio *et al.*, 1994), although it is likely that the proportion of luteal cells present at each stage of the oestrous cycle will influence the function of the CL *in vivo*. In addition to minimising damage to luteal cells during dispersion, it is also important to produce

cell populations which are representative of the proportions of cells present in the intact CL, so that the conclusions reached reflect the physiology of the CL *in vivo*.

Collagenase preparations have been widely used to dissociate luteal tissue for experiments *in vitro*. However, morphometric studies of CL in both the cow and the sheep have suggested that collagenase dissociation does not always produce cell populations representative of intact luteal tissue. In particular, there is a selective loss of large luteal cells (Rodgers *et al.*, 1984; O'Shea *et al.*, 1989). We have observed differences in cell populations following dissociation of equine luteal tissue with collagenase (Broadley *et al.*, 1994) compared to cell populations produced by mechanical dissociation without the use of any collagenase enzymes.

The first aim of this study was to compare dispersed bovine luteal cell populations, prepared using a range of commonly used collagenase enzymes, with those prepared by mechanical dissociation. The second aim was to determine whether the type of collagenase used had an effect on the cell membrane of dissociated cells, using LH receptor populations to indicate cell membrane integrity. The data obtained from this study would allow us to select the conditions which were most appropriate for the preparation of dispersed luteal cell populations for cell culture and, in particular, for flow cytometry studies which would be used to investigate immune cell populations within the bovine CL.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Seven collagenase preparations were used in this study: Collagenase IA, Collagenase V and Collagenase XI (Sigma Chemical Co., Poole, Dorset, UK.), Worthington I and Worthington IV (Cambridge Bioscience, Cambridge, UK.) and Collagenase D and Collagenase/dispase (Boehringer-Mannheim, East Sussex, UK.). The collagenase enzymes selected are commonly used in studies involving dissociation of bovine CL

and have a range of enzymatic activity (Table 3.1). Variations in collagenase activities are known to occur between different batches of the same collagenase. Therefore sufficient enzyme was obtained to enable one batch to be used throughout the study. Hepes buffer was purchased from Flow Laboratories, Irvine, U.K.

TABLE 3.1 Enzymatic activities of collagenase preparations*

Enzyme	Coll. XI	Coll. V	W1	Coll. Disp.	Coll. IA	Coll. D
COLLAGENASE (FALGPA hydrolysis)	2.7	1.7		>0.1	1.2	
COLLAGENASE (collagen digestion)	2030	510	196		425	
NEUTRAL PROTEASE (caseinase)	110	65	331		43	33.2
CLOSTRIPAIN	0.9	0.7	2.6		0.4	0.7
TRYPSIN			0.29			0.1
DISPASE				>0.8		

*Enzymatic activity in units/mg dry weight.

3.2.2. Dissociation of luteal tissue and analysis.

3.2.2.1. Collection and preparation of luteal tissue

Ovaries from non-pregnant cows were collected at the abattoir within 15 minutes of slaughter and transported on ice to the laboratory. Stage III CL (days 11-17 of the oestrous cycle) were selected according to the criteria described by Ireland *et al.* (1980) and dissected from the surrounding ovarian tissue. The selection criteria are described in Chapter 2 (Section 2.2.2).

Three or four Stage III CL were combined, and the tissue was minced finely using a scalpel blade before being washed by gentle repeated inversion in buffered $HBSS^-$

(Hanks balanced salt solution without divalent cations, containing 20mM Hepes, 100U penicillin/ml, 100μg streptomycin/ml) and filtered through a 100 μm stainless steel mesh. The filtrate from this wash represented the cell population produced by mechanical dissociation (MD) of luteal tissue. In order to assess the effect, if any, of washing the tissue prior to collagenase treatment, samples of minced luteal tissue were collected on two occasions before and after washing and fixed in 4% buffered formalin. Sections from paraffin blocks prepared from these samples were stained with haematoxylin and eosin and the cell morphology examined by light microscopy. In particular the proportion of large cells within the tissue was assessed as these cells are more susceptible to damage because of their extensive cytoplasm. Washing of the tissue prior to collagenase treatment did not alter tissue morphology although it did result in less red blood cell contamination after dispersion.

3.2.2.2. Collagenase dissociation of luteal tissue

The washed tissue was then divided into 5g portions and placed in 50ml plastic centrifuge tubes. The collagenase treatment of luteal tissue was based on the method described by Simmons *et al.* (1976) as follows;

A collagenase enzyme made up in dissociation media (buffered HBSS containing divalent cations, 10% foetal calf serum, 0.02% DNase, 100U penicillin/ml and 100μg/ml streptomycin) was added to each tube (3ml/g) and mixed gently. The centrifuge tubes were then placed in a shaking water bath at 37°C for 50 minutes. Following collagenase digestion or mechanical dissociation the cells were filtered through a 100μm stainless steel mesh to remove undigested tissue, washed twice in buffered $HBSS^-$ and collected by gentle centrifugation (10 min 200 *g*). Any undigested luteal tissue remaining was frozen at -20°C for LH binding assay.

3.2.2.3. Cell counts

The dissociated cells were resuspended in buffer and the total cell yields for 5g of luteal tissue calculated using a haemocytometer. Cytospin cell preparations were

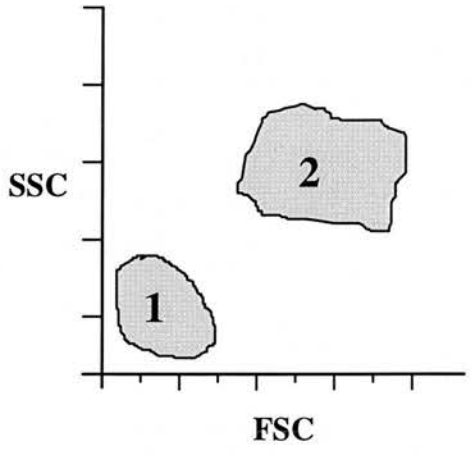
produced using a cytocentrifuge (10 min, 100 *g*) and stained with Diff-Quik™ (Baxter, Thetford, Norfolk, UK). The number of large cells present in the cytospin preparations was expressed as a percentage of the total cells present rather than as a ratio of large:small cells because of the difficulty in clearly differentiating small luteal cells from the other cell types present such as endothelial cells. In particular, there were many cells which appeared to have distinct nuclei and small portions of uneven cytoplasm attached. It was difficult to assess whether these cells were small luteal cells or large luteal cells that had lost their cytoplasm. The number of intact large cells (identified on the basis of their size, central nucleus, distinct nucleolus and intact cytoplasm) in a total of 200 cells was calculated. The remaining cells were frozen at -20^{0}C for LH binding assay. There was a minimum of 10 replicates for each of the four collagenase treatments and mechanical dissociation.

3.2.3. Analysis of cell populations by flow cytometry.

3.2.3.1. Principles of flow cytometry

Flow cytometry is a technique which allows simultaneous measurement of a variety of physical characteristics of a single cell within a large population of cells. Between 500 and 4000 cells in a fluid stream may be measured per second. A flow cytometer can measure the relative size and granularity (or surface complexity) of a cell by recording how it interacts with a focused laser beam. The degree of light diffraction caused by an individual cell is proportional to the cell surface area and the degree of light refraction plus reflection is proportional to cell granularity. The cells are then presented as a series of dots on a graph (dot-plot) as shown in Fig. 3.1. The X-axis represents light diffraction (forward light scatter, FSC), related to cell size, and the Y-axis represents light refraction and reflection (90^0 light side scatter, SSC), related to cell granularity. Therefore the smallest, least granular cells, such as lymphocytes will be present in Region 1 of the dot plot (Fig 3.1) and large, granular cells, such as macrophages will be found in Region 2 (Fig 3.1).

Fig. 3.1. Regions of dot plot associated with cells of a specific size and complexity. The X-axis represents forward light scatter (FSC) and the Y-axis represents light refraction and reflection, side scatter (SSC).



In addition to measuring cell size and granularity the flow cytometer can also measure the fluorescence intensity of a cell. For this function cells are first stained with a monoclonal antibody and then linked to a fluorescein-conjugated second antibody. The fluorimeter detects fluorescent cells within a large population which allows different cell types to be identified (for example, CD4+ T-lymphocytes in a mixed population of white blood cells). Fluorescent cells can be expressed as a percentage of the total population or, where the properties of the cell types involved are known, the fluorimeter can be programmed to count cells only within a range of size and complexity by setting specific 'gates'.

The use of the flow cytometer to detect cells that have been stained with specific antibodies and fluorescent markers is decribed in Chapter 6, Section 6.2.8. However, in this study flow cytometry was used to compare the proportions of different luteal cells, based on their size and granularity, within dispersed cell populations produced by collagenase and mechanical dissociation.

3.2.3.2. Flow cytometry of luteal cell populations

Aliquots of luteal cells in buffer from the dissociation procedure described above were washed in cold PBS (containing 0.1% azide and 1% BSA). The resulting cell pellet was resuspended in PBS to give a concentration of cells in the region of $2x10^6$ cells/ml and kept on ice. PBS was used at this stage because HBSS fluoresces, and this would affect the results. The flow cytometer (FACScan flow cytometer, Becton Dickinson, Mountain View, USA) was then used to analyse $1x10^4$ cells from each sample using settings of FSC 2.0 and SSC 1.5.

3.2.4. LH Binding Assay

The samples were assayed using the method described by Bramley *et al.* (1987) which can be found in Chapter 2 (Section 2.3.1.).

3.2.5. DNA Assay

DNA in each sample was assayed fluorometrically using the method described by West *et al.* (1985) which can be found in Chapter 2 (Section 2.4.).

3.2.6. Statistical Analysis

Differences between cell populations produced using the various collagenase enzymes and mechanical dissociation were analysed using the Kruskal-Wallis test for one way non-parametric analysis of variance. Where statistically significant differences were apparent, a Mann Whitney *U* test was used to compare the data between groups. Groups were considered to be significantly different where $p<0.05$.

3.3. Results

3.3.1. Preliminary results- comparison of 6 collagenase enzymes

Initially six collagenase enzymes were compared by visual assessment of dissociated cell populations and measurement of LH binding.

3.3.1.1. Visual assessment

Dispersed cell populations were examined under a microscope to investigate whether there were obvious differences between cells prepared using different enzymes. In particular the appearance of the large cells was studied as these cells are most susceptible to damage;

1. Worthington 1 (W1)

   There initially appeared to have been good dispersion but on closer examination the cell types present were all of similar size. It was difficult to distinguish small cells from damaged large cells or non-luteal cells. The large cells were ragged in appearance with portions of cytoplasm missing.

2. Worthington 4 (W4)

   Few intact large cells were present, most large cells appeared ragged or had a vacuolated cytoplasm. There were few clumps but, as in cell dispersions from Worthington I, the various cell types were difficult to differentiate.

3. Collagenase IA (CIA)

   The different cell populations within the cell dispersions were easier to identify, with a higher percentage of intact large cells present (although there were more clumps of cells present than either of the Worthington collagenases).

4. Collagenase V (CV)

   There was good dispersion with distinct populations of large and small luteal cells. The small cells tended to appear in clumps but not to the extent seen with Collagenase IA.

5. Collagenase XI (CXI)

   Similar in appearance to Collagenase V with good dispersion of distinct cell populations.

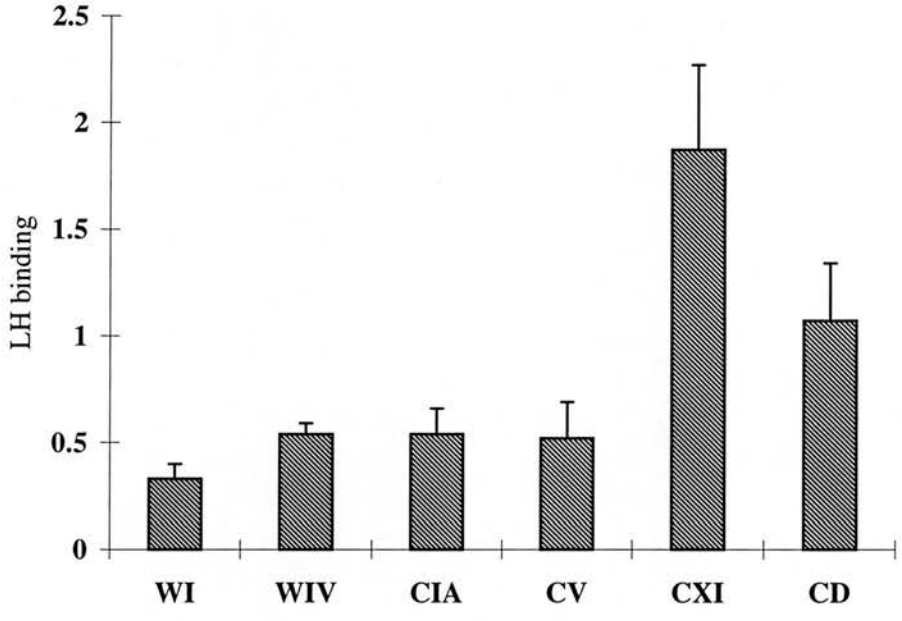
6. Collagenase D (CD)

   Intact large cells and good dispersion with little clumping. Similar appearance to Coll V and XI.

3.3.1.2. LH Binding /Preliminary Study

Results of the LH binding assay can be seen in Fig 3.2. LH binding varied quite considerably between dispersed cell populations produced using different collagenase enzymes (0.33-1.87/per μg DNA).

FIGURE 3.2 **Preliminary Study**- LH binding (pg/μg DNA) to luteal cells dispersed using 6 collagenase enzymes; Worthington 1 (W1), Worthington 4 (W4), Collagenase IA (CIA), Collagenase V (CV), Collagenase XI (CXI) and Collagenase D (CD)[1]



[1]Each figure represents mean (+/- s.e.m.) from 3-5 dispersions.

From the LH binding results and visual assessment of dispersed populations of cells 3 enzymes were chosen (Worthington 1, Collagenase XI and Collagenase V) for further study. An additional enzyme, Collagenase/dispase (Boehringer Mannheim), was also selected, although it was not included in preliminary work. Collagenase/dispase is a relatively "gentle" enzyme, containing a highly purified collagenase and is recommended by the manufacturers for studies where preservation of functionality and surface protein integrity are important.

3.3.2. Comparison of four collagenase enzymes and mechanical dissociation

Following on from the preliminary study, cells prepared by four collagenase preparations were compared with each other and mechanical dissociation. Cell populations produced by these treatments were compared by recording the total cell yield from 5g of mid-cycle luteal tissue, the number of large luteal cells in dissociated cell populations, the number of intact LH receptors (and analysis of dispersed cell populations by flow cytometry).

Abbreviations used in the Results and Discussion sections are as follows;

1. Mechanical dissociation (MD),
2. Collagenase V (CV),
3. Collagenase XI (CXI),
4. Collagenase dispase (CDp),
5. Worthington 1 (W1).

3.3.2.1. Total Cell Yield

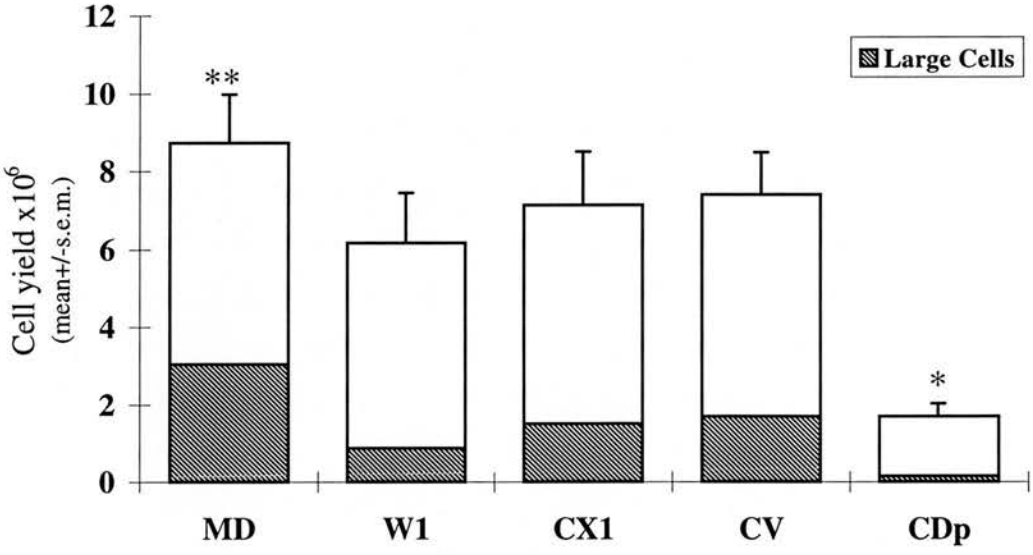
The cell yields produced by the different dispersion treatments are shown in Figure 3.3. There were no significant differences between CXI, CV, W1 and MD. However CDp had a significantly smaller ($p<0.005$) yield of cells from 5g luteal tissue. This result was not surprising as the cell pellets produced after treatment with CDp were visibly smaller than any of the others throughout the study.

3.3.2.2. Large luteal cell numbers

The MD cell preparations contained a significantly higher number of large cells ($p<0.05$) than the other groups with levels around 35-40% (Fig. 3.3) although there were obvious clumps of mixed cell types in these cytospin preparations (Fig. 3.4a). Direct smears of MD cell preparations on glass slides confirmed that these clumps were not a result of cytocentrifugation. There were no statistically significant differences between CXI, CV and W1, although the number of large cells present in W1 preparations was always lower than CXI and CV. Cell populations produced by

CXI and CV appeared to be similar on examination of cytospin preparations, there was good dissociation of cells with limited clumping and large cells remained intact (Fig. 3.4b/3.4c). The cells produced using W1, particularly the large cells, tended to have a ragged appearance and there were often portions of detached cytoplasm within the cell populations (Fig. 3.4d). CDp produced significantly lower numbers of large cells ($p<0.05$) compared to the other collagenase enzymes and MD, although the large cells seen in cytospin preparations from CDp were usually intact with very little cell debris.

FIGURE 3.3 Total cell yield and number of large luteal cells from 5g of luteal tissue after mechanical dissociation (MD) or collagenase dissociation using Worthington 1(W1), Collagenase XI (CXI), Collagenase V (CV) or Collagenase Dispase (CDp).



* There were significantly fewer cells in total ($p<0.005$) and significantly fewer large luteal cells ($p<0.05$) in cell populations produced using CDp compared to all other treatments.

** There were significantly more large luteal cells ($p<0.05$) in luteal cell populations produced by MD compared to all other treatments.

FIGURE 3.4a. Cytospin appearance of luteal cell populations prepared by mechanical dissociation (MD x218).

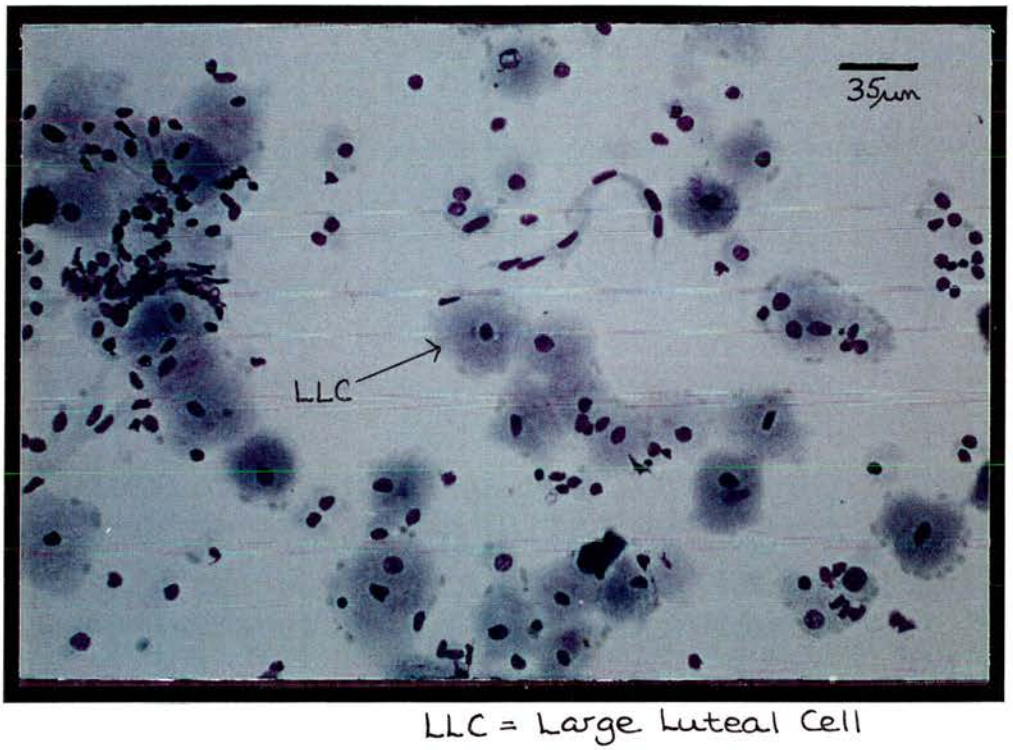


FIGURE 3.4b. Cytospin appearance of luteal cell populations prepared by collagenase dissociation using Collagenase V (CV x218).

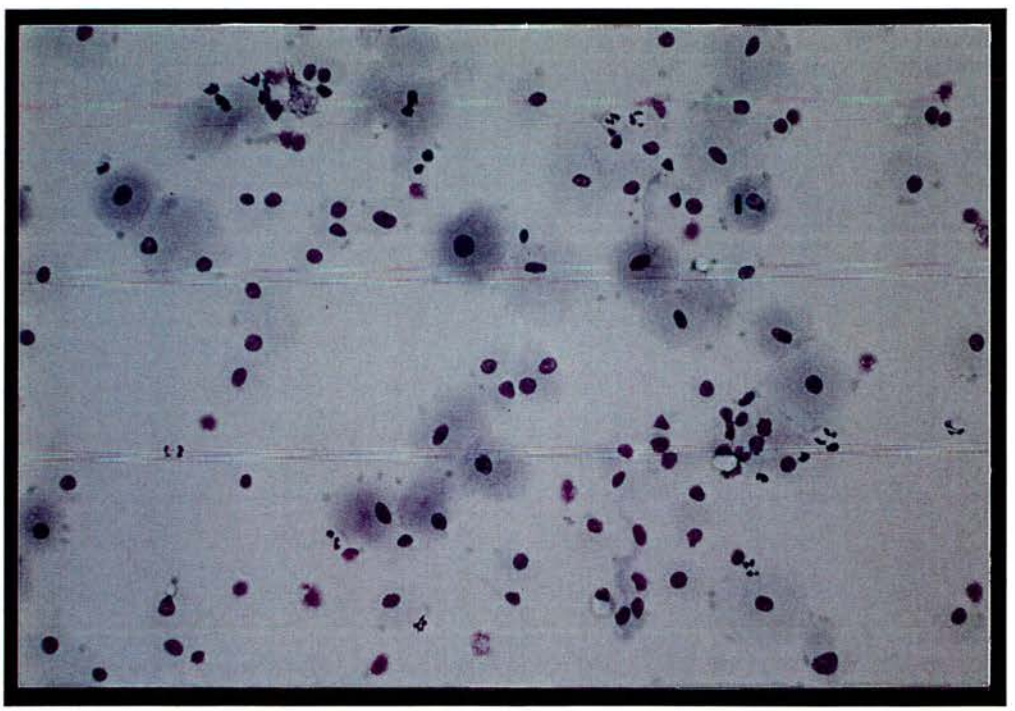


FIGURE 3.4c. Cytospin appearance of luteal cell populations prepared by collagenase dissociation using Collagenase XI (CXI x218).

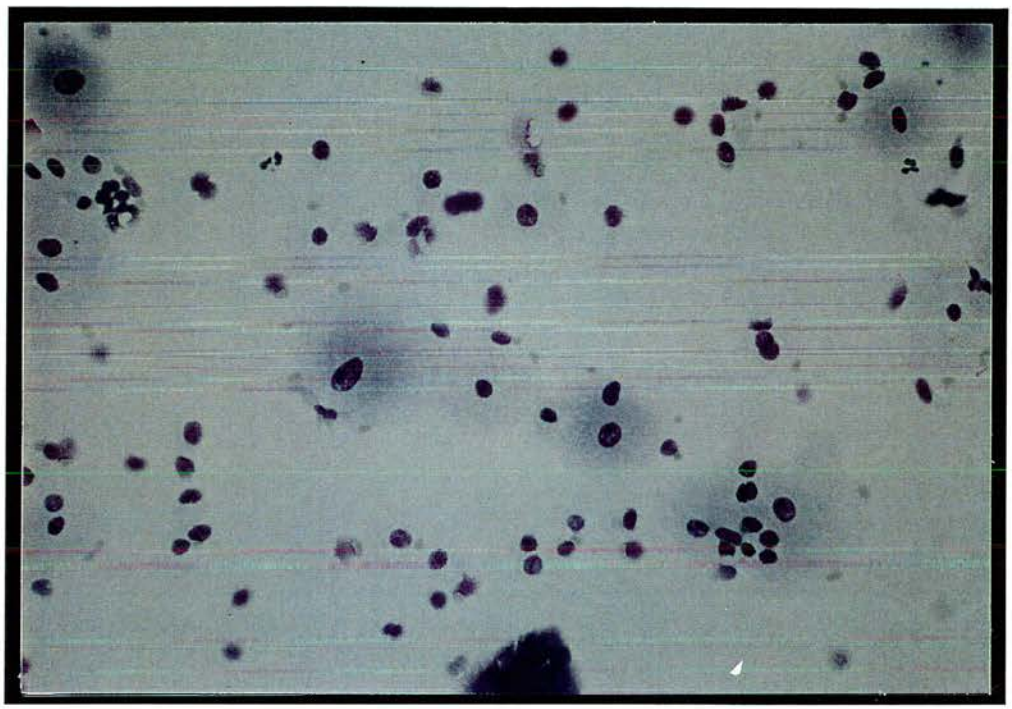
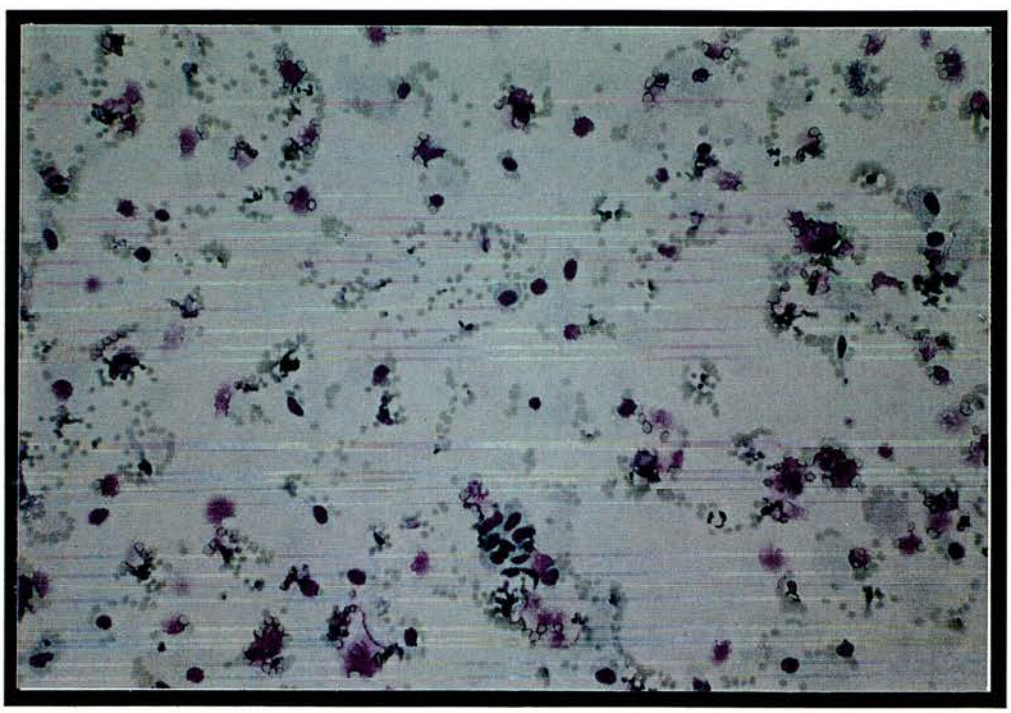


FIGURE 3.4d. Cytospin appearance of luteal cell populations prepared by collagenase dissociation using Worthington 1 (W1 x218).



3.3.2.3. Flow cytometry

Initial dot plots showed some distinct differences in appearance in particular between mechanically dissociated cell populations and those produced by the various collagenase enzymes (Fig. 3.5 a-d).

Successful use of the flow cytometer is dependent on a good cell dispersion and the presence of distinct cell types if specific cells are to be identified. Unfortunately, because of the nature of the cell populations involved in this study, neither of these requirements were fulfilled. Dispersed luteal cell preparations were not made up completely of distinct cells and there was a high incidence of clumps of mixed cell types, as well as damaged cells and other cell debris within the cell populations. A clump of small cells would appear to be larger and more complex than a single small cell and would therefore be recorded as a single complex large cell, whereas large cells with portions of cytoplasm missing may be identified as similar in size to small cells. Moreover, since there is some evidence of small luteal cells differentiating into large luteal cells during the oestrous cycle (Alila and Hansel, 1984; Niswender *et al.*, 1985), small and large luteal cells may not be distinct enough to be separated by size alone.

It was therefore decided that flow cytometry would be of little use in accurately defining luteal cell types and proportions within dispersed cell preparations and the technique was not used further in this study.

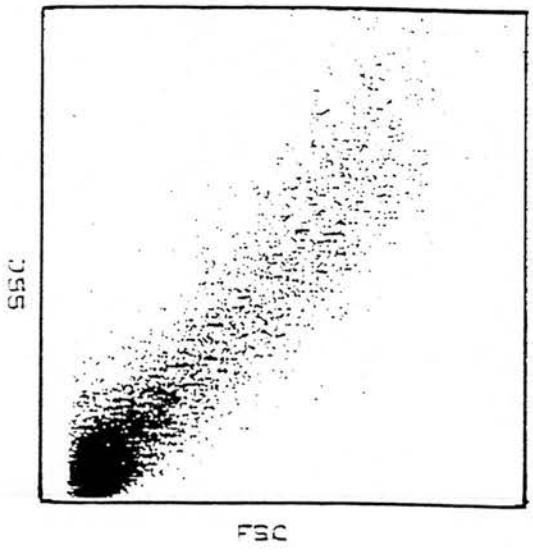
3.3.2.4. LH Binding Studies

LH binding to luteal cells

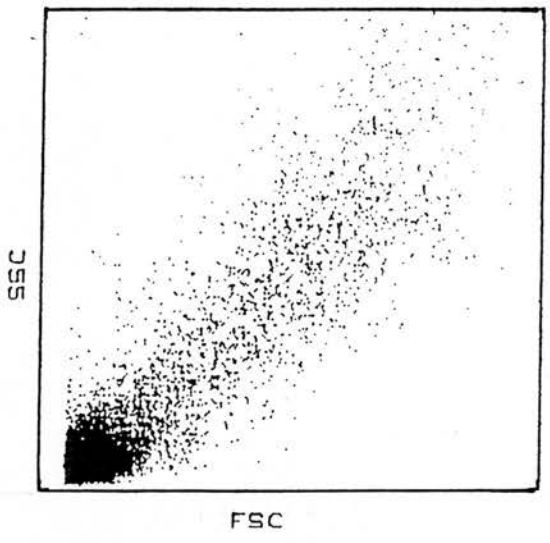
There were no significant differences in LH binding per μg of DNA between MD, CXI, CV or CDp, although CDp exhibited the highest levels of binding (Fig. 3.6). LH binding levels were significantly lower ($p<0.005$) in cell populations produced using W1.

Figure 3.5. Flow cytometry: Dot plot appearance of cell populations produced after mechanical and collagenase dissociation of luteal tissue.

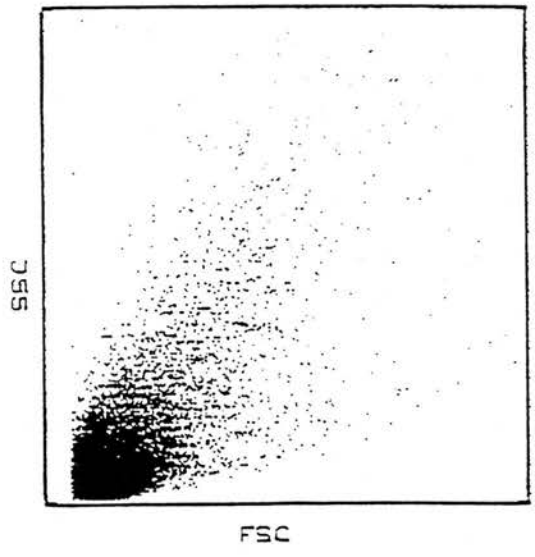
a) Mechanical dissociation.



b) Collagenase XI.



c) Worthington 1.



d) Collagenase/dispase.

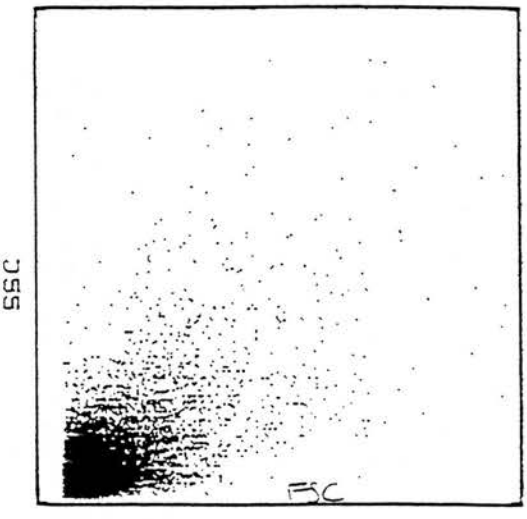
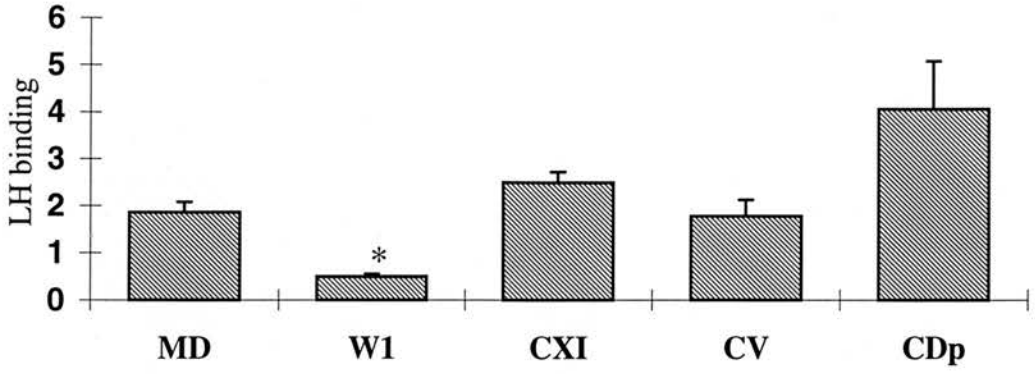


FIGURE 3.6 LH binding (pg/μg DNA) to luteal cells after mechanical dissociation (MD) or collagenase dissociation using Worthington 1(W1), Collagenase XI (CXI), Collagenase V (CV) or Collagenase Dispase (CDp)[1].



[1]Each figure represents the mean (+/-s.e.m.) for a minimum of 8 treatments.

*LH binding was significantly lower ($p<0.005$) in cell populations produced using W1 compared to all other treatments.

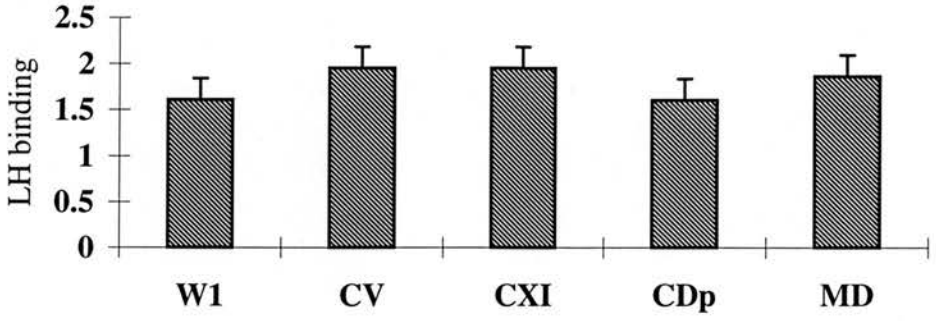
LH binding to luteal tissue

LH binding to luteal tissue after mincing and washing of the various batches of corpora lutea (prior to mechanical or collagenase dissociation) from different days varied between 1.31-2.1 per μg DNA (Table 3.2) and there were no significant differences in LH binding in tissue remaining after any of the collagenase treatments and mechanical dissociation (Fig. 3.7).

Table 3.2 LH binding to different batches of luteal tissue, prior to mechanical or collagenase dissociation.

Run No.	1	2	3	4	5	6
LH Binding (pg/μg DNA)	1.37	1.61	1.31	1.76	1.92	2.1

FIGURE 3.7 LH binding (pg/μg DNA) to luteal tissue remaining after collagenase dissociation using Worthington 1 (W1), Collagenase V (CV), Collagenase XI (CXI), Collagenase Dispase (CDp) or mechanical dissociation (MD)[1].



[1] Each figure represents mean (+/- s.e.m.) from 4-8 experiments.

3.4. Discussion

A variety of collagenase preparations have been used to prepare dispersed luteal cells in a range of studies investigating their properties. Differences in the large:small cell ratios produced by collagenase dissociation (Hansel *et al.*, 1987) compared to morphometric analysis of luteal tissue (O'Shea *et al.*, 1989) led us to investigate the possibility that the type of collagenase used may affect the cell population produced by dissociation and hence influence the conclusions of some *in vitro* studies. In this study, we were interested in damage that might also occur during luteal cell dispersion at the level of the luteal cell membrane. We used numbers of LH receptors per cell as an indicator of such damage.

It is not possible to draw conclusions about specific collagenase enzymes, nor to describe conclusively the properties of individual collagenase preparations because of the variation in enzymatic activity between different batches of the same enzyme. Moreover, the degree to which each enzyme affects cells will also depend on the dissociation conditions including temperature, time, concentration of enzyme and the use of repeated incubations. However, we minimised the effects of these variables in

this study by using the same batches of collagenases throughout and a single incubation in a shaking water bath at 37°C. The results confirmed that different collagenase enzymes produced luteal cell populations that differed in cell yield, proportion of large cells and cell membrane characteristics.

Cell yields from MD, CV, CXI and W1 were similar, but CDp produced a significantly smaller number of cells. This was probably due to the low collagenase activity of this enzyme which reduced the rate of collagen breakdown and, therefore, increased the time necessary to release cells from intact tissue. Longer and/or repeated incubations of luteal tissue with this collagenase may improve the cell yield from this particular enzyme preparation.

There was marked variation in the percentage of large cells produced using the different treatments. Mechanical dissociation produced a high yield of large cells; however they tended to be found in mixed clumps of large and small cells, making the technique unsuitable for most luteal cell studies. The collagenase enzymes, CV and CXI, produced cell populations with around 22% large cells. The large cells obtained were easy to identify, had intact cytoplasm and appeared to have suffered little cell damage. This was in contrast to the cell populations produced using W1 which were much more disrupted in appearance with portions of detached cytoplasm making identification of individual cells more difficult.

The selective loss of large cells during collagenase dissociation has been recognised by several workers (O'Shea *et al.*, 1989; Hansel *et al.*, 1991). The extensive cytoplasm of the large cells may render them more susceptible to structural damage during dissociation than the more compact cytoplasm of the small cell (Koos and Hansel, 1981). Hansel *et al.*. (1987) used collagenase to disperse luteal tissue from CL at various stages of the oestrous cycle. Their preparations had ratios of small cells:large cells between 20:1 and 40:1. The low numbers of large cells seen in their study, [using the dissociation technique described by Gospodarowicz and Gospodarowicz (1972)], compared to the present study may be due to their repeated

incubations with collagenase during dissociation compared to our use of a single incubation. Repeated exposure to collagenase may increase damage to large cells, particularly where crude collagenase enzymes with high tryptic activity are employed. Compared to results from dissociated cell populations, morphometric studies of intact luteal tissue have reported much lower small:large cell ratios in the region of 7.6:1 to 10.2:1 (Weber *et al.,* 1987; O'Shea *et al.,* 1989). Since the cell ratios obtained in this study using either of the Sigma collagenase enzymes, CX1 or CV, were reasonably representative of luteal cells proportions *in vivo*, it would appear that either of these enzymes would yield luteal cell populations in physiological proportion for techniques such as cell culture.

Luteal cell LH binding was used in this study as an indicator of damage occurring to the cell membrane. Luteinising hormone is the main luteotropic hormone in the cow, although large and small luteal cells differ in their responsiveness to LH (see Hansel *et al.*, 1991 for a review). Small cells show a dramatic increase in progesterone production in response to LH (Koos and Hansel, 1981; Weber *et al.*, 1987). In contrast, LH responsiveness is much reduced in large cells. There is some debate in the literature as to the reason for this difference in responsiveness. Some studies have described the presence of more LH receptors on small cells in comparison to large cells (Fitz *et al.*, 1982) whereas others have found equal numbers of LH receptors or more to be present on large cells (Chegini *et al.*, 1991; Harrison *et al.*, 1987; Smith *et al.*, 1996). LH receptor numbers vary according to the stage of cycle (Diekman *et al.*, 1978; Chegini *et al.*, 1991). However, since the LH receptor is found at all stages of luteal lifespan until luteolysis, it is a good candidate to be used as an indicator of membrane damage to the luteal cell.

Differences in LH binding were not only the result of use of collagenase, as two collagenase dispersions (CDp and CXI) gave cell preparations which demonstrated higher levels of binding than mechanically dispersed cells. Differences may also have been affected by variation in the total numbers of large and small luteal cells present after dispersion. Mechanically dispersed populations of cells contained large clumps

of mixed cell types, as well as fibrous strands possibly originating from the capsule or trabeculae, which may have contributed less to the total LH binding, although there is some evidence that binding sites may be present on cells other than luteal cells in the CL including blood vessels (Chegini *et al.*, 1991).

The high LH binding demonstrated by cell populations produced by CDp compared to other treatments may result from the low enzymatic activity of this enzyme, so causing less damage to receptors than other higher activity collagenase preparations. Conversely, the relatively crude collagenase W1, which has high proteinase activity, produced luteal cell populations with significantly lower numbers of intact LH binding sites compared to MD. This collagenase has been widely used in luteal cell studies though we have shown here that not only are the large cells visibly damaged during dissociation with W1, but that cell membrane LH receptors are also affected. It is likely therefore that receptor sites for other hormones and/or proteins (Chegini *et al.*, 1991) may also be affected by treatment with W1 collagenase and this should be taken into consideration when studies involving receptor sites or hormonal responsiveness are undertaken, and when comparing results from studies using different collagenase treatments.

In conclusion, we have demonstrated firstly that mechanical dissociation produces a high yield of intact large cells but mixed clumps of large and small cells probably make this approach unsuitable for most luteal cell studies. Secondly, dispersion of bovine corpora lutea using different collagenase enzymes produces luteal cell populations which vary in cell yield, proportion of large cells and LH binding. These results led us to select Collagenase XI to be used in our own studies where dispersed luteal cell populations were required. Finally, since the type of collagenase used to dissociate luteal tissue is an important variable, it is imperative to take this into account when studying luteal cell properties and interactions *in vitro*, as this could markedly influence the results.

Chapter 4

Immune cell populations in the bovine corpus luteum throughout the oestrous cycle.

4.1. Introduction

The cow corpus luteum, in common with other species, is made up of a variety of cell types. These include large and small luteal cells as well as significant numbers of non-luteal cells including fibroblasts and endothelial cells and immune cells such as lymphocytes and macrophages (see review by Fields and Fields, 1996). Although the proportions of cell types present within luteal tissue vary during the oestrous cycle and pregnancy, non-luteal cell populations make up a significant number of the total cell types in the CL at all stages (O'Shea *et al.*, 1989; Lei *et al.*, 1991).

Many of the non-luteal cells, such as endothelial cells and fibroblasts, are associated with the large number of blood vessels which are present within luteal tissue. The mature CL receives one of the highest rates of blood flow of any organ, relative to its size, within the body (Ford and Chenault, 1981; Smith *et al.*, 1994). This blood supply, in combination with the extensive network of capillaries, allows efficient transport of luteal cell products including progesterone, oxytocin and prostaglandin as well as delivery of lipoprotein substrates and oxygen to the CL. It may be therefore that immune cell populations are present within the ovary for most of the cycle purely as a result of the volume of blood circulating through the tissue. However there is some evidence that the migration of immune cells, particularly lymphocytes, into the ovary is an active process as numbers of lymphocytes in utero-venous lymph are higher than those in jugular blood (Alders and Shelton, 1990).

Immune cells, in particular macrophages, have a specific role during luteolysis when they are involved in the dramatic destruction of luteal tissue which occurs at this time

(Paavola, 1979; Bagavandoss *et al.*, 1988). Their role in this situation is similar to the function of immune cells within the body where they are involved in recognition and destruction of 'foreign' or damaged tissue. However, in recent years increasing information about the potential effects and actions of immune cells and their products, outwith their accepted function, has led some workers to consider a more active role for these cells within the ovary. (Adashi, 1990; Brannstrom and Norman, 1993; Pate and Townson, 1994).

It would seem likely that, if immune cells do play a significant role within the CL, then there will be some variation in their number and/or cell type at different stages of the oestrous cycle which are not associated with actual destruction of luteal tissue. However there is limited information on immune cells types and numbers present within the cow CL. As early as 1968, Lobel and Levey recorded the presence of immune cells within the bovine CL. Since then others have studied cell populations within the cow CL but immune cells have often been included under a general description of 'non-luteal cells' (Parry *et al.*, 1980; O'Shea *et al.*, 1985; Lei *et al.*, 1991). There are no details on the proportions of specific immune cell types in the cow CL throughout the oestrous cycle. Populations of immune cells have been identified, and in some cases quantified, within CL from other species including the rat (Brannstrom *et al.*, 1994a), rabbit (Bagavandoss *et al.*, 1988), pig (Standaert *et al.*, 1991) and human (Wang *et al.*, 1992c). These studies have shown variations in immune cell types present within luteal tissue which appear to be species-specific.
The aim of this study was to identify and quantify immune cell populations within the bovine CL throughout the oestrous cycle.

There are obviously very many different groups and sub-groups of immune cell populations that can be identified by immunohistochemistry. We selected a range of cell types, partly based on work in other species but in particular using information from studies which have looked at the effects of a range of cytokines, produced mainly by T-lymphocytes and macrophages, on bovine luteal cell populations *in vitro*

(see Pate, 1995 for review). These were of interest to us because it was our intention in the longer term to investigate production of these cytokines in the ovary *in vivo*.

We chose to investigate CD8+ (cytotoxic/suppressor cells) and CD4+ (helper/inducer cells) T-lymphocytes as well as CD5+ T-lymphocytes (includes the majority of T-lymphocyte sub-populations) to give a more general indication of changes within the total T-lymphocyte population. We were also interested in macrophage numbers in the CL because these cells are not only a potent source of cytokines themselves, but are also attracted and activated by the cytokine products of T-lymphocytes. In addition, they play a vital role in the destruction of luteal tissue during luteolysis. B-lymphocytes were selected out of general interest although, as they are primarily involved with the antibody response to antigen in the body, they would be less likely to have an active role in the CL.

Major histocompatability complexes (MHC) are glycoprotein molecules found on the surface of cells. They are required for recognition of antigen by T-lymphocytes. Class I MHC is expressed on virtually all cells and is associated with the response of cytotoxic T-cells to cell surface antigens on foreign or infected cells. MHC class II molecules are also associated with the T-cell immune response but their expression is more limited. Recently cells outwith the immune system have been shown to express MHC II, including cells from the human and sheep CL (Khoury and Marshall, 1990; Kenny *et al.*, 1991) and altered MHC II expression could be important if the immune system does have significant effects on CL function.

MHC II expression on cultured bovine luteal cells from day 10-14 CL was first described by Fairchild and Pate (1989). They used flow cytometry to assess MHC II expression throughout the oestrous cycle and during early pregnancy (Fairchild Benyo *et al.*, 1991). However, this method yields limited information as to which cells are the actual source of the MHC II expression because of the difficulty in identifying individual cell types accurately. In addition, the collagenase dissociation of CL and repeated incubations required to prepare luteal cells for flow cytometry

may not only result in cell populations that are not representative of the intact CL (O'Shea *et al.*, 1989; Hansel *et al.*, 1991) but may also alter MHC II expression. We therefore decided to study MHC II expression using immunohistochemistry as well as flow cytometry.

Eosinophils have a variety of functions in both immune and inflammatory responses. They are highly phagocytic for cells bearing antigen/antibody complexes. Increased systemic populations of eosinophils are particularly associated with parasitic infestations and allergic reactions but high numbers of eosinophils have also been observed in the sheep CL. Increased numbers of eosinophils have been seen particularly after induced luteolysis (Nett *et al.*, 1976) and the existence of a luteal chemoattractant for eosinophils as well as the role of eosinophils in luteal function in the sheep has been investigated (Murdoch *et al.*, 1987; Murdoch and Steadman, 1991). Since there were no descriptions of eosinophils in the cow CL, we looked for these cells using a carbol chromotrope stain which stains the granular cytoplasm of the eosinophil.

4.2. Materials and methods

4.2.1. Collection and preparation of tissue

All CL were collected, staged and snap-frozen in dry ice/isopentane at a local abattoir. The majority of CL were from Holstein-Friesian cross or continental cross heifers. In order to assess more accurately the stage of the oestrous cycle, both ovaries were collected and the uterus was examined for signs of oestrus, such as increased mucus and hyperaemia, and to confirm that there was no pregnancy. The stage of the oestrous cycle was then assessed by examination of the CL and other ovarian structures using the criteria described by Ireland *et al.* (1980), summarised in Chapter 2, (Section 2.2.2.). This method allocates CL into 4 groups based on luteal age: Stage I (days 1-4), Stage II (days 5-10), Stage III (days 11-17) and Stage IV

(days 18-20). Any CL which did not clearly fall into any of the described categories were discarded.

After staging and within 30 minutes of slaughter, the CL were blunt-dissected from the ovary and cut into 3-6 pieces, depending on size. One or two pieces from each CL were then placed onto OCT on individual cork discs and snap frozen in a mixture of dry ice/isopentane. All samples were stored at -70°C prior to sectioning for immunohistochemistry.

4.2.2. Tissue collected

1. Four stage II or III CL were collected to optimise the techniques that would be used.
2. CL were selected from stages I-IV of the oestrous cycle for the primary experiment. Six CL were collected for each stage of the cycle.

4.2.3. Selection of monoclonal antibodies.

A wide range of ruminant-specific antibodies are available. However, there is some variability in the staining properties of these antibodies on frozen tissue. Moreover the high fat content of the CL can cause problems with background staining, and it was therefore necessary to test several different antibodies to select a panel which would give specific staining. Table 4.1 lists the monoclonal antibodies that were tested. Sections of bovine gut and/or lymph node were used as positive controls throughout. The distribution of staining on positive control sections allowed us to to determine that the monoclonal antibody was staining the correct cell types.

Finding a monoclonal antibody that would consistently and clearly detect macrophages proved to be difficult. Prior to finding a suitable monoclonal antibody, a non-specific esterase stain for macrophages was tested (Chapter 2, Section 2.7) but this was also unsuccessful because of excessive background staining in luteal tissue. This was also a problem with many of the monoclonal antibodies that we tried. In particular sheep macrophage markers did not produce good staining despite the fact

that some of these monoclonals are known to cross-react with cattle tissue. These monoclonals may have worked more successfully on paraffin sections. Eventually, clear identification of macrophages was achieved using the VMRD monoclonal, DH59B.

The panel of monoclonal antibodies selected is shown in Table 4.2.

Table 4.1

Monoclonal antibodies which were tested during the course of the study.

Monoclonal	Specificity	Source	Isotype	Form[1]
CC17	CD5	IAH, Compton,UK	IgG_1	S
CACT80-C	CD8	VMRD, Pullman, USA	IgG_1	A/F
GC50A1	CD4	VMRD, Pullman, USA	IgM	A/F
IL-A12	CD4	ILRAD	IgG_{2a}	S
CC8	CD4	IAH, Compton, UK	IgG_{2a}	S
CC21	B-cell (WC30)	IAH, Compton, UK	IgG_1	S
DU2104	B-cell	Basel Institute Switzerland	IgM	S
VPM36	MHC II (DQ)	Dept. Vet. Path. Edinburgh University	IgG_1	S
VPM54	MHC II (DR)	Dept. Vet. Path. Edinburgh University	IgG_1	S
TH14B	MHC II (DR)	VMRD, Pullman, USA	IgG_{2a}	A/F
VPM32	Macrophage (CD14)	Dept. Vet. Path. Edinburgh University	IgG_{2a}	S
VPM65	Macrophage (CD14)	Dept. Vet. Path. Edinburgh University	IgG_1	S
VPM66	Macrophage (CD14)	Dept. Vet. Path. Edinburgh University	IgG_1	S
VPM67	Macrophage (CD14)	Dept. Vet. Path. Edinburgh University	IgG_1	S
CH137A	Macrophage	VMRD, Pullman, USA	IgM	A/F
DH59B	Macrophage (GM1)	VMRD, Pullman, USA	IgG_1	A/F

[1] Supernatant (S) or Ascites Fluid (A/F)

Table 4.2

Monoclonal Antibodies (MoAb)-Final panel

MoAb	Specificity	Isotype	Source	Form[3]	Dilution
CC17[1]	CD5	IgG_1	IAH Compton, UK	S	1/10
CACT80-C	CD8 T-suppressor/cytotoxic	IgG_1	VMRD, Pullman USA	A/F	1/200
CC8[2]	CD4 T-helper/inducer	IgG_{2a}	IAH, Compton , UK	S	1/5
DU2104	B-cell	IgM	Basel Institute, Switzerland	S	1/5
DH59B	GM1	IgG_1	VMRD, Pullman, USA	A/F	1/2000
TH14B	MHC II (DR)	IgG_{2a}	VMRD, Pullman, USA	A/F	1/1500
VPM36	MHC II (DQ)	IgG_1	University of Edinburgh	S	1/20

[1] Howard *et al.* (1988).

[2] Howard and Morrison (1991).

[3] Supernatant (S) or Ascites Fluid (A/F)

4.2.4. Immunohistochemistry protocol

The basic protocol for immunohistochemical staining of luteal tissue is described in Chapter 2 (Section 2.5). The technique was based on an avidin-biotin complex (ABC) method described by Hsu *et al.*(1981) and optimised as described below.

4.2.5. Optimisation of immunohistochemical technique.

4.2.5.1. Blocking of endogenous peroxidase

Since endogenous peroxidase staining was very intense in luteal tissue, the blocking of endogenous activity had to be optimised for successful immunohistochemistry. Incubation with glucose oxidase ($5U/ml^{-1}$) was compared with hydrogen peroxide (0.3% H_2O_2 in methanol) for 30 minutes prior to addition of primary antibody.

Hydrogen peroxide was extremely effective at blocking endogenous peroxidase activity but caused a significant loss of luteal cell structure. Although some endogenous staining remained after glucose oxidase treatment, the luteal tissue appeared intact, making quantification of positively stained cells, particularly cells expressing MHC II, much easier. For this reason, glucose oxidase was used throughout.

4.2.5.2. Dilution of primary antibody and incubation time

The panel of monoclonal antibodies was used at a range of dilutions to select optimal dilutions for experimental work. Phosphate-buffered saline (pH 7.3) containing 0.1% sodium azide was used as diluent. In general, ascites fluid was used at dilutions of 1/200-1/2000 and supernatant was used at dilutions of 1/5 to 1/20. Various incubation times and temperatures for primary antibody binding were compared. Incubation overnight at 4°C was found to give clearer staining than 1 hour at 37°C for all monoclonal antibodies tested.

4.2.5.3. Visualisation of product

We compared two peroxidase substrates from Vector Laboratories (Burlingame, USA) that are widely used to visualise the product. Diaminobenzidine (DAB) produced a brown reaction product which was long lasting and did not fade on exposure to light. A red reaction product was obtained by incubating with 3-amino-9-ethyl carbazole (AEC) which resulted in a more distinct stain than DAB against the background of luteal cells. AEC had the disadvantage of the colour on the section fading within one month of preparation however. To overcome this, AEC was used and positive cells on sections were counted as soon as each batch of sections was processed. Incubation with AEC for 10-15 minutes at room temperature was found to be optimal. However, there was some variation depending on which monoclonal antibody was being used. Slides were therefore examined during the incubation and the reaction stopped before 10 minutes if necessary.

4.2.6. Assessment of immune cell distribution in luteal tissue

Prior to counting of positively stained cells, the general distribution of different cell types and MHC II expression was assessed in luteal tissue. Individual lymphocytes and macrophages were found scattered amongst the luteal cells (Fig. 4.1a) and there were also occasional clumps of lymphocytes which tended to be associated with larger blood vessels (Fig. 4.1b). T-lymphocytes and macrophages were found in greater numbers in the capsule of the CL (Fig 4.1c) and in trabeculae extending from the capsule. MHC II expression (DQ and DR) was the most difficult to evaluate because staining was less clearly associated with individual cells.

The primary aim of this study was to investigate the populations of immune cells within luteal tissue at different stages of the oestrous cycle as a first step towards assessing the potential effects of immune cells and their cytokine products within the CL. If the cytokine products of immune cells have a significant role within the CL it is most likely that they will act directly on the surrounding cells. Therefore, after this preliminary investigation, it was decided that positively stained cells would be counted in regions of the CL that were primarily made up of luteal cells. The capsule and associated fibrous areas were therefore avoided, as were fields dominated by large blood vessels. For statistical analysis the mean of counts (40x objective of a binocular microscope) from 6 fields were calculated for each luteal section. Results from each CL were then combined to give a mean figure for each stage of the oestrous cycle (n=6 CL per group) which were then analysed. When assessing MHC II expression only cells with a clear positive stain were included.

4.2.7. Carbol chromotrope stain for eosinophils

Eosinophils were detected on sections from frozen tissue using the carbol chromotrope method described in Chapter 2 (Section 2.6). Sections of gut were used as positive controls.

4.2.8. Non-specific esterase staining of macrophages.

Non-specific esterase staining of macrophages was attempted using the method described by Hudson and Hay (1989) which is described in Chapter 2 (Section 2.7).

FIGURE 4.1a. Individual T-lymphocytes (CD5+) within luteal tissue (x 70).

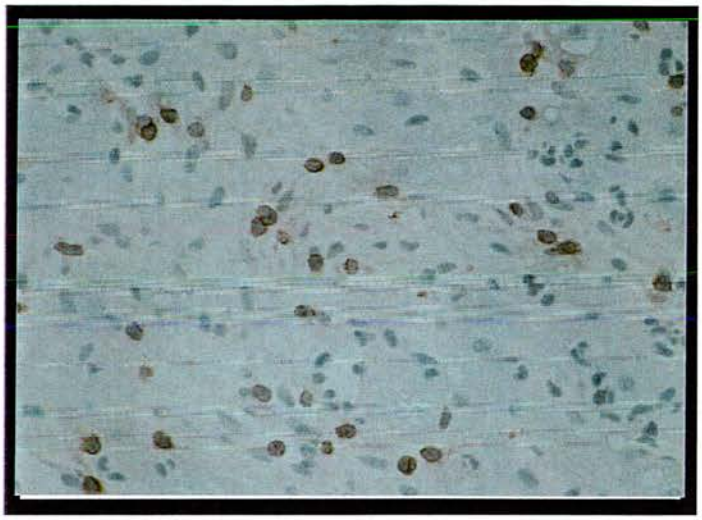


FIGURE 4.1b. T-lymphocytes (CD5+) present around large blood vessels (x 70).

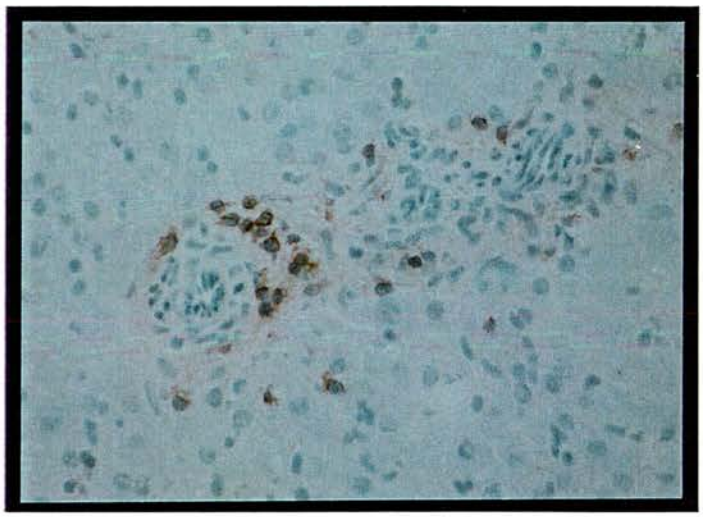
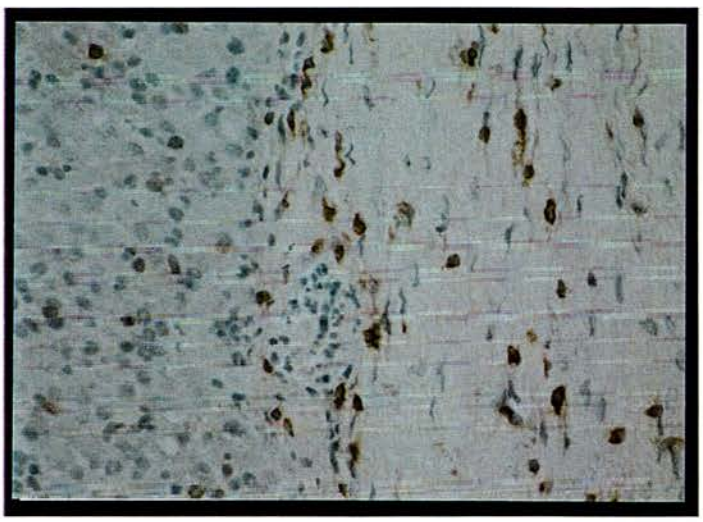


FIGURE 4.1c. T-lymphocytes (CD5+) in large numbers in the CL capsule (x 70).



4.2.9. Statistical analysis of data.

Results were analysed using the Kruskal-Wallis test for non-parametric data and the Mann-Whitney test was then used to analyse differences between samples where present. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Minitab software (Pennsylvania State University).

4.3. Results

4.3.1. T-lymphocytes

T-lymphocytes were found in the CL at all stages of the oestrous cycle but there were some variations in the different sub-groups investigated (Fig. 4.2). There were significantly higher numbers of CD5+ and CD8+ T-lymphocytes ($p<0.01$) at Stage IV of the oestrous cycle compared to stage II. Populations of CD4+ lymphocytes did not vary significantly throughout the oestrous cycle (Fig. 4.2).

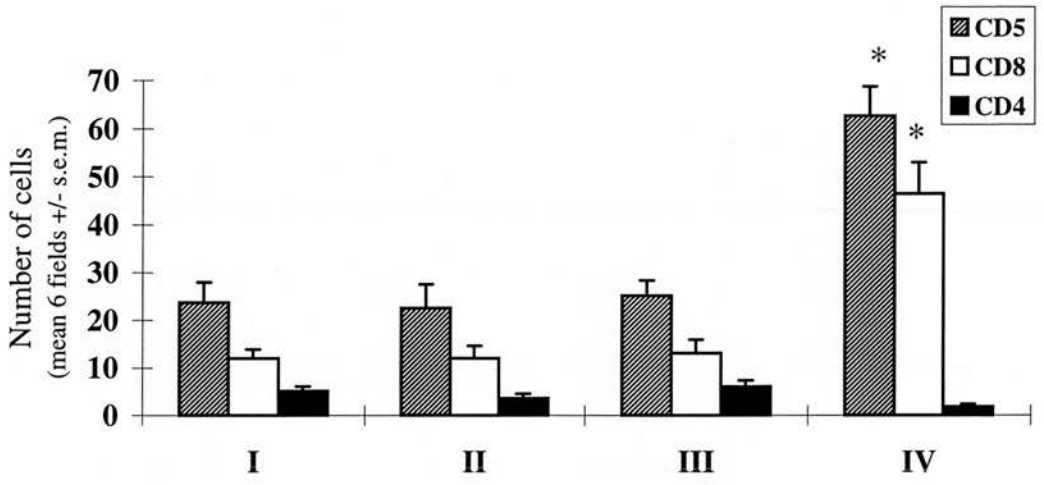
4.3.2. B-lymphocytes

There were few B-lymphocytes in CL at any stages of the oestrous cycle, with most fields containing no positively-stained cells with a maximum of 1-3 cells per 6 areas counted.

4.3.3. Macrophages

Macrophages showed the most variation in numbers compared to the other cell types investigated. There were significantly more macrophages in stage I, III, and IV CL ($p<0.01$) compared to stage II with the highest numbers of cells at stages I and IV (Fig. 4.3).

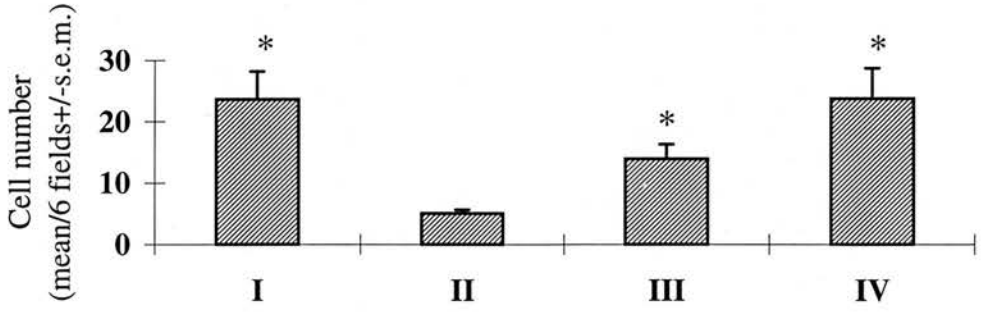
FIGURE 4.2. T-lymphocytes (CD5+, CD8+, CD4+) at Stage I (d1-4), Stage II (d5-10), Stage III (d11-17) and Stage IV (d18-20) of the oestrous cycle.



[1]Each figure represents mean figures from 6 CL.

*There were significantly ($p<0.01$) more CD5+ and CD8+ T-lymphocytes in CL from Stage IV of the oestrous cycle compared to Stage II.

FIGURE 4.3. Number of macrophages in luteal tissue at Stages I (d1-4), II (d5-10), III (d11-17) and IV (d18-20) of the oestrous cycle[1]



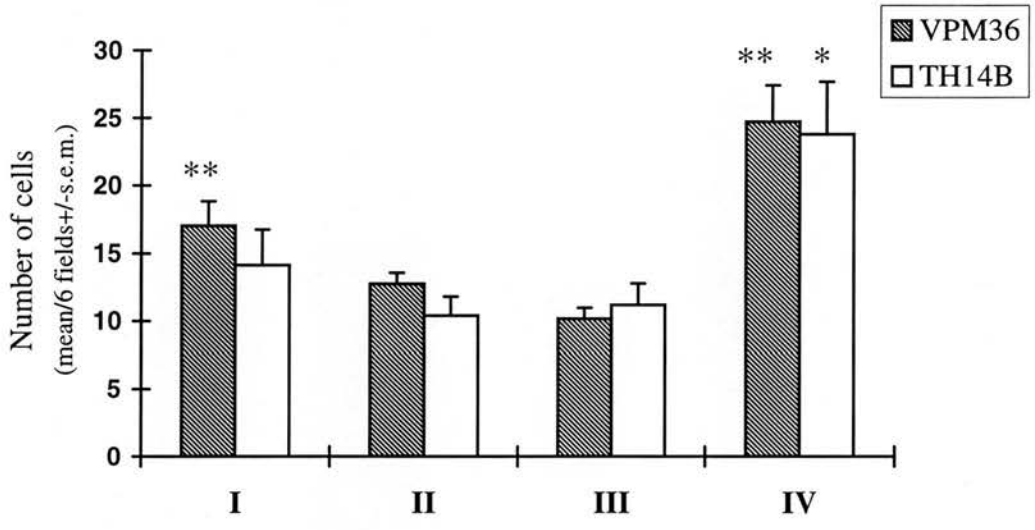
[1]Each figure represents mean for 6 CL.

*There were significantly more ($p<0.01$) macrophages at Stages I, II and IV compared to Stage II.

4.3.4. MHC II Expression

Expression of both MHC II/DR (TH14B) and MHC II/DQ (VPM36) was recorded (Fig. 4.4). MHC II/DR was significantly higher ($p<0.01$) in Stage IV CL compared to stages I-III. MHC II/DQ was significantly higher in both Stage I ($p<0.05$) and Stage IV CL ($p<0.01$) compared to Stages II and III.

FIGURE 4.4. Expression of MHC II/DQ (VPM 36) and MHC II/DR (TH14B) at Stages I (d1-4), II (d5-10), III (d11-17) and IV (d18-20) of the oestrous cycle[1]



[1]Each figure represents mean for 6 CL.

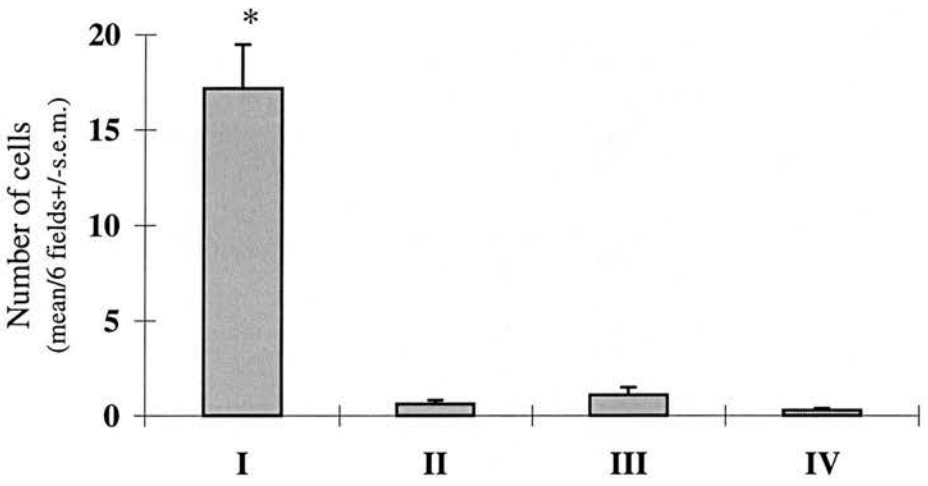
*MHC II/DR expression was significantly higher ($p<0.01$) at Stage IV compared to Stages I-III.

**MHC II/DQ expression was significantly higher at Stages I ($p<0.05$) and IV ($p<0.01$) compared to Stages II and III.

4.3.5. Eosinophils

Eosinophil numbers were significantly higher in stage I CL ($p<0.01$) compared to any other stage (Fig 4.5). The highest numbers of eosinophils were found around the central cavity of the early developing CL and they could easily be seen in sections stained with haematoxylin and eosin (Fig. 4.6a).The presence of these cells could be very dramatic with between 10-75 cells recorded per field in Stage I CL (Fig. 4.6b) compared to 0-3 cells per field in Stages II-IV (Fig 4.6c).

FIGURE 4.5. Number of eosinophils in luteal tissue at Stages I (d1-4), II (d5-10), III (d11-17) and IV (d18-20) of the oestrous cycle[1]



[1]Each figure represents the mean for 6 CL.

*There were significantly more ($p<0.01$) eosinophils in Stage I CL compared to all other stages.

FIGURE 4.6a. Eosinophils within Stage I luteal tissue (H&E, x 70).

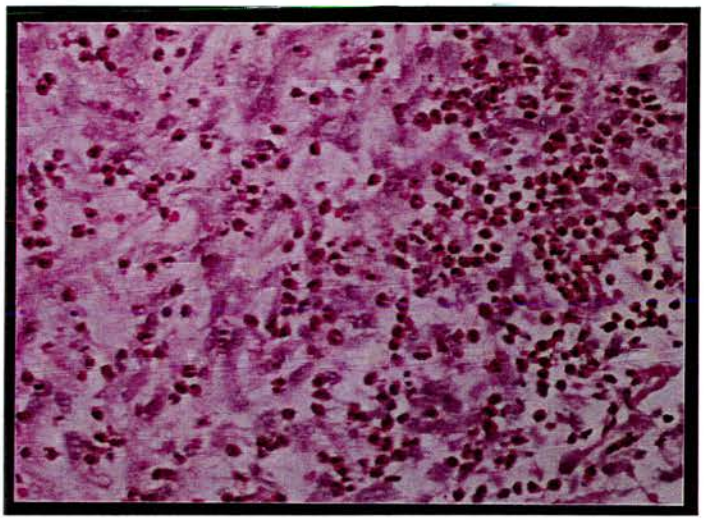


FIGURE 4.6b. Stage I (days 1-4) luteal tissue (carbol chromotrope, x 218).

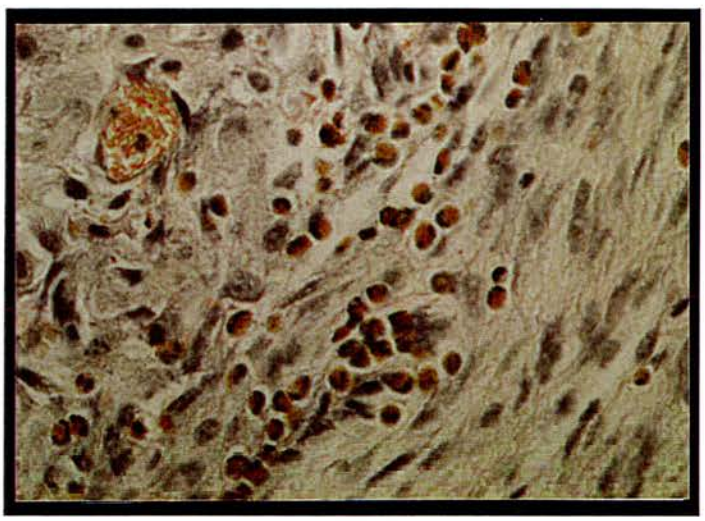
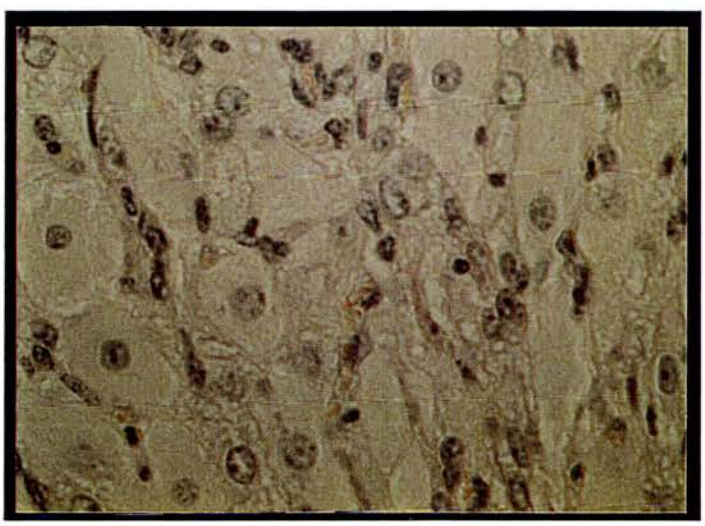


FIGURE 4.6c. Stage II (days 5-10) luteal tissue (carbol chromotrope, x 218).



4.4. Discussion

We have shown that populations of immune cells and MHC II expression vary during the oestrous cycle of the cow, with the most dramatic alterations observed in stage IV CL. At this stage there were significant increases in CD5+and CD8+ lymphocytes, macrophages and expression of both MHC II/DQ and MHC II/DR. These results appear to support the theory that immune cells have a significant role only in the rapid structural dissolution of the CL (structural luteolysis) after release of $PGF_{2\alpha}$ from the uterus has induced functional luteolysis.

This theory was put forward for the cow as early as 1968 when Lobel and Levey reported that macrophages were most abundant in degenerating CL. Our results, which also show an increase in T-lymphocytes and MHC II expression in the cow CL, may give a more complete picture of events occurring at this time compared to the findings of Lobel and Levey, due to advances in immunohistochemical techniques and knowledge of immunological processes. T-lymphocytes, (in particular CD8+, so-called cytotoxic T-cells) also increase during destruction of luteal tissue and are associated with recruitment and activation of macrophages. In comparison B-lymphocytes appear to be insignificant within the cow CL. The lack of B-lymphocytes in cow CL was similar to data from the human CL which reported no B-cells at any stage of the cycle (Wang *et al.*, 1992c).

MHC II expression is now known to be required as part of the recognition processes involved in response of T-lymphocytes to antigen (Roitt, 1991). Therefore, it could be hypothesised that a cycle of attraction of T-lymphocytes and increased MHC II expression, leading to increased attraction and activation of macrophages takes place in the CL after functional luteolysis. Bagavandoss *et al.*, (1988) also suggested that this might be the possible order of cellular events within the rabbit CL at luteolysis although the distinct rise in numbers of T-lymphocytes after luteolysis was not observed in the rabbit.

It would appear from the results described above that we have failed to find any clear evidence of a role for immune cells, apart from the events occurring after luteolysis. However, our results are based on the assumption that gross morphological assessment is sufficiently accurate to allocate CL to different stages of the luteal phase. This is particularly important when investigating events occurring within the CL around key times such as luteolysis, when immune cells are most likely to have some influence.

Some stages of the CL lifespan can be recognised visually with confidence using the criteria described in Chapter 2, Section 2.2.2. (Ireland *et al.*, 1980). Following ovulation, the early CL is easily recognised because of its small size, soft texture and haemorrhagic appearance. Likewise, Stage II CL are relatively easy to identify because of the creamy pink colour and soft pleated appearance of the body of the CL and lack of blood vessels over the surface. After this stage however it becomes progressively more difficult to accurately assess the stage of the luteal phase without the use of individual progesterone profiles which were not used in the present study.

Events within the CL around luteolysis occur very rapidly and there is no way of visually differentiating CL that are in the process of undergoing structural luteolysis from those that are approaching this stage, although events at the cellular level would be expected to be dramatically different. It is likely that any CL that appear to be Stage IV by the criteria of Ireland *et al.* (1980) will have undergone functional luteolysis at least 24 hours previously. Although structural luteolysis also occurs rapidly, there is a time lapse before the gross appearance of the CL changes. Thus, although this method of CL categorisation is widely used and is extremely useful for many studies, we conclude that, for studies of the critical events around luteolysis, it is necessary to collect tissue from animals whose stage of cycle is known accurately from sequential serum progesterone profiles.

There is other evidence which opposes the conclusion that immune cells within the CL play no role around the time of luteolysis until after $PGF_{2\alpha}$ is released from the

uterus, in particular the effects of the cytokine products of immune cells on luteal cells in culture. The cytokine products of T-lymphocytes and macrophages have been shown to have a variety of effects on bovine luteal cells *in vitro*. Interferon-γ (IFN-γ), interleukin-1β (Il-1β) and tumour necrosis-α (TNF-α) all stimulate luteal $PGF_{2\alpha}$ production (Nothnick and Pate, 1990; Fairchild and Pate, 1991; Fairchild Benyo and Pate, 1992). The same studies demonstrated that IFN-γ inhibits basal progesterone production by luteal cells and IFN-γ and TNF-α inhibit LH-stimulated progesterone production by luteal cells in culture. Both of these processes, namely decreased progesterone production and increased $PGF_{2\alpha}$ production, could be associated with events occurring in the time leading up to functional luteolysis implying a role for these immune cell products at this stage.

In addition to the changes in populations of T-lymphocytes, macrophages and MHC II expression in regressing luteal tissue we also observed some significant changes in cell populations at Stage I, *i.e.* during luteinisation and CL formation. Significantly more eosinophils were observed in Stage I CL than at any other stage. This apparent eosinophilia is an interesting finding which has not been described in other species. An increase in eosinophils within the sheep CL was associated with $PGF_{2\alpha}$-induced luteolysis (Murdoch, 1987) and it has been suggested that a chemoattractant may be involved in this process. In the cow this increase in eosinophil numbers occurred during CL formation rather than luteolysis, and it is difficult to suggest a role for these cells at this time. However, it is interesting to note that large numbers of mast cells have been recorded in the pre-ovulatory follicle of the cow which are not present in such numbers in the early CL (Nakamura *et al.*, 1987). This decrease in mast cell numbers may be due to degranulation of the mast cells by the LH surge prior to ovulation, a process which has been described in the hamster by Krishna and Terranova (1985), and could explain the presence of large numbers of eosinophils in early CL, as these cells are attracted by substances released from mast cells as part of the inflammatory response. This process might also explain the high numbers of macrophages found in the CL at this stage.

Finally, in addition to the problems caused by lack of accuracy in visual staging of luteal tissue, the methods of counting for immunohistochemical studies in the CL should also be considered. As discussed in Section 4.2.6. we spent some time considering the distribution of immune cell populations within luteal tissue. T-lymphocytes and macrophages were found in greater numbers in the CL capsule and trabeculae and around large blood vessels, in comparison to areas of CL predominated by luteal cells. In addition, T-lymphocytes were found both individually and in clumps. This lack of homogeneity in cell distribution makes accurate cell counting very difficult. However it is noticeable that few authors appear to take this irregular distribution in to account, making no mention of the problems it presents. We overcame the difficulty by avoiding areas of luteal tissue where the capsule or large blood vessels predominated. The approach to cell counts should be considered when comparing results from different studies.

In conclusion, we have shown that the numbers of some immune cells and MHC II expression vary throughout the oestrous cycle. This appears to be most significant in Stage IV CL, suggesting that the main role of the immune system is in luteal destruction at this stage. However gross luteal morphology is not sufficiently accurate to assess the exact stage of the luteal phase around the time of luteolysis. We conclude that it will be necessary to study immune cell populations in accurately staged luteal tissues before a possible role for immune cells prior to functional luteolysis can be definitely ruled out.

Chapter 5

Immune cell populations in the bovine corpus luteum around the time of luteolysis.

5.1. Introduction

The results from the previous chapter demonstrate that, as in many other species, there are variations in the numbers of immune cells and MHC II expression in the cow CL throughout the oestrous cycle. In particular, we demonstrated an increase in T-lymphocytes, macrophages and MHC II expression in CL described as stage IV (days 18-20). However, in order to assess when exactly these changes in cell populations took place, we concluded that it would be necessary to look in detail at CL collected around luteolysis because the methods used to stage CL visually are not accurate enough to differentiate between CL immediately before or immediately after luteolysis.

The potential role of immune cells around luteolysis is of particular interest because so little is known about what is happening at the cellular level at this time. $PGF_{2\alpha}$ from the uterus has been shown to be the primary luteolytic substance in the cow (Knickerbocker *et al.*, 1988, for review) and luteolysis can be artificially induced using synthetic prostaglandin from around day 5 of the oestrous cycle (Hafs *et al.*, 1974). In the light of this knowledge the potential role of any other cells might seem somewhat irrelevant. However, although uterine $PGF_{2\alpha}$ causes luteolysis, it is not clearly understood how this prostaglandin causes luteolysis of the CL, nor how the release of $PGF_{2\alpha}$ from the uterus is controlled.

Immune cells and their cytokine products have recently been the centre of much debate as to their function in modulation of ovarian activity (Adashi, 1989, 1990; Brannstrom and Norman 1993; Pate, 1995). Rapidly advancing knowledge about the immune cells and their products suggests that these cells are not only involved in

destruction of the CL after luteolysis, but may also play an active role within luteal tissue at other stages, particularly around the key events of ovulation and luteolysis. However, because of the potential number of events and cell interactions involved it will be difficult to clarify events occurring *in vivo.*

Lobel and Levey (1968) recorded an increase in lymphocytes from day 14 of the oestrous cycle in the cow, a finding that has not been investigated further. Studies in the pig, human and rat (Standaert *et al.*, 1991; Brannstrom *et al.*, 1994c; Brannstrom *et al.*, 1994a) have not shown a similar increase in T-lymphocytes but there appears to be a large inter-species variation in immune cell populations within the CL. In order to investigate immune cell populations in bovine luteal tissue further we looked more specifically at CL around the time of luteolysis in the non-pregnant cycle, between days 16 and 20 of the oestrous cycle. The various immune cell types and MHC II expression present were detected by immunohistochemistry and the results were compared with tissue from CL collected on days 13 and 14 of the oestrous cycle.

5.2. Materials and methods

5.2.1 Oestrous Synchronisation

Twenty seven mature Friesian-cross heifers were selected from the Roslin Institute herd and were examined *per rectum* to ensure that they had normal reproductive tracts. Oestrus was synchronised in groups of 6-7 animals by insertion of progesterone-impregnated intravaginal devices (PRID, Sanofi Animal Health, Watford, U.K.) for 12 days. Cloprostenol (50μg), a synthetic $PGF_{2\alpha}$ analogue (Estrumate, Pitman-Moore LTD, Crewe, U.K.) was given by intramuscular injection on day 10. Following PRID removal, the animals were observed for signs of oestrus 3 times daily and the date and time of standing oestrus recorded. Blood samples were collected for progesterone assay to confirm oestrus at this time.

Throughout oestrous synchronisation and prior to slaughter the animals were housed in slatted courts and fed a maintenance ration of silage and mineral supplement with free access to water. One animal was excluded during the trial as she developed mastitis. Animals were then sent for slaughter in four groups, varying the time from oestrus to slaughter in order to collect CL at different stages around luteolysis. Blood samples (7ml) for progesterone analysis were collected daily by coccygeal venepuncture for 1 or 2 days prior to slaughter. Plasma was harvested after centrifugation (30 min, 1000 *g*) and stored at -20°C. The cows were again observed 2-3 times daily for signs of oestrous behaviour in the days leading up to slaughter.

5.2.2 Tissue collection and preparation.

Both ovaries were collected within 20-30 minutes of slaughter along with a 10ml blood sample (Chapter 2, Section 2.2.1.). The ovaries were examined and any structures (CL or follicles >10mm) present were recorded and measured. The CL was removed from the body of the ovary as quickly as possible, the stage of cycle visually assessed as previously (Ireland *et al.*, 1980, Chapter 2, Section 2.2.2.) and then cut into four pieces using a scalpel blade. The tissue was then either snap frozen in OCT on a cork disc for immunohistochemistry (1 piece), snap frozen in foil for molecular studies (2 pieces) or placed in 10% buffered neutral formalin for preparation of paraffin blocks (1 piece). Blood samples were transported to the laboratory on ice, centrifuged (30 min, 1000 *g*), plasma collected and stored at -20°C until progesterone assay. The snap frozen tissues were transported to the laboratory on dry ice and stored at-70°C. Samples in buffered formalin were removed from the formalin after 24 hours and then processed through to paraffin wax on a Tissue-Tek VIP 2000.

5.2.3 Haematoxylin and eosin stain for general morphology

Sections (8μm) were cut from each block of paraffin-embedded tissue and processed automatically using a Linistain GLX 22 for staining with haematoxylin and eosin as described in Chapter 2 (Section 2.8). All sections were examined using high power microscopy (magnification 40x) to assess general morphology of the tissue.

The tissue sections from animals around the time of luteolysis (days 16-20) were assessed in more detail in an attempt to decide if it was possible to tell by morphology alone whether functional luteolysis had occurred. Four criteria were used to compare sections; 1) loss of cell structure, 2) vacuolation of large cells, 3) intensity of cellular appearance (*i.e.* numbers of cells between luteal cells, within blood vessels or capsule) and 4) any other distinctive features. The first three categories were graded individually between 0 and 3+ (where 0 indicated intact morphology, absence of vacuoles and lack of intense cellular reaction in the tissue and 3+ indicated severe loss of cellular structure, associated with structural luteolysis, extensive vacuolation of large cells or an intense cellular appearance). The fourth category included more specific features not included in the other categories such as the appearance of tissue whorls or unusual staining patterns. This part of the study was completed before any progesterone or $PGF_{2\alpha}$ metabolite assay results were known.

5.2.4. Progesterone assay

Progesterone concentrations were used to monitor whether CL had undergone functional luteolysis in the days leading up to slaughter. As the decrease in progesterone concentrations occurs prior to structural regression, this is the most useful indicator of alterations in luteal function.

Progesterone was assayed as described in Chapter 2 (Section 2.3.2.). All samples were assayed in a single assay with an intra-assay coefficient of variation (CV) of 13.9%. The assay sensitivity was 0.1ng/ml and values of less than 1ng/ml were considered to indicate that luteolysis had occurred.

5.2.5. 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) assay

In order to give some indication of the systemic concentrations of $PGF_{2\alpha}$ around the time of slaughter, plasma samples were also assayed by non-extraction radioimmunoassay for 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) which is the stable metabolite of $PGF_{2\alpha}$ (Kindahl *et al.*, 1976a; Grannstrom and Kindahl, 1982). The

limit of detection was 8.9-10.6 pg/ml when assaying 0.5ml of plasma. The samples were kindly assayed by Professor Hans Kindahl at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

5.2.6 Immunohistochemistry

Tissue was processed using the method described in detail in Chapter 2 (Section 2.5.). The same monoclonal antibodies as those described previously (Chapter 4, Table 4.2) were used in this study.

5.2.7 Eosinophils

Eosinophils were detected using the carbol chromotrope method described in Chapter 2 (Section 2.6.). Paraffin-embedded tissue was used instead of frozen tissue. This required the inclusion of a deparaffinisation and hydration step prior to staining with haematoxylin.

5.2.8 Cell counts

As described in Chapter 4 (Section 4.2.6.) 6 fields of predominantly luteal cells were counted for each section, avoiding the capsule, trabeculae and major blood vessels.

5.2.9 Statistical analysis

The results from immunohistochemistry were analysed using the Kruskal-Wallis test for non-parametric data. The Mann-Whitney test was then used to analyse differences between groups where present. A p value of less than 0.05 was considered to be significant. All statistical analysis was performed using Minitab software (Pennsylvania State University).

5.3. Results

5.3.1. Stage of oestrous cycle and ovarian structures

In this experiment variation in time span from oestrus (day 0) to slaughter resulted in CL collected between days 13 and 20 of the oestrous cycle. For ease of comparison these animals were initially divided into 3 groups; days 13-14 (n=7), days 16-17 (n=6) and days 18-20 (n=12).

CL were visually assessed using the criteria described by Ireland *et al.* (1980), in order to estimate the stage of cycle. All CL, except 2 discussed below, were identified as stage III CL. Luteal tissue was orange in colour throughout with surface capillaries visible on the external surface. There was some variation in size, (between 1.5 and 3.5cm diameter), which is a larger range than that described by Ireland.

The ovarian structures recorded at time of slaughter are presented in Table 5.1. As expected there was some variation in the structures present at different stages and the findings were consistent with the known stage of the oestrous cycle in all but 2 animals. Cow 988 had a fluid-filled cystic structure of 4cm diameter on one ovary and cow 961 had a CL with a red apex and soft texture, findings consistent with a CL from stage II (days 5-10) of the oestrous cycle. This was not unexpected as this cow had not been recorded to be in oestrus at any time during oestrous synchronisation. Both of these animals were excluded from the study.

The most obvious structure on the ovaries from animals around day 13-14 of the oestrous cycle was a CL of around 2.5-3.5cm diameter. There was some variation in the number and size of follicular structures present on the ovaries at this stage of the cycle. Follicular waves have been shown to begin on days 2, 9 and 16 in heifers with three follicular waves, and days 2 and 11 in heifers with two waves of follicular development (Sirois and Fortune, 1988). Between days 16 and 20 there was a 2-3cm CL and at least 1 follicle of 1-1.5cm diameter in 13 out of 18 animals.

In late-luteal stage animals at day 19-20 (especially cow 957, which was later found to have low progesterone for 2 days before slaughter) large follicles (>10mm) or collapsed follicles/ very early luteal structures were not recorded although these might have been expected to be present.

Table 5.1. Cow number, day of cycle (oestrus=day 0) and ovarian structures on ovaries collected from synchronised heifers.

COW No	Age of CL	Ovary 1	Ovary 2
11030	d13	3cm CL / 2x1.5cm follicles	NSS[1]
11108	d13	2.5cm CL / 1.5cm follicle	NSS
7333	d13	3.5cm CL	NSS
11671	d14	3cm CL	1cm follicle
10766	d14	2.5cm CL / 2cm follicle	NSS
1238	d14	3cm CL / 1.5cm follicle	MSF[2] / degen CL
10861	d14	3cm CL	NSS
972	d16-17	2.2cm CL	1.5cm follicle / MSF
958	d17	3cm CL/ 1.5cm follicle	MSF
956	d17	1.5cm CL / 2cm CL	1cm follicle/ MSF
964	d17	1.5cm CL / 1cm follicle	2.5cm CL / 1cm follicle
970	d17	2.5cm CL	NSS
980	d17	2cm CL	1cm follicle / MSF
987	d18	NSS	2.5cm CL / 1.5cm follicle
962	d18	MSF	2cm CL / 2x1cm follicles
974	d18	1.5cm follicle / MSF	2cm CL
959	d19	2.5cm CL / 1cm follicle	MSF
971	d19	2cm CL	MSF
983	d19	2.5cm CL	MSF
978	d19	1.5cm CL / 3cm follicle	2x1.5cm follicle
979	d19	2.5cm CL / 1.75cm follicle	MSF
982	d19	2cm CL	MSF
955	d19	MSF	2cm CL / 1cm follicle
957	d20	MSF	1.5cm CL
984	d20	1cm follicle / MSF	2cm CL

[1] NSS= no specific structures

[2] MSF= multiple small follicles (<0.5cm)

5.3.2. Examination of H & E stained sections.

General tissue morphology was studied to assess whether H&E staining of sections might allow more accurate staging of luteal tissue than visual assessment alone, particularly around luteolysis. If so, this technique might be a useful addition to staging CL by external appearance alone.

General examination of tissue morphology showed clear differences between the day 13-14 CL and CL from days 16-20. Day 13-14 tissue appeared very homogenous with luteal cells, in particular large luteal cells, the most immediately obvious structures. The stain was taken up evenly and luteal cells appeared intact (Fig. 5.1a). Luteal tissue collected between days 16-20 appeared more cellular, luteal cells were more intensely stained and large luteal cells were not the obviously predominant cell type. There were many clear, small, darkly-staining cells (probably leukocytes) between the luteal cells. Their appearance was sometimes quite dramatic (Fig. 5.1b). The blood vessels also appeared more prominent, sometimes because of clumps of cells either within or close by the lumen. (Fig. 5.1c) The intensity of staining was more uneven, especially on the large luteal cells which took up the stain very strongly. Any capsule or trabeculae extending into the tissue appeared highly cellular, again with clumps of darkly-staining cells. Obviously there was some variation, as sections were not cut from exactly the same region of CL, but in general the whole picture at this stage was quite different from day 13-14 CL.

We also attempted to predict whether functional luteolysis had taken place in any of the later stage CL (from day 16 of the oestrous cycle onwards) using morphology alone (Table 5.2). There was only one animal (cow 957) with a CL morphology that was distinctly different from the other animals in this group. This animal was predicted already to have undergone functional luteolysis. The assay results later confirmed this, along with functional luteolysis in another 4 cows (979, 955, 982 and 984) that we had not predicted.

FIGURE 5.1a. Day13-14 luteal tissue (H&E, x 218)

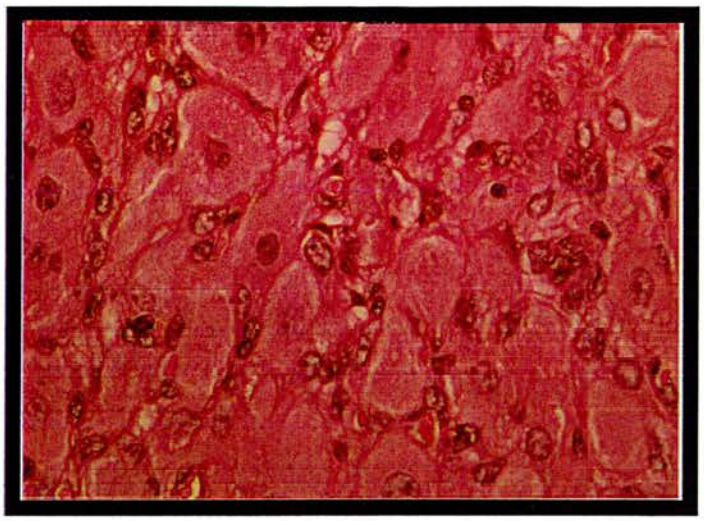


FIGURE 5.1b. Day 19 luteal tissue containing large numbers of lymphocytes (H&E, x 70).

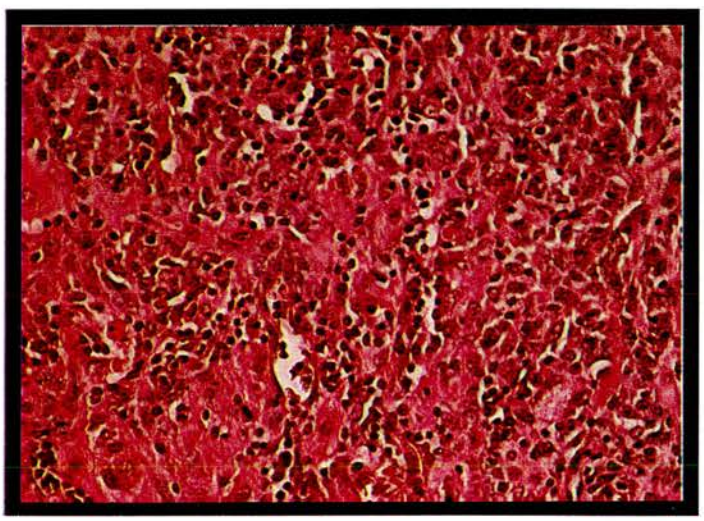


FIGURE 5.1c. Day 18-19 luteal tissue. Lymphocytes in blood vessel (H&E, x 218)

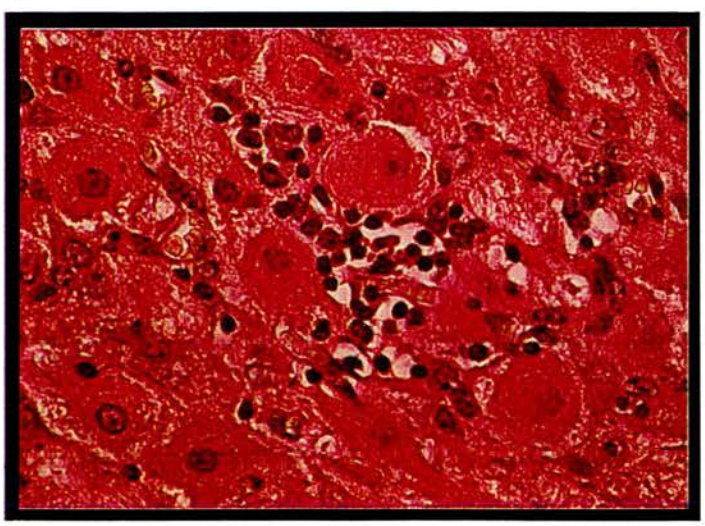


Table 5.2. Appearance of sections stained with haematoxylin and eosin

COW No	Age of CL	Loss of cell structure[1]	Vacuolated large cells[1]	Cellular intensity[2]	Distinctive features[2]	Luteolysis based on morphology	Luteolysis based on P_4
972	d16-17	3+	2+	1+	Immune cells clear between luteal cells	-	-
958	d17	2+	0	1+	? whorls forming	-	-
956	d17	2+	1+	3+	Very vascular, start of whorls.	-	-
964	d17	2+	1+	1+	large luteal cells-uneven staining	-	-
970	d17	2+	0	2+		-	-
980	d17	3+	0	2+	Immune cells close to blood vessels	-	-
987	d18	2+	1+	1+	Multiple obvious blood vessels	-	-
962	d18	3+	0	2+	Intense stain	-	-
974	d18	3+	1+	1+		-	-
959	d19	3+	0	2+		-	-
971	d19	2+	1+	2+	Uneven staining on large luteal cells	-	-
983	d19	3+	0	1+	Multiple obvious blood vessels	-	-
978	d19	1+	0	3+	? start of whorls	-	-
979	d19	3+	0	2+		-	+
982	d19	3+	0	1+	Immune cells present in distinct trabeculae	-	+
955	d19	2+	0	2+		-	+
957	d20	0	1+	4+	Presence of whorls	+	+
984	d20	1+	0	3+	Immune cells close to blood vessels	-	+

[1]Refers to luteal cells.

[2]Includes all cell types

5.3.3. Progesterone profiles

Progesterone concentrations are shown in Table 5.3. All animals slaughtered between days 13-14, 16-17 and 7 cows slaughtered between days 18-20 had progesterone concentrations consistent with a functional CL prior to and at the time of slaughter (progesterone >1ng/ml). Five animals slaughtered between days 18 and 20 had undergone functional luteolysis as indicated by low progesterone concentrations at the time of slaughter, although there was some variation in the exact time that this decrease took place.

5.3.4. PGFM assay

PGFM concentrations are shown in Table 5.3. The results show considerable variation between samples from the same animal on different days and between animals. This is related to many of the cows being around the time of luteolysis which could occur at any point after day 17 of the oestrous cycle although there is some variation between animals.

PGFM concentrations greater than 100pg/ml were measured in 13 out of the 18 animals which were between days 16 and 20 of the oestrous cycle on the day of slaughter. Only one of these animals (cow 955) had a concentration of greater than 100pg/ml in a previous sample and this animal had undergone luteolysis at the time of slaughter, as demonstrated by the low progesterone concentration. Concentrations greater than 150pg/ml were measured in 3 animals at slaughter, 2 animals had high progesterone concentrations (974, 959) and 1 animal had a low progesterone concentrations (982). Such high levels are only likely to be found during the peaks of $PGF_{2\alpha}$ release at luteolysis. However, as would be expected at so late a stage in the lifespan of the CL, it also appears that many of the other animals were also coming under the influence of increasing concentrations of $PGF_{2\alpha}$ at this time.

Table 5.3. Progesterone (P4, ng/ml) and PGFM (PG, pg/ml) profiles around slaughter. Results are presented as progesterone results in normal type for all animals and PGFM results from animals from day 16 onwards bracketed in bold type.

Cow No	Age of CL	-2 days P4 (**PG**)	-1 day P4 (**PG**)	Day 0 P4 (**PG**)
11030	d13	n/s[1]	n/s	5.5
11108	d13	n/s	n/s	2.3
7333	d13	n/s	n/s	3.2
11671	d14	n/s	n/s	2.2
10766	d14	n/s	n/s	3.1
1238	d14	n/s	n/s	1.4
10861	d14	n/s	n/s	2.3
972	d16-17	2.8 (**68.4**)	2.0 (**50.4**)	3.5 (**114.9**)
958	d17	4.1 (**72.0**)	4.6 (**57.4**)	4.3 (**80.8**)
956	d17	5.3 (**58.5**)	4.1 (**43.9**)	6.1 (**111.3**)
964	d17	7.0 (**44.7**)	4.7 (**44.3**)	6.9 (**69.9**)
970	d17	3.3 (**55.3**)	5.1 (**43.6**)	3.6 (**91.5**)
980	d17	4.13 (**77.6**)	3.0 (**57.1**)	4.1 (**115.2**)
987	d18	n/s	3.3 (**46.1**)	2.9 (**138.3**)
962	d18	n/s	3.3 (**58.2**)	4.7 (**73.0**)
974	d18	n/s	3.7 (**45.4**)	3.3 (**242.9**)
959	d19	3.1 (**58.5**)	1.2 (**59.9**)	2.7 (**197.5**)
971	d19	5.5 (**49.6**)	2.3 (**78.0**)	2.6 (**117.7**)
983	d19	5.1 (**57.8**)	2.3 (**57.4**)	3.6 (**114.5**)
978	d19	n/s	1.81 (**71.3**)	1.9 (**109.6**)
979	d19	n/s	0.6* (**53.9**)	0.3* (**144.7**)
982	d19	2.5 (**47.9**)	0.7* (**51.8**)	0.7* (**231.9**)
955	d19	2.3 (**123.8**)	0.9* (**66.3**)	0.9* (**112.1**)
957	d20	0.6* (**50.7**)	0.4* (**63.1**)	0.2* (**78.7**)
984	d20	n/s	1.5 (**63.5**)	0.7* (**116.7**)

[1]n/s = no sample collected.

* progesterone <1ng/ml

5.3.5. Immunohistochemistry

Using the above information, particularly progesterone concentrations, the animals were divided into four groups for comparison of immunohistochemistry results;

Group A -Days 13-14 (n=7),

Group B -Days 16-17 (n=6),

Group C1 -Days 18-20, high progesterone at slaughter (n=7),

Group C2- Days 18-20, low progesterone at slaughter (n=5).

Immune cell populations and MHC II expression in CL were compared (Table 5.4).

Table 5.4. Immune cell populations (mean ± s.e.m.) around luteolysis. Each figure represents mean results from a minimum of 5 animals.

CELL TYPE	Group A (d13-14)	Group B (d16-17)	Group C1 (d18-20/highP4)	Group C2 (d18-20/low P4)
CD5+ T-lymphocytes	25.66 ± 1.9	49.75** ± 2.3	43.17* ± 3.7	50.50** ± 1.2
CD8+ T-lymphocytes	16.06 ± 3.1	31.78* ± 6.7	34.95* ± 6.6	40.67* ± 11.2
CD4+ T-lymphocytes	5.82 ± 0.8	3.36 ± 0.3	5.07 ± 0.9	4.63 ± 1.5
Macrophages	9.74 ± 2.7	7.81 ± 1.1	5.09 ± 1.0	10.7 ± 3.9
MHC II+ (DQ)	10.33 ± 1.7	10.06 ± 1.4	8.57 ± 0.5	10.03 ± 1.7
MHC II+ (DR)	7.48 ± 0.7	12.11 ± 3.1	9.09 ± 1.4	10.63 ± 1.4
Eosinophils	0.38 ± 0.2	0.94 ± 0.2	0.64 ± 0.2	5.03 ± 2.9
B-cells	0	0.28 ± 0.1	0.02 ± 0.05	0

* $p < 0.05$ compared to day 13-14

** $p < 0.01$ compared to day 13-14

There were significantly higher numbers of CD5+ T-lymphocytes in Groups B ($p<0.01$), C1 ($p<0.05$) and C2 ($p<0.01$) *i.e.* all CL collected from day 16 of the oestrous cycle onwards, compared to group A. There were also significantly higher

numbers of CD8+ T-lymphocytes in groups B ($p<0.05$), C1 ($p<0.05$) and C2 ($p<0.05$) compared to group A. There were no significant differences in CD5+and CD8+ lymphocyte numbers between C1 and C2. CD4+ cell numbers were not significantly different between any of the groups, and B-cells were recorded only very occasionally in any of the sections counted (Table 5.4).

There were no significant differences in numbers of macrophages, eosinophils nor in MHC II expression (DQ or DR) between groups (Table 5.4).

5.4. Discussion

In this study cell morphology and immune cell populations were investigated in the bovine corpus luteum around the time of luteolysis. It was not possible to assess whether luteolysis has taken place by visual appearance of the CL or cell morphology alone, but progesterone concentrations were a useful additional technique when staging CL. The most significant finding when studying immune cell populations was a significant increase in the numbers of CD5+ and CD8+ T-lymphocytes in luteal tissue collected from all cows after day 16 of the oestrous cycle compared to CL collected between days 13-14. Cell numbers were not significantly different in animals that had undergone functional luteolysis and those that had not.

In the previous chapter we concluded that visual assessment of CL to differentiate between stages III (days 11-17) and IV (days 18-20) of the oestrous cycle was not sufficiently accurate. This study, in which all CL used were visually categorised as stage III, confirms that it is not possible to predict whether luteolysis has taken place by gross appearance alone and that structural luteolysis may have been underway for some time before the gross appearance of the CL changes.

We found that morphological changes assessed by examination of tissue sections stained with H&E were a useful addition to visual staging only when differentiating early stage III (days 13-14) luteal tissue from CL collected between days 16-20. It

was not possible by examining cell morphology to differentiate animals which had undergone luteolysis from those that had not when studying CL collected between days 16 and 20 of the oestrous cycle. Only one animal (cow 957) had a sufficiently distinct luteal morphological appearance to indicate that this cow had already undergone luteolysis at the time of slaughter. It is interesting to note that cow 957 had undergone functional luteolysis at least 48 hours before slaughter, as P4 levels were less than 1ng/ml in all 3 plasma samples. This time lapse between functional luteolysis and collection of the CL explains the obvious structural regresssion apparent on examination of cell morphology.

PGFM concentrations alone were also of limited use in establishing whether luteolysis had taken place. At luteolysis $PGF_{2\alpha}$ is released in a pulsatile fashion with peaks of increasing frequency and magnitude, prior to the decline in progesterone concentrations (Silvia *et al.*, 1991; Kindahl *et al.*, 1976b). Therefore it is difficult to interpret results from single samples at this stage. However, concentrations of PGFM prior to luteolysis (around days 14-16) have been recorded by various workers as being in the region of 60-70pg/ml (Kindahl *et al.*, 1981),100pg/ml (Vighio and Liptrap, 1986) and 70pg/ml (Basu and Kindahl, 1987). In these studies, the concentrations of PGFM are relatively constant prior to luteolysis with only small peaks and these figures can therefore be used as a guide whereby it can be assumed that concentrations above 100pg/ml are likely to be associated with luteolysis. It is not possible however to draw conclusions from figures less than 100pg/ml because these could be associated with either pre-luteolytic concentrations or could be concentrations in the troughs of luteolytic pulses.

The less pulsatile nature of progesterone release meant that progesterone assay of a sample collected at slaughter appeared to be the single most useful method for differentiating between animals that had or had not undergone luteolysis. Categorisation of the stage of CL by visual assessment is a widely-used technique in studies using tissue from animals with unknown reproductive histories. Obvious changes in the gross appearance of the CL make staging stage I, II and late stage IV

CL relatively easy. This is not the case at stage III or early stage IV, when collection of a blood sample at the time of slaughter followed by progesterone assay would significantly improve the accuracy of visual assessment.

The most interesting finding in this study was the increase in T-lymphocytes (CD5+ and CD8+) from day 16 of the oestrous cycle which occurred in animals prior to functional luteolysis. The presence of T-lymphocytes has been described in various species at different stages of the cycle including the rabbit (Bagavandoss *et al.*, 1990), rat (Brannstrom *et al.*, 1994a), pig (Standaert *et al.*, 1991) and human (Brannstrom *et al.*, 1994c; Bukovsky *et al.*, 1995a; Wang *et al.*, 1992c). There are distinct differences between species but this may be due partly to differences in the methods and range of monoclonal antibodies used in each study. None of these studies describe T-lymphocyte populations in detail around the time of luteolysis.

As discussed in Chapter 4, the presence of lymphocytes prior to luteolysis has been described previously (Lobel and Levey, 1968). It is certainly likely that these cells play a key role in events occurring after luteolysis but their presence several days prior to this event might also suggest a more specific role for these cells within the functional CL at this stage. T-lymphocytes and their cytokine products have been shown to have a variety of effects on hormone production by luteal cells in culture which might suggest a role for these cells within luteal tissue before $PGF_{2\alpha}$ release from the uterus (see Pate and Townson, 1994 for a review). Lymphocytes have been shown to increase progesterone production by human granulosa luteal cells *in vitro* (Emi *et al.*, 1991) whereas Adashi *et al.*, (1990) found that TNF-α inhibited hCG-supported progesterone production in luteinised granulosa cells from the rat.

The role of these cells and other immune cell types has been much discussed in recent years but few clear conclusions have been drawn (Pate, 1994; Brannstrom and Norman, 1993), mainly because T-lymphocytes have a range of potential effects on surrounding tissues through the production of a variety of cytokines. Such is the

potency of these cell products that very few cells would be required to have a dramatic local effect within the CL.

CD4+ T-lymphocytes, eosinophils, macrophages and MHC II expression were not significantly different between groups. These findings for CD4+ lymphocytes and eosinophils were similar to the previous study in which we also found no change in CD4+ cells and showed that eosinophils were significantly higher only between days 1-5 of the oestrous cycle compared to all other stages (Chapter 4). However, the lack of differences in macrophage populations and MHC II expression between groups was unexpected when compared with our previous results. Using visually staged tissue, as described in Chapter 2, we have shown that MHC II (DQ and DR) expression was significantly higher in stage IV CL (days 18-20) when compared to stage III CL (days 13-18). Populations of macrophages were highest at Stage IV, although this was not significantly greater than Stage III. As we were working with the same range of age of tissue, we might have expected similar results in the present study. This assumes however that visual staging of luteal tissue is accurate and it is likely that the apparent differences between the two studies are due to a wider variation in the age of tissue used in the first study.

In particular, it is likely that the Stage IV CL (days 19-20) selected by visual assessment of ovarian structures in the first study were older than those described as Stage IV (days 19-20) in the present study. CL in our previous study were staged on a variety of characteristics, including the presence of a follicle (greater then 10mm) on either ovary at slaughter. In reality, the follicles present on ovaries described previously as stage IV were often larger than 10mm and were more likely to be preovulatory follicles. However, it takes several days for the ovulatory follicle to develop after luteolysis. We commented earlier on the apparent lack of large follicles in ovaries collected from animals around day 19-20 of the oestrous cycle in the present study. However, this may indicate collection of CL prior to maturation of the final follicular wave.

In conclusion, there is an increase in T-lymphocytes (CD5+, CD8+) within bovine luteal tissue from day 16 of the oestrous cycle onwards. Although these cells are actively involved in destruction of luteal tissue after luteolysis these results also suggest a potential role for them in the CL prior to luteolysis. It is likely that these cells will exert any effect at this stage through release of their cytokine products. However, the variety of these substances and their range of potential effects on luteal cells at low concentrations make accurate analysis of events occurring at the cellular level very difficult. The mechanism by which the lymphocytes are attracted into luteal tissue at this stage of the cycle is also not known.

Chapter 6

Immune cell populations in the bovine corpus luteum following luteolysis artificially induced using exogenous $PGF_{2\alpha}$

6.1. Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is released from the uterus and transported to the ovary via a venoarterial countercurrent system, has been shown to be the luteolytic substance in the cow (Knickerbocker *et al.*, 1988; Silvia *et al.*, 1991). However the exact order of events that take place within the CL itself during luteolysis is not clear (Auletta and Flint, 1988). There is some evidence that a positive feedback mechanism is involved between prostaglandin release from the uterus and oxytocin release from the CL (Silvia *et al.*, 1991), although local production of various types of prostaglandin by luteal cells could also be involved (Milvae, 1986). In recent years there has also been increased interest in the potential role of immune cell populations within the CL at this time (Pate, 1995).

In order to investigate these events further, it is necessary to collect luteal tissue at specific time periods around luteolysis. However this is no easy task because of the difficulty in anticipating luteolysis *in vivo* and the rapid progression of events which take place at the level of the CL once luteolysis is initiated. For this reason induced luteolysis has been used as a model for studying events within the CL around natural luteolysis. Natural and artificially-derived prostaglandins can be used in the cow to induce luteolysis during the normal oestrous cycle from around day 5 onwards (Hafs *et al.*, 1974; Henricks *et al.*, 1974).

There is evidence that induced luteolysis is an appropriate model to be used for studies looking at luteolysis in the cow and sheep. Stacey *et al.* (1976) showed that the morphological changes associated with induced luteolysis in the sheep were similar to those occurring during natural luteolysis and in the cow. Spicer *et al.* (1981) found that similar hormonal changes occurred during induced and natural luteolysis. Changes in blood flow are also similar between the two processes in sheep (Niswender *et al.*, 1976). There would appear to be no difference between the hormonal response induced by using $PGF_{2\alpha}$ or a synthetic product to induce luteolysis (Baishya *et al.*, 1994).

Although the hormonal changes in the cow during induced and natural luteolysis are similar, it is not known whether the morphological changes within the CL are also comparable. This is an important question if induced luteolysis is to be used successfully as a model to study cellular events around luteolysis and, in particular, changes in immune cell populations within the CL at this time. The purpose of this study therefore was to collect luteal tissue at several time periods after artificially inducing luteolysis and examine tissue morphology, immune cell populations and MHC II expression within the CL to assess whether induced luteolysis resulted in similar changes to cell populations to those previously described following natural luteolysis (Chapter 5). In addition, the results of analysis of MHC II expression using flow cytometry were compared with those of immunohistochemistry to assess whether this technique would be useful in future studies.

6.2. Materials and methods

6.2.1. Oestrous synchronisation and induction of luteolysis

Sixteen mature Friesian or Ayrshire cows were obtained from two dairy herds. Each cow was examined *per rectum* to assess the state of the reproductive tract. The oestrous cyles of these animals were then synchronised using progesterone-releasing intravaginal devices (PRID, Sanofi Animal Health,Watford, U.K.) for 12 days.

Cloprostenol (50μg), a synthetic $PGF_{2\alpha}$ analogue (Estrumate, Pitman-Moore Ltd, Crewe, U.K.) was given by intramuscular injection on day 10 of the PRID treatment. Following PRID removal the animals were observed for signs of oestrus 3 times daily. On the day that each animal showed standing oestrous behaviour coccygeal blood samples (7ml) were collected into lithium heparin (143U/tube). After centrifugation (30 min, 1000 *g*) plasma was collected and stored at -20°C for progesterone analysis. Throughout the time of synchronisation and prior to collection of the CL the cows were housed in a straw court and fed a maintenance ration of silage and concentrate with free access to water.

On day 10 (standing oestrus = day 0) the ovary bearing the CL was identified by transrectal ultrasound examination. Animals were assigned to one of 4 groups and luteolysis was induced in 3 of the groups using an intramuscular injection of 25mg dinoprost, a synthetic form of natural $PGF_{2\alpha}$ (Lutalyse, Upjohn Ltd, Crawley, U.K.) 6, 12 or 24 hours prior to removal of the CL. Coccygeal blood samples were collected for progesterone analysis immediately before injection of $PGF_{2\alpha}$. The fourth group were control animals and did not receive any $PGF_{2\alpha}$.

6.2.2. Collection of the CL

Prior to surgery the cows were starved for 24 hours although water intake was not restricted. At the time of surgery each animal was sedated using 0.05-0.1mg/kg intravenous xylazine (Rompun, Bayer plc, Suffolk, U.K.). The left flank was clipped and local anaesthesia achieved by infiltration of the body wall with 2% lignocaine (Lignavet, C-Vet, Leyland, Lancs., U.K.). The skin was prepared for surgery and a 10cm vertical incision was made through the body wall. The uterus and ovary bearing the CL was identified and the CL was removed from the ovary by enucleation. This technique was used rather than ovariectomy, as originally intended, because of the ease with which enucleation was performed, particularly in the $PGF_{2\alpha}$-treated animals. This was probably due to alterations in blood supply and shrinkage of tissue which takes place very rapidly after induction of luteolysis. The main risk with this procedure is bleeding from the ovary after removal of the CL. This was of more

concern with control animals. In order to detect any bleeding, a dry swab was applied to the ovary to check for excessive haemorrhage immediately after removal of the CL and prior to inserting final sutures in the abdominal wall. The peritoneum and abdominal muscles were closed in two layers using interrupted sutures of 5 metric coated vicryl (Ethicon Ltd) and the skin was sutured using 4 metric mersilk (Ethicon Ltd). Duplocillin LA (20-25ml, i.m. Mycofarm UK Ltd, Cambridge) was given to each animal as prophylactic antibiotic cover at this time. Blood samples (7ml) for progesterone analysis were collected from the jugular vein at the time of surgery.

After surgery the cattle were housed separately in loose boxes and observed for 24 hours before returning to the straw court. Rectal temperatures were recorded daily for a period of 3 days after surgery.

6.2.3. Treatment of luteal tissue

All CL collected were immediately placed on a sterile petri dish sitting in crushed ice. The CL were weighed and measured before cutting into 4-5 sections using a sterile scalpel blade. One section for immunohistochemistry was snap-frozen onto a cork disc using a slurry of dry ice and isopentane. Two sections were snap-frozen in aluminium foil for molecular studies and the remaining sections were placed either in 10% paraformaldehyde for preparation of paraffin blocks or Hanks Buffered Salt (without divalent cations) prior to dissociation of tissue for analysis by FACS.

6.2.4. Haematoxylin and eosin staining of sections

Sections (8μm) were cut from paraffin-embedded tissues and stained with haematoxylin and eosin (H&E) as described in Chapter 2 (Section 2.8.). These sections of CL were examined by high power microscopy (x40) to assess the general appearance of the cells present in tissue from $PGF_{2\alpha}$-treated animals in comparison to control animals. The presence of shrunken cells, fibrous whorls of tissue and vacuolated cells was noted and each section from the $PGF_{2\alpha}$-treated cows were assessed for loss of structure and graded between 1+ and 5+, where 1+ indicates

minimal change in luteal structure and 5+ indicates complete loss of structure in comparison to CL from control animals (day 10).

6.2.5. Immunohistochemistry

Sections (6μm) of frozen tissue were stained by immunohistochemistry as described in Chapter 2 (Section 2.5.). The luteal tissue sections were stained using the same panel of monoclonal antibodies as described in Chapter 4 (Table 4.2.). Positively stained cells were counted in 6 fields as described in Chapter 4 (Section 4.2.6.). The capsule and areas predominated by blood vessels were avoided.

6.2.6. Carbol chromotrope stain for eosinophils.

Individual sections (8μm) from paraffin-embedded tissues were stained as described in Chapter 2 (Section 2.7).

6.2.7. Progesterone assay

All samples were analysed using the progesterone assay described in Chapter 2 (Section 2.3.2.). 0.1ng/per ml was the limit of detection and concentrations of less than 1ng/ml were taken to indicate that luteolysis had taken place. The intra-assay coefficient of variation (CV) was 13.9%.

6.2.8. Flow cytometry of dispersed luteal cells

The flow cytometer can be used to count positively stained cells from a population of dispersed cells, expressing them as a percentage of the total cell number. Dispersed cells are first stained with a specific monoclonal antibody and the secondary antibody is conjugated with a fluorescent marker. This technique has been used previously in CL studies, most often to study MHC II expression (Fairchild Benyo *et al.*, 1991; Kenny *et al.*, 1991). Dispersed portions of luteal tissue were stained for MHC II expression using monoclonal antibodies that had also been used for immunohistochemistry (VPM36 and TH14B). MHC II expression in luteal cell

populations obtained by both collagenase and mechanical dissociation were compared with each other and with results from immunohistochemistry.

6.2.8.1. Dispersion of luteal tissue

Luteal tissue was dispersed as described in detail in Chapter 3 (Section 3.2.2.) and summarised as follows:

The portions of CL were weighed and chopped finely using a scalpel blade prior to mechanical dissociation by repeated inversion of the chopped tissue suspended in 6mls $HBSS^-$/g. The solution was then filtered and the filtrate collected to recover cells dispersed by mechanical dissociation (MD) and the washed tissue was resuspended in a solution containing collagenase (Collagenase XI, Sigma, 3ml/g tissue) and incubated for 50 minutes at 37°C in a shaking water bath. The solution was filtered and the filtrate collected represented cells dispersed by collagenase dissociation (CD). The dispersed populations of luteal cells (MD and CD) were then washed twice in $HBSS^-$.

6.2.8.2. Preparation of blood cells for flow cytometry

White blood cells, collected as described below, were used as a positive control throughout:

A blood sample (10ml) was collected into lithium heparin (200 U) and the red blood cells were lysed as follows; 90ml NH_4Cl (0.16M) were added to 10ml of Tris (0.17M, pH 7) and the solution (pH 7.2) was heated to 37°C. Heparin (0.5%) was added and 35 ml of the lysis solution was then mixed with 10ml of whole blood. The solution was centrifuged at 250 *g* for 5 minutes, lysed cells poured off and the remaining cells resuspended in lysis solution and centrifuged. This process was repeated once more and the cells were finally suspended in FACS buffer (0.1% azide, 1% BSA in PBS) containing 0.5% heparin.

6.2.8.3. Incubation with monoclonal antibody

The dispersed cells were washed in FACS buffer, counted and resuspended in FACS buffer to give a concentration of approximately 2 x 10^6 cells/ml. Monoclonal antibodies TH14B and VPM 36 (25µl) at concentrations of 3/2000 and 3/10 respectively (diluted in PBS containing 0.1% azide) were added to 50µl dispersed cells, mixed gently and incubated at 4°C for 1 hour.

6.2.8.4. Fluorescence staining of cells

The cells were washed twice in FACS buffer and were then re-incubated with 25µl fluorescein isothiocyanate-labelled second antibody (FITC, 1/250 in FACS buffer) at 4°C for 30 min. After incubation the cells were washed twice in buffer and resuspended in 200µl FACS buffer + 200µl 1% paraformaldehyde.

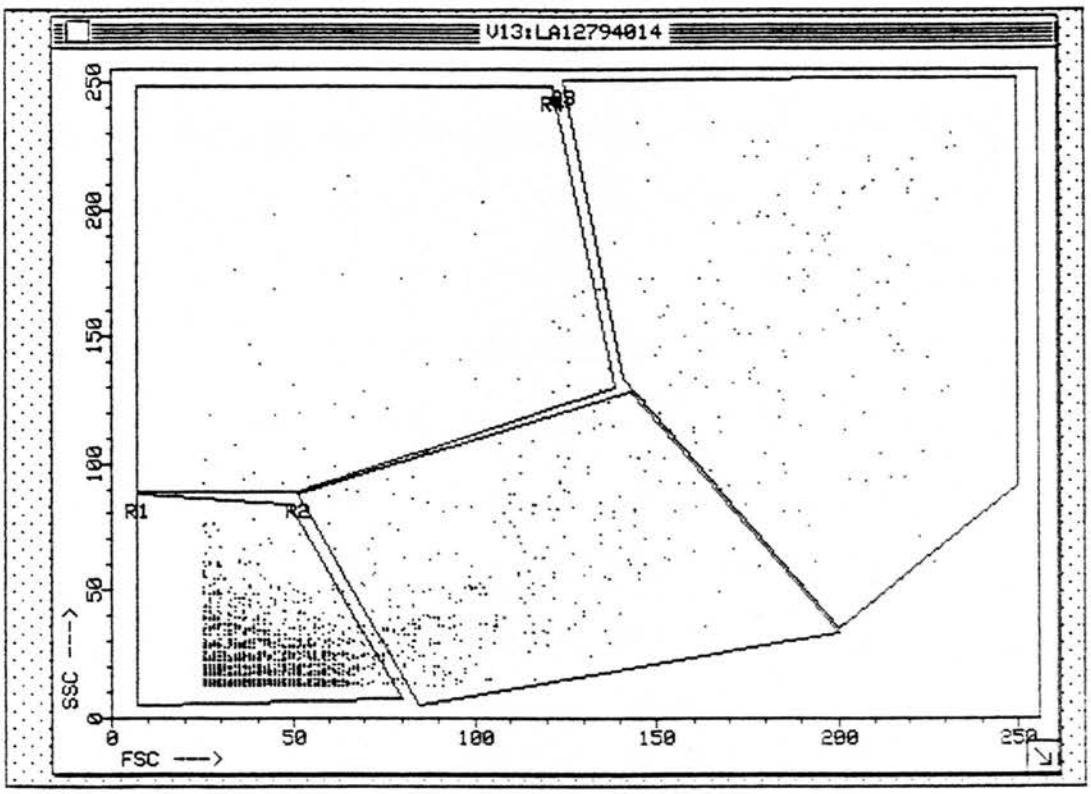
6.2.8.5. Flow cytometry

Positively-stained cells were counted using the FACSCAN (Becton Dickinson UK). For each sample a total of $1x10^4$ cells were analysed. Specific gates were not set at this time to select specific cell populations. Gate settings included all cells. The data collected were then divided into four regions at the time of data analysis (Figure 6.1) which was performed using Consort 30 (Becton Dickinson, UK).

6.2.9. Statistical analysis

Results from immunohistochemistry and FACS were analysed using the Kruskal-Wallis test for non-parametric data and the Mann-Whitney test was then used to analyse differences between samples where present. A p value of less than 0.05 was taken to be statistically significant. All statistical analyses were performed using Minitab software (Pennsylvania State University).

Figure 6.1. Flow cytometry: Dot-plot appearance of luteal cell populations showing regions (gates 1-4) that were used during analysis of results.



6.3. Results

6.3.1. Tissue assessment

There were differences in size and weight of CL collected (Table 6.1). As there is always a great deal of variation in CL size between cows, especially older animals, it is not possible to draw major conclusions from these results except to note that most animals treated with $PGF_{2\alpha}$ 12 and 24 hours before tissue collection had CL that were smaller and lighter than those of the control animals. There was also an alteration in the texture of luteal tissue which became much more fibrous and difficult to cut in CL obtained 24 hours after treatment.

6.3.2. Progesterone assay

Progesterone concentrations had decreased in all $PGF_{2\alpha}$-treated animals by the time of surgery in comparison to pre-treatment samples (Table 6.1). However, all cows treated with $PGF_{2\alpha}$ 6 hours before surgery had progesterone concentrations consistent with a CL that was still functional at surgery (P_4> 1ng/ml). Three cows treated 12 hours before surgery and 2 cows treated 24 hours before surgery also had progesterone concentrations higher than those associated with functional luteolysis at the time of surgery, although the concentrations were lower than those seen 6 hours after treatment.

6.3.3. Morphological examination of H&E stained tissue

All CL from prostaglandin-treated animals showed some alteration in luteal structure but the degree of alteration varied from animal to animal and was not consistent within each group (Table 6.2). Although there was significant loss of structure in all CL after 24 hours, obvious changes were detectable in all cows even after 6 hours. The most frequently observed change was the presence of fibrous whorls and strands of cellular tissue in between luteal cells (Fig. 6.2b-6.2d) which often dominated the fields in comparison to tissue from control animals which was predominantly made up of luteal cells (Fig. 6.2a).

FIGURE 6.2a. Luteal tissue collected on day 10 of the oestrous cycle (H&E x218).

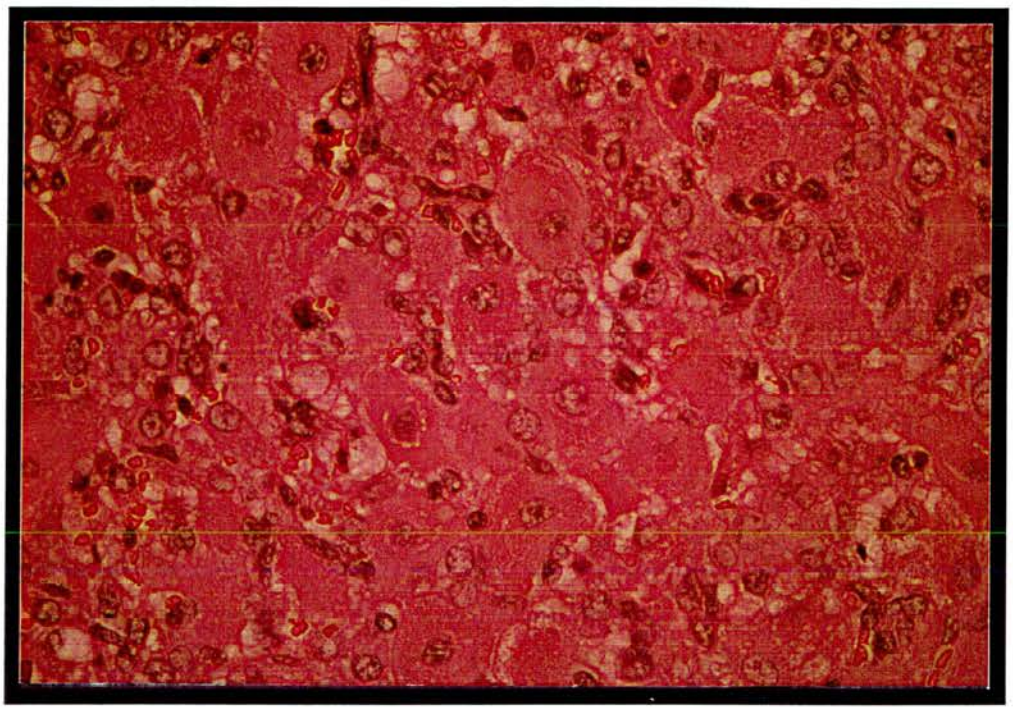


FIGURE 6.2b. Day 10 luteal tissue 6 hours after $PGF_{2\alpha}$ injection (H&E x218).

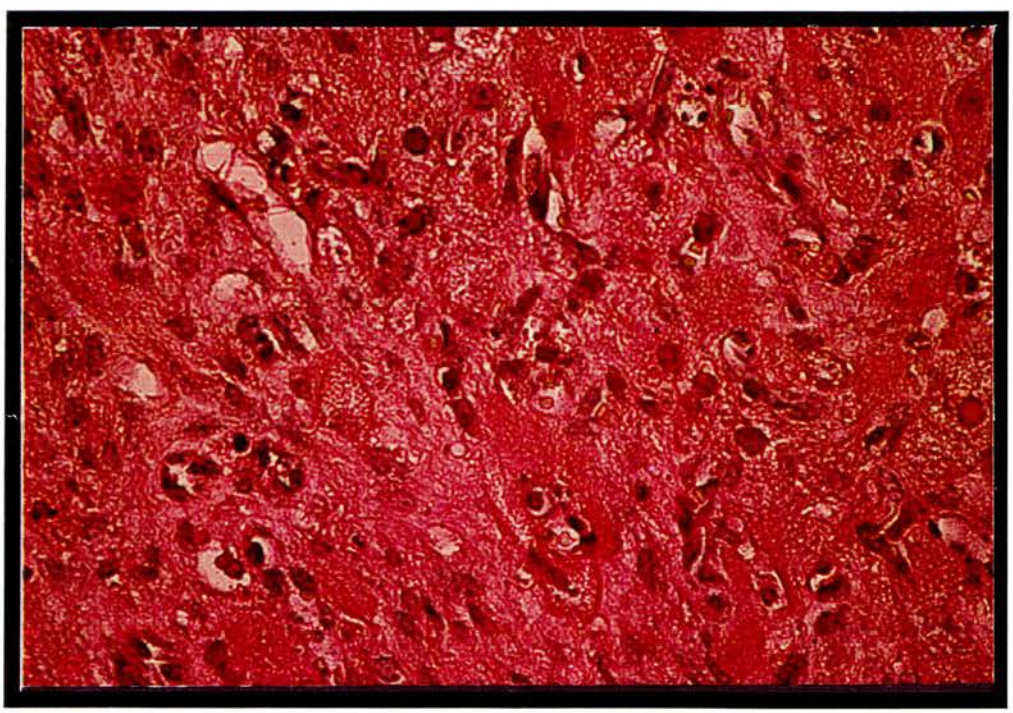


FIGURE 6.2c. Day 10 luteal tissue 12 hours after $PGF_{2\alpha}$ injection (H&E x218).

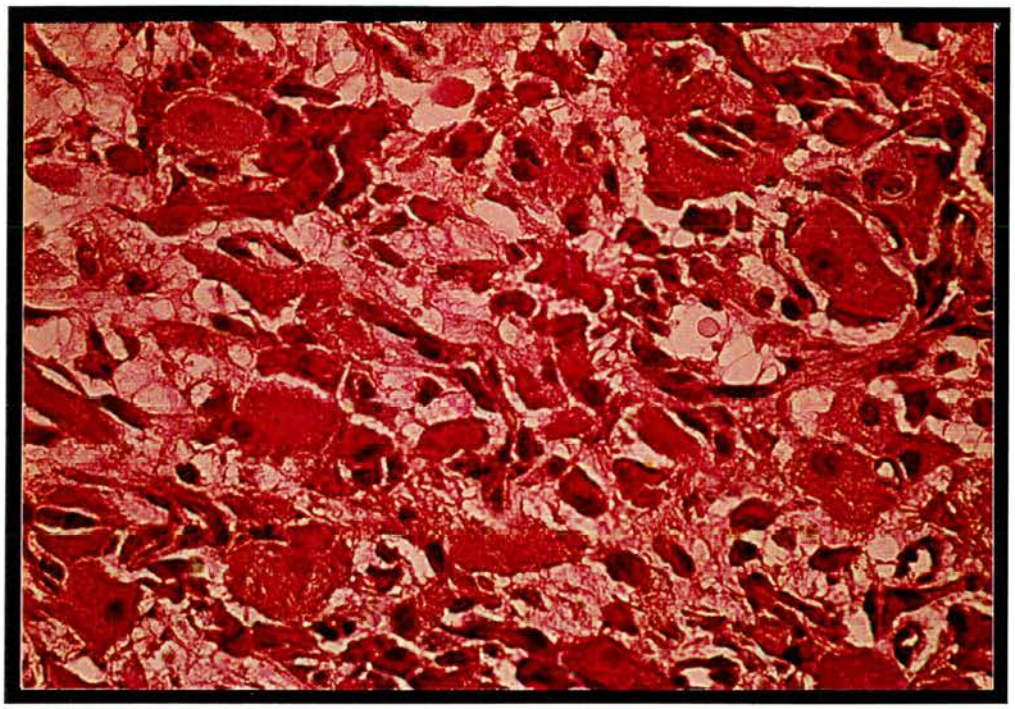


FIGURE 6.2d. Day 10 luteal tissue 24 hours after $PGF_{2\alpha}$ injection (H&E x218).

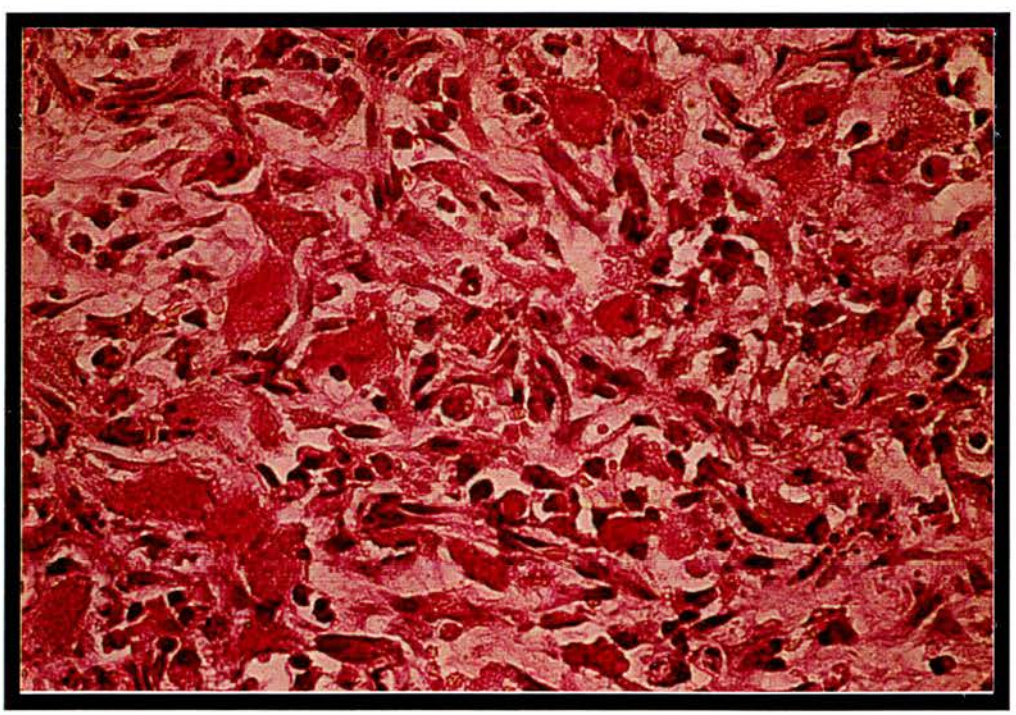


Table 6.1. Weight and size of CL at collection and progesterone (P4) results.

COW No	Time after $PGF_{2\alpha}$	CL Weight	CL size (Apex to base)	P4 (ng/ml) pre-$PGF_{2\alpha}$	P4 (ng/ml) pre-surgery
45	Control	8.1g	4cm	-	2.89
T71	Control	13.0g	3.5cm	-	1.01
X63	Control	9.8g	4cm	-	1.18
X2	Control	6.1g	2cm	-	2.28
36	6 hours	6.0g	2.3cm	4.6	2.79
646	6 hours	3.3g	1.9cm	2.8	1.0
T18	6 hours	10.0g	4cm	5.7	3.1
M39	6 hours	11.0g	5cm	7.5	3.48
652	12 hours	4.2g	2.5cm	3.1	2.46
496	12 hours	3.8g	2cm	1.1	1.05
511	12 hours	2.6g	2.2cm	1.1	0.77
186	12 hours	4.4g	2.5cm	2.2	1.38
235	24 hours	3.7g	2.4cm	3.4	0.81
S6	24 hours	4.3g	2cm	4.4	1.11
S48	24 hours	4.8g	2cm	2.8	1.17
X381	24 hours	7.0g	2.5cm	4.1	0.62

Table 6.2. Results from morphological examination of luteal tissue.

COW No	Time after $PGF_{2\alpha}$	Loss of structure	Morphological appearance
45	Control	-	distinct luteal cells predominate. Even staining of tissue.
T71	Control	-	as 45
X63	Control	-	as 45
X2	Control	-	as 45
36	6 hours	1+	small blood vessels obvious, whorls of fibrous tissue. Weak staining
646	6 hours	1+	luteal cells obvious, strong even stain, no whorls
T18	6 hours	3+	luteal cells appeared shrunken, some normal and some whorls of cells
M39	6 hours	2+	luteal cells appear shrunken
652	12 hours	2+	whorls of vacuolated fibrous tissue between luteal cells
496	12 hours	3+	uneven staining of LLC[1], intercellular tissue appears vacuolated. Fibrous cellular whorls present
511	12 hours	4+	intensely cellular, loss of individual cell outline. Whorls in some areas.
186	12 hours	1+	Appeared intact, lots of intact LLC
235	24 hours	4+	Loss of luteal cell outline, obvious fibrous intercellular tissue present.
S6	24 hours	4+	Fibrous areas dominate areas of luteal cells. Luteal cells shrunken
S48	24 hours	2+	Loss of luteal cell outline. Fibrous tissue between cells limited.
X381	24 hours	3+	Whorls of fibrous tissue throughout. Strongly staining.

[1] LLC=large luteal cells

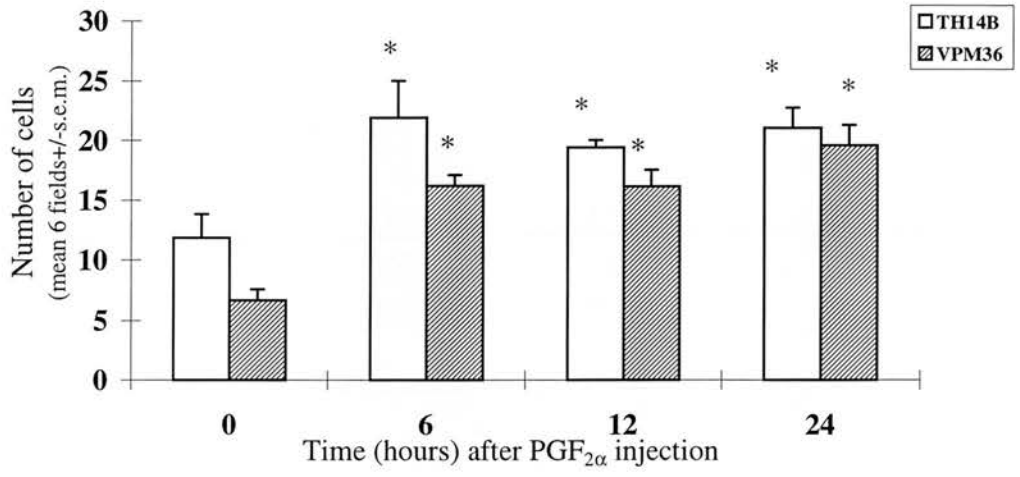
6.3.4. Immunohistochemistry

There were no significant differences between the numbers of T-lymphocytes (CD5+, CD8+ or CD4+) or macrophages counted in luteal tissue from control or treated animals (Table 6.3). However, MHC II/DR and MHC II/DQ expression was significantly higher ($p<0.05$) at all time periods after injection of $PGF_{2\alpha}$ in comparison to controls (Figure 6.3).

TABLE 6.3. Immune cells in the CL after $PGF_{2\alpha}$ treatment (Each figure represents the mean result +/- s.e.m. from CL collected from 4 animals per group, counting 6 fields per CL).

	Control	**6 hours**	**12 hours**	**24 hours**
CD5+	27.92 ± 3.2	30.67 ± 8.6	21.67 ± 3.9	51.38 ± 17.3
CD8+	12.11 ± 4.2	11.08 ± 3.3	8.17 ± 2.2	11.96 ± 1.9
CD4+	7.38 ± 0.9	5.16 ± 0.5	1.71 ± 0.6	13.67 ± 6.5
B-cells	0.04 ± 0.05	0	0.04 ± 0.05	0.04 ± 0.05
Macrophages	9.21 ± 2.6	24.25 ± 4.9	11.5 ± 2.2	24.21 ± 7.9
Eosinophils	0.38 ± 0.1	3.75 ± 1.4	1 ± 0.3	5.63 ± 3.5

FIGURE 6.3. MHC II/DR (TH14B) and MHC II/DQ (VPM36) expression in luteal tissue 0, 6, 12 and 24 hours after $PGF_{2\alpha}$ injection on day 10 of the oestrous cycle[1].



*MHC II expression was significantly higher ($p<0.05$) at all time periods after injection of $PGF_{2\alpha}$ compared to control animals.
[1]Each figure represents the mean result +/-s.e.m. for CL collected from 4 animals per group, counting 6 fields per CL.

6.3.5. Flow cytometry

Cells expressing MHC II (DQ+DR) were counted in the four regions of the dot-plot and there were no significant differences between control and treated cows at any of the time periods after $PGF_{2\alpha}$ injection using cells that were mechanically dissociated or dispersed using collagenase. The results from flow cytometry of luteal cell populations produced by both mechanical dissociation and collagenase dispersion then stained with TH14B (MHC II/DR) are shown in Figures 6.4a and 6.4b.

FIGURE 6.4a. MHC II/DR (TH14B) expression in luteal tissue collected 0, 6, 12 or 24 hours after treatment with $PGF_{2\alpha}$, dispersed by mechanical dissociation and analysed in 4 regions of the cell population (gates 1-4) by flow cytometry. Each bar represents mean ± s.e.m. for 4 animals.

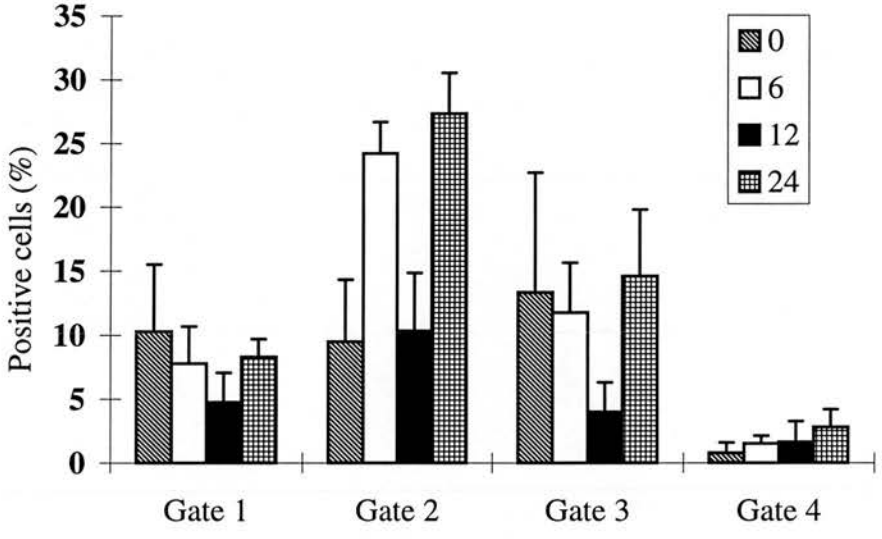
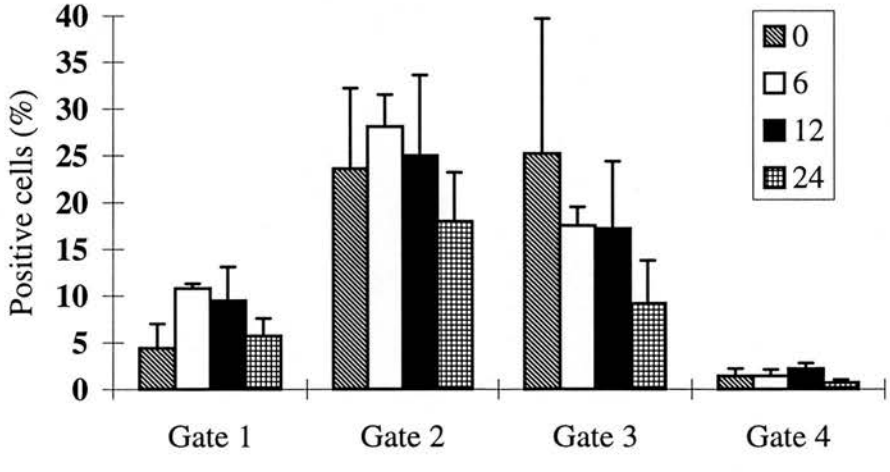


FIGURE 6.4b. MHC II/DR (TH14B) expression in luteal tissue collected 0, 6, 12 or 24 hours after treatment with $PGF_{2\alpha}$, dispersed with collagenase and analysed in 4 regions of the cell population (gates 1-4) by flow cytometry. Each bar represents mean ± s.e.m. for 4 animals.



6.4. Discussion

In order to investigate whether induced luteolysis resulted in cell populations and morphology similar to those seen after natural luteolysis (Chapter 5) CL were examined at 3 time periods after injection with a standard dose of $PGF_{2\alpha}$. MHC II expression within the CL was significantly higher at all time periods after luteolysis artificially induced on day 10 of the oestrous cycle compared to day 10 CL where luteolysis had not been artificially induced. The numbers of lymphocytes and macrophages did not differ significantly between groups although both six and twenty four hours after treatment the numbers of macrophages in luteal tissue were considerably higher than those of the control group. CL from all time periods after $PGF_{2\alpha}$ treatment differed morphologically from the control CL with significant loss of luteal structure in some animals after only 6 hours, although progesterone concentrations in the majority of animals were still representative of a functional CL at the time of surgery.

Induced luteolysis has been described by some workers to be useful as a model for studying events that occur during natural luteolysis particularly as the hormonal changes which take place during both processes have been found to be similar in heifers (Spicer *et al.*, 1981). However, the present study did not find similar cellular events to those seen around the time of natural luteolysis (Chapter 5). There was no significant alteration in the number of T-lymphocytes at any time period after induced luteolysis, a process that appears to accompany natural luteolysis in the cow (Lobel and Levey, 1968; Chapter 5). MHC II expression however was significantly increased at all time periods after induced luteolysis, a change that was not observed in 5 animals between 1-2 days after natural luteolysis (Chapter 5) but was observed in abattoir-derived Stage IV luteal tissue, which (as discussed in Chapter 4) is likely to represent CL several days after luteolysis. Although not statistically significant, the increased numbers of macrophages seen 6 or 24 hours after treatment were also observed in stage IV luteal tissue but not immediately after natural luteolysis.

The morphological changes in appearance of luteal tissue after induced luteolysis also differed from those seen after natural luteolysis. Alterations to tissue morphology took place rapidly and, unlike the order of events during natural luteolysis, occurred prior to a significant decrease in the progesterone concentrations of each animal. Natural luteolysis could only be detected by changes in cellular morphology alone in one animal although a total of 5 animals had undergone luteolysis during the course of the study (Chapter 5, Section 5.3.2.). However, in the present study, signs of structural regression were apparent in animals at all time periods after injection of $PGF_{2\alpha}$. These findings differ from those of Tian *et al.* (1994) who did not observe morphological changes in CL until 24 hours after $PGF_{2\alpha}$-induced luteolysis. However the different results may be due to the use of heifers in this study as well as inducing luteolysis at an earlier stage of the cycle (days 6-7).

It would appear that luteolysis induced with a standard dose of $PGF_{2\alpha}$ in the cow results in a much more rapid alteration to luteal structure than that seen in the first two days of natural luteolysis. This is in contrast to findings in the sheep where the morphological changes which take place during induced luteolysis are described as being similar to natural luteolysis (Gemmell *et al.*,1976; Stacy *et al.*,1976). However these studies were based on luteolysis induced by infusion of $PGF_{2\alpha}$ directly into the uterine vein at a considerably lower dose rate than that used to induce luteolysis by intramuscular injection. This technique of inducing luteolysis, although technically more difficult, is bound to result in PGFM profiles that are far more representative of the pulses of $PGF_{2\alpha}$ release that occur during natural luteolysis (Kindahl *et al.*, 1976a) than a single high dose of $PGF_{2\alpha}$.

The use of luteolysis induced by a single bolus of exogenous $PGF_{2\alpha}$ as a model for events occurring at natural luteolysis is debatable for other reasons. The concentrations of plasma $PGF_{2\alpha}$ achieved by treatment with 25mg of exogenous $PGF_{2\alpha}$ are considerably higher than the concentrations of $PGF_{2\alpha}$ in peripheral plasma during natural luteolysis in the cow (Chapter 5, Section 5.3.4.). It is also impossible, without cannulation of the ovarian vessels, to know exactly what concentration of

$PGF_{2\alpha}$ is actually reaching the ovary after intramuscular injection. In addition, where luteolysis is induced before approximately day 15 of the oestrous cycle the luteal cell populations in CL will differ from those found around the time of natural luteolysis. There is variation in luteal cell types, particularly the ratio of large:small luteal cells, during the oestrous cycle (Hansel *et al.*, 1987). The relative numbers of large and small luteal cells are significant because the two cell types respond differently to $PGF_{2\alpha}$ (Koos and Hansel, 1981; Hansel *et al.*, 1991).

In contrast to immunohistochemistry, analysis of luteal cells by fluorimetry did not detect any differences in MHC II expression between any of the groups. There are various possible reasons for this. It may be that the dispersion and preparation of cells resulted in cell damage and loss of MHC II cell surface markers. The production of well dispersed populations of intact luteal cells appropriate for analysis by fluorimetry in itself is not easy (O'Shea *et al.*, 1990). Mechanical dissociation produces intact cell types but they are often not completely dissociated and there is some evidence that the process of collagenase dispersion results in damage to cell surface receptors (Chapter 3). However, as LH-receptor levels in mechanically and enzymatically dissociated cells were similar in this study, the lack of variation between animals does not appear to be related specifically to the use of collagenase. Paraformaldehyde fixation of stained cells, required because samples could not be immediately analysed, is another possible source of cell damage. Other workers have successfully used unfixed luteal cells for analysis (Fairchild Benyo *et al.*, 1991).

There was a large variability in the results from individual animals within groups and this was compounded by the small group size. It is possible that larger numbers of animals may have shown significant differences by fluorimetry. Finally, in this study, cells were not sorted into specific cell populations prior to data analysis. In contrast Fairchild Benyo *et al.* (1991) did observe increased MHC II expression on sorted luteal cells 12 hours after injection of $PGF_{2\alpha}$. It is also possible to sort dispersed luteal cells into separate populations of large and small cells of greater than 90% purity prior to analysis by fluorimetry (Alila *et al.*, 1988). This might allow detection

of MHC II on specific cell types such as large or small cells, instead of including all cell types present.

However, even using effective cell dispersion and computerised cell separation techniques prior to cell counting fluorimetry is still not always an accurate technique for counting cells in luteal tissue. As discussed in Chapter 3, there are problems working with dispersed CL caused by cell clumping because counts are based on size and complexity. For example, any large clumps of small luteal cells will be counted as large cells. For these reasons it would appear that there is little to be gained in the use of FACS in comparison to immunohistochemistry which involves less processing of tissue and better identification of specifically stained cells.

At the time of surgery, all cows at 6 hours after injection of $PGF_{2\alpha}$, 3 cows at 12 hours and 2 cows at 24 hours still had progesterone concentrations consistent with a functional CL (P_4> 1ng/ml). These relatively high concentrations of progesterone were unexpected, particularly 24 hours after injection. However the results are similar to those of Berardinelli and Adair (1989) who found that it took around 32 hours before concentrations of progesterone decreased significantly (<1ng/ml) in heifers injected with $PGF_{2\alpha}$ (25mg, Lutalyse) at mid-cycle (days 10-14) in comparison to twenty four hours in animals injected either in the early (days 5-9) or late (days 15-19) luteal phase. Similarly Schallenberger *et al.* (1984) found that progesterone concentrations did not reach basal concentrations until 24-36 hours after induced luteolysis. This apparent variation in speed of response to exogenous $PGF_{2\alpha}$ at different stages of the CL lifespan may be related to the differing cell populations, particularly large:small cell ratios, in the CL at these times as more $PGF_{2\alpha}$ receptors are found on large cells than small cells (Fitz *et al.*, 1982; Niswender *et al.*, 1985).

In conclusion, the results of this study indicate that the cellular events which occur after induced luteolysis are considerably more dramatic and rapid than those seen during natural luteolysis. For this reason it has to be concluded that the artificial induction of luteolysis by a single high dose injection of $PGF_{2\alpha}$ does not result in

morphological changes that are representative of natural luteolysis. Studies using animals during a normal cycle or employing pulsatile infusion techniques during the late luteal phase, preferably directly into the uterine vein, are likely to be of far more use in analysing events that occur *in vivo*.

Chapter 7

The effect of prostaglandin inhibition on immune cell populations in the bovine corpus luteum around the time of luteolysis.

7.1. INTRODUCTION

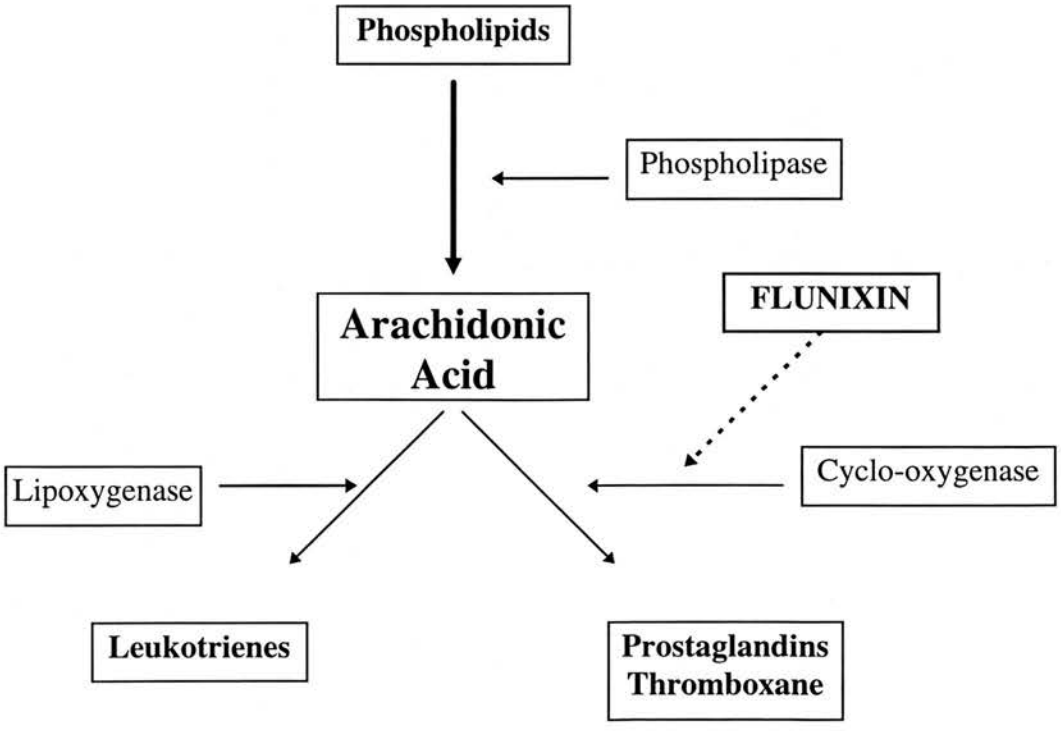
The numbers of T-lymphocytes (CD8+, CD5+), macrophages, eosinophils and MHC II expression vary within the cow CL throughout the oestrous cycle (Chapter 4 and Chapter 5). The most interesting finding is a significant increase in CD5+ and CD8+ T-lymphocytes within luteal tissue from day 16 of the oestrous cycle onwards which occurred prior to functional luteolysis (see chapter 5).

The role, if any, of this apparent influx of T lymphocytes within the CL in the days leading up to luteolysis is not known and it is also unclear what factor(s), systemic or intraovarian, are involved in stimulating the movement of T-lymphocytes into the CL around day 16 of the oestrous cycle. Uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), released in large pulses around day 18, has been established as the luteolytic substance in the cow (Inskeep and Murdoch,1980; Auletta and Flint, 1988). Several workers, measuring the stable metabolite of $PGF_{2\alpha}$, 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM), have also observed that there is an increase in concentrations of PGFM between days 14 and 17 of the oestrous cycle, prior to luteolytic concentrations being recorded. Shemesh and Hansel (1975) noted increased concentrations of $PGF_{2\alpha}$ within the endometrium and uterine vein from around day 15 and episodic increases in systemic PGFM from day 14 were recorded by Parkinson and Lamming (1990) and day 17 (Basu and Kindahl, 1987). It is possible that this pre-luteolytic increase in the concentration of $PGF_{2\alpha}$ may be involved as a signal

to T-lymphocytes or may activate cells already present within the CL to recruit other leukocyte populations.

One way in which this hypothesis can be tested is by inhibiting $PGF_{2\alpha}$ production in the days leading up to luteolysis. Various substances have been used to inhibit $PGF_{2\alpha}$ including intrauterine indomethacin, a cyclo-oxygenase inhibitor (Lewis and Warren 1977; Milvae and Hansel, 1985) and intrauterine nordihydroguaiaretic acid, a cyclo-oxygenase and lipoxygenase inhibitor (Hamilton *et al.*, 1990). More recently flunixin meglumine, a potent non-steroidal anti-inflammatory drug which is licensed for use in cattle, has been used. Flunixin is a cyclo-oxygenase inhibitor acting within the arachidonic acid cascade (Fig. 7.1). It is widely used in veterinary medicine as an anti-inflammatory / antipyrexic drug; however it also inhibits $PGF_{2\alpha}$ produced by the reproductive tract and, at four times the therapeutic dose rate, has been shown to delay luteolysis (Aiumlamai *et al.*, 1990; Odensvik and Gustafsson, 1994; Odensvik, 1995).

Figure 7.1. Site of action of flunixin within the arachidonic acid cascade.



In this study, in order to investigate whether $PGF_{2\alpha}$ is involved in stimulating an influx of T-lymphocytes into the cow CL prior to functional luteolysis, we used high dose flunixin meglumine to suppress $PGF_{2\alpha}$ production by 12 cows in the days leading up to luteolysis, replacing $PGF_{2\alpha}$ artificially in six of the flunixin-treated animals. Immune cell types and MHC II expression present within luteal tissue were quantified using immunohistochemistry and the cell populations observed in the two treatment groups and a control group were compared.

7.2. Materials and methods

7.2.1 Experimental Animals

7.2.1.1. Experiment 1; Prostaglandin trial

The purpose of this preliminary experiment was to calculate the dose of exogenous $PGF_{2\alpha}$ that would give systemic PGFM concentrations similar to those seen in the days leading up to luteolysis, without inducing luteolysis itself. This dose would then be used to artificially replace prostaglandin in one group of flunixin-treated cows in the main experiment.

Experimental protocol

The oestrous cycles of 6 mature Holstein-Friesian dairy cows from the Roslin Institute herd were synchronised using progesterone-impregnated intravaginal devices (PRID, Sanofi Animal Health, Watford, U.K.) for 12 days. 50μg of cloprostonol, a synthetic $PGF_{2\alpha}$ analogue (Estrumate, Pitman-Moore Ltd, Crewe, UK) was given by intramuscular injection 2 days before PRID removal. The cows were observed for signs of oestrus three times daily for the next three days.

On day 9-10 of the following oestrous cycle intravenous cannulae were inserted into the jugular vein of each animal under local anaesthetic. The cows were then allocated to one

of 3 groups and 3 different dose rates (0.01mg, 0.1mg or 1mg) of a naturally-occurring $PGF_{2\alpha}$ (Lutalyse, Upjohn Ltd, Crawley, UK.) were given in a 5ml volume of sterile saline through the jugular cannula over a period of 1 minute. Blood samples (7ml) were collected into heparinised glass tubes (Vacutainer, Becton Dickinson 143U heparin /tube) at 5 minute intervals for 15 minutes prior to $PGF_{2\alpha}$ injection and then every 2 minutes for 6 minutes; 5 minute intervals for 30 minutes, 10 minute intervals for 30 minutes,15 minute intervals for 30 minutes and then 30 minute intervals for 1.5 hours. Catheters were flushed with sterile saline after treatment and between samples. Daily blood samples were collected by coccygeal venepuncture for the 3 days following treatment in order to assess whether any of the dose rates had been sufficient to induce luteolysis. Blood samples were centrifuged at 1000 *g* for 30 min at 4°C and the plasma was then collected and stored at -20°C prior to being assayed for PGFM and progesterone concentrations.

7.2.1.2. Experiment 2; Flunixin inhibition of prostaglandin production

Oestrous synchronisation

Mature Holstein Friesian cows were selected from the Roslin Institute dairy herd at Blyth Bank Farm and the reproductive tract of each animal was examined *per rectum* for any detectable abnormalities prior to the start of the experiment. Oestrus was synchronised as described in Experiment 1 although, in addition to frequent observation for signs of oestrous behaviour, blood samples were collected 2 days after PRID removal for progesterone assay to confirm oestrus at this time. On day 6 after oestrus the ovaries were examined by ultrasound to locate the corpus luteum. During the time of oestrous synchronisation the cows were kept indoors in an open straw-bedded court with natural light and maintained on a diet of hay and a small quantity of concentrate with free access to water at all times.

Flunixin treatment

A cannula was inserted into the left jugular vein of each cow on day 15 of the oestrous cycle under local anaesthetic. Each cannula was made from sterile silastic tubing because of its flexibility and resistance to kinking which makes it suitable for long-term

cannulation. The cannulae were stitched to the skin at the entrance to the jugular vein then attached to the halter by tape and to the hair along the neck of the animal to the shoulder using umbilical clips. This allowed blood samples to be collected and treatments to be administered with minimal disturbance to the animal. All animals were haltered in separate stalls on wood shavings during treatment with individual access to water and hay. After cannulation the cows were put in their stalls and left for 12-18 hours to settle before commencing treatment.

The cows were assigned to one of three groups of six animals;

Group 1; Flunixin

These cows were treated 4 times daily with 2.2mg/kg flunixin meglumine (Finadyne, generously supplied by Schering Plough, UK) from day 16-18, following a protocol previously described by Odensvik, (1995). Following infusion of flunixin the cannulae were flushed with 10ml saline which contained heparin (50U/ml).

Group 2; Flunixin and $PGF_{2\alpha}$

This group were treated with flunixin as in group 1 but for the final 12 hours of treatment, immediately prior to surgery, $PGF_{2\alpha}$ (Lutalyse, UpJohn, Crawley, UK) was infused using a pump system at a rate of 170μg/hour in a volume of 5ml sterile saline/hour. During $PGF_{2\alpha}$ infusion, flunixin was also administered through the jugular cannula but blood samples were collected from the coccygeal vein.

Group 3; Control

The animals in this group were given 20ml of sterile saline 4 times daily.

7.2.1.3. Blood sampling

Throughout treatments for all groups blood samples (10ml) were collected 8 times daily into heparin, all samples were centrifuged at 1000 *g* for 30 min and the plasma was then collected and stored at -20°C.

All cows had access to hay and water *ad libitum* throughout the treatment period, although initially it had been intended to restrict hay and water in the 24 hours before ovariectomy to reduce problems at surgery caused by excessive gut contents. The change of plan was caused by the side-effects of high dose flunixin treatment. Prolonged, or in this case, high dose flunixin meglumine, in common with other non-steroidal anti-inflammatory drugs (NSAIDS) can cause irritation and erosions in the wall of the gut. This is thought to be due to inhibition of prostaglandin E_2-mediated bicarbonate and mucus secretion with reduced control of, and protection against, gastric acid secretion (Boothe, 1995). It became apparent early in treatment (within 18-24 hours) that this was going to be a problem as all flunixin-treated cows developed diarrhoea which sometimes contained blood. However, the animals did not actually appear ill, were normal on clinical examination and continued to eat. It was decided at this stage to keep all the animals on hay as an empty intestinal tract would be much more prone to damage. After the first 36 hours of treatment most animals seemed to 'adapt' to the flunixin and the diarrhoea was less severe.

7.2.1.4. Ovariectomy

On day 18 of the oestrous cycle after three days of treatment the ovaries were removed by ovariectomy. At the time of surgery each animal was sedated using 0.05mg/kg intramuscular xylazine (Rompun, Bayer plc, Suffolk, UK). The left flank was then clipped, infiltrated with 100ml 2% lignocaine (Lignavet, C-Vet, Leyland, Lancs, UK) and prepared for surgery. An incision was made in the left flank into the abdominal cavity, the uterus located and both ovaries were removed using umbilical clamps to achieve haemostasis of the ovarian stump. The peritoneum and abdominal muscles were closed in two layers using interrupted sutures of 5 metric coated vicryl (Ethicon Ltd) and the skin was sutured using 4 metric mersilk (Ethicon Ltd). Long-acting penicillin (Duplocillin LA, 20-25ml, Mycofarm UK,Ltd, Cambridge) was given to each animal as prophylactic antibiotic cover at this time.

7.2.1.5. Tissue collection

The ovary bearing the corpus luteum was immediately placed in a sterile petri dish on ice. The CL was dissected free of the surrounding ovarian tissue and then cut into four portions using a scalpel. One section was placed in 4% paraformaldehyde for paraffin blocking and the other sections were snap frozen in dry ice/isopentane for immunohistochemistry and molecular studies. The remaining ovarian tissue and second ovary were used for follicular studies.

7.2.2. Sample analysis

7.2.2.1. Progesterone assay

Plasma progesterone concentrations were measured as described in Chapter 2 (Section 2.3.2.). All samples were measured in a single assay with an intra-assay coefficient of variation (CV) of 16.1%.

7.2.2.2. PGFM assay

Experiment 1 These plasma samples were analysed for PGFM using a protocol developed by Dr R. Kelly, MRC Centre for Reproductive Biology, Edinburgh. The protocol is described in Chapter 2 (Section 2.3.3.).

Experiment 2

All samples from experiment 2 were analysed by Professor Hans Kindahl at the Swedish University of Agricultural Sciences, Uppsala, Sweden. PGFM was analysed in unextracted plasma by radioimmunoassay (Kindahl *et al.*, 1976b; Grannstrom and Kindahl, 1982). The antiserum cross-reacted 16% against 15-ketodihydro-$PGF_{2\alpha}$, 4% against 13,14-dihydro- $PGF_{2\alpha}$, 0.4% against $PGF_{2\alpha}$ and 1.7% against 15-ketodihydro-PGE_2, a major metabolite of PGE_2. For other prostaglandins tested the cross-reaction was less than 0.1%. The detection limit of the assay was between 25-30pmol/l. The inter-assay

coefficient of variation was 14% and the intra-assay coefficient of variation ranged between 6.6% and 11% at different points on the standard curve.

7.2.2.3. Immunohistochemistry

Sections of luteal tissue were stained as described in Chapter 2 (Section 2.5.) using the same panel of monoclonal antibodies as previously (Chapter 4, Table 4.2).

7.2.2.4. Quantification of stained cells

The number of positively stained cells in each of 6 fields per section were counted as described in Chapter 4 (Section 4.2.6.).

7.2.3. Statistical analysis

The results were analysed using the Kruskal-Wallis test for non-parametric data. Differences between groups were then analysed by the Mann-Whitney test. A p value of less than 0.05 was taken to be significant. All statistical analysis was performed using Minitab software (Pennsylvania State University).

7.3. Results

7.3.1 Experiment 1

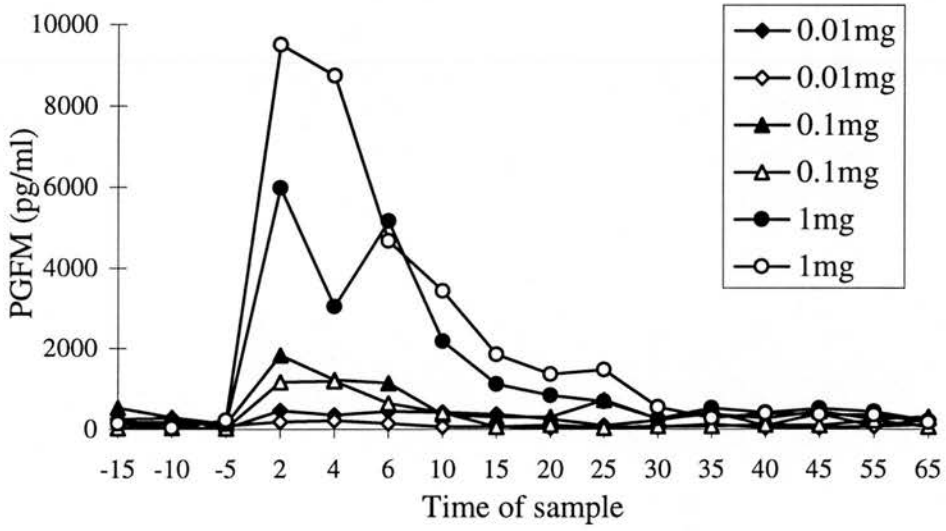
7.3.1.1. Progesterone

Luteolysis was not induced in any of the animals as demonstrated by plasma progesterone concentrations of greater than 1ng/ml in the 3 days following treatment.

7.3.1.2. PGFM

An immediate increase in peripheral concentrations of PGFM was seen using all 3 dose rates of prostaglandin (Fig. 7.2). The amount of time taken to return to pre-trial levels of PGFM varied from around 20 minutes at the lowest dose rate to over 90 minutes at a dose of 1mg.

FIGURE 7.2. PGFM concentrations after treatment with 3 doses (0.01mg, 0.1mg, 1mg) of $PGF_{2\alpha}$, 2 animals per treatment.



7.3.2 Selection of PGF_{2a} dose rate for main experiment;

Previous studies have shown a range of concentrations of PGFM in the cow around luteolysis. Parkinson and Lamming (1990) reported concentrations of between 60-100pg/ml (170-282pmol/l) prior to luteolysis with luteolytic peaks of around 400pg/ml (1128pmol/l). Vighio and Liptrap (1986) reported peaks ranging from 300pg/ml (846pmol/l) in the days leading up to luteolysis and luteolytic peaks of 500-600pg/ml (1400-1700pmol/l) which are similar results to those of Kindahl *et al.* (1976a) and Basu and Kindahl, (1987) Any variation in results from these studies is likely to be due to differences in frequency of sampling, methods and sensitivity of assay as well as variation between animals. In addition to the results of the studies mentioned and our own results, we also had to take into account the fact that baseline production of $PGF_{2\alpha}$ would be suppressed in flunixin-treated animals. We elected to use a dose rate of 170μg $PGF_{2\alpha}$/hour given by constant infusion through the jugular cannula.

7.3.3. Experiment 2

7.3.3.1. Progesterone

2 animals from the control group (542, 185) underwent luteolysis during the course of the trial as shown by an increase in $PGF_{2\alpha}$ metabolite and an associated decrease in progesterone concentrations (Figure 7.3a / 7.3b). Progesterone concentrations in the remaining control cows and all flunixin-treated animals indicated the presence of a functional CL throughout the trial (P4>1ng/ml). There were no significant differences in progesterone concentrations between any of the groups (excluding the 2 cows in which luteolysis occurred).

FIGURE 7.3a. Cow 542 (Control Group). Plasma PGFM and progesterone concentrations during the trial period (days 16-18 of the oestrous cycle).

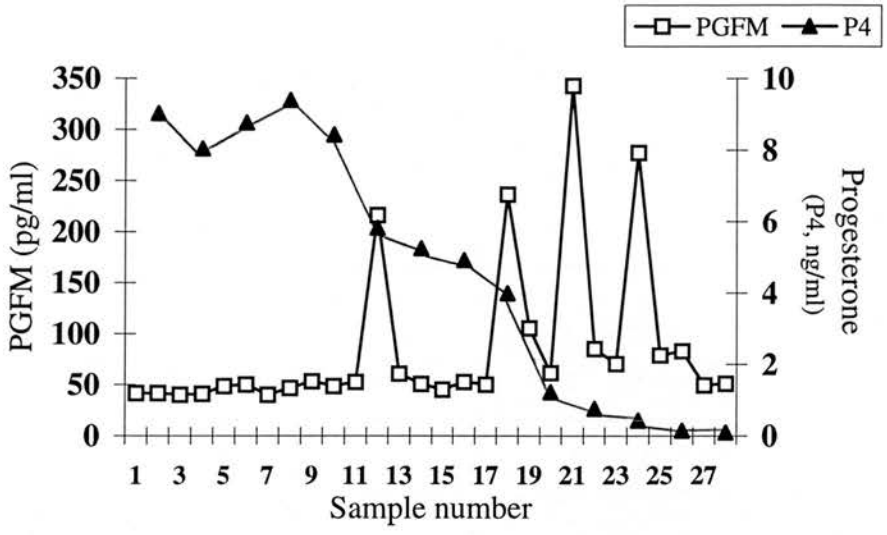
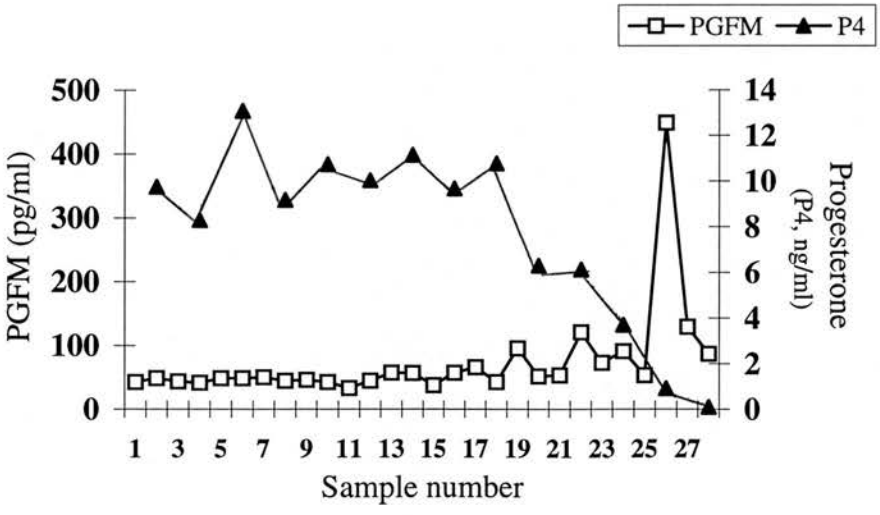


FIGURE 7.3b. Cow 185 (Control group). Plasma PGFM and progesterone concentrations during the trial period (days 16-18 of the oestrous cycle).



7.3.3.2. PGFM concentrations

Mean concentrations of PGFM are shown in Figures 7.4a-7.4c. It can be seen that synthesis of $PGF_{2\alpha}$ was inhibited in all flunixin-treated animals resulting in PGFM concentrations that were approximately 50% of those of the control cows. This result is similar to previous studies (Aiulamai *et al.*, 1990, Odensvik, 1995). The pulses of PGFM, associated with luteolysis, which were clearly observed in 2 control cows (542, 185) were also absent in flunixin-treated animals (Fig 7.4b). During infusion of $PGF_{2\alpha}$ in group 3 (Fig. 7.4c) concentrations of PGFM in jugular plasma were elevated throughout the 12 hour treatment to levels of around 300-600pg/ml, although in one animal (cow 574, Fig. 7.4c) concentrations as high as 1600 pg/ml were recorded..

FIGURE 7.4a. Plasma PGFM concentrations for control animals in flunixin trial.

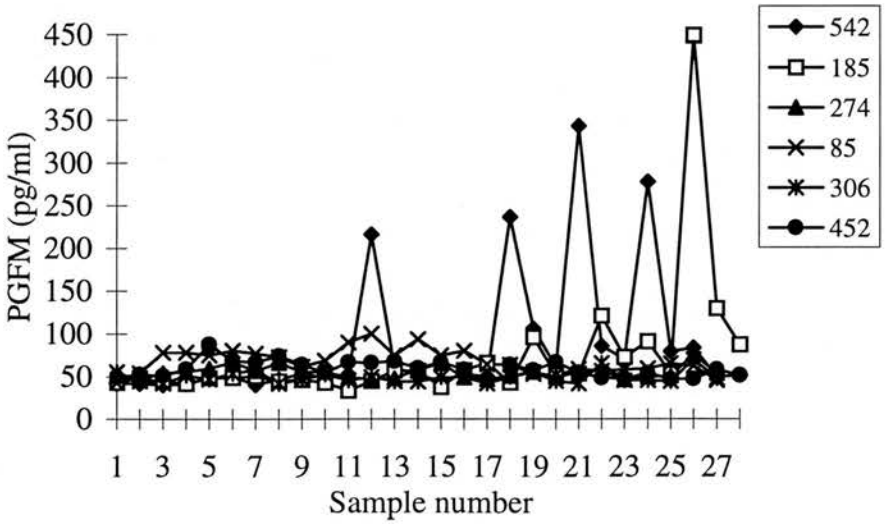


FIGURE 7.4b. Plasma PGFM concentrations for flunixin-only treated animals.

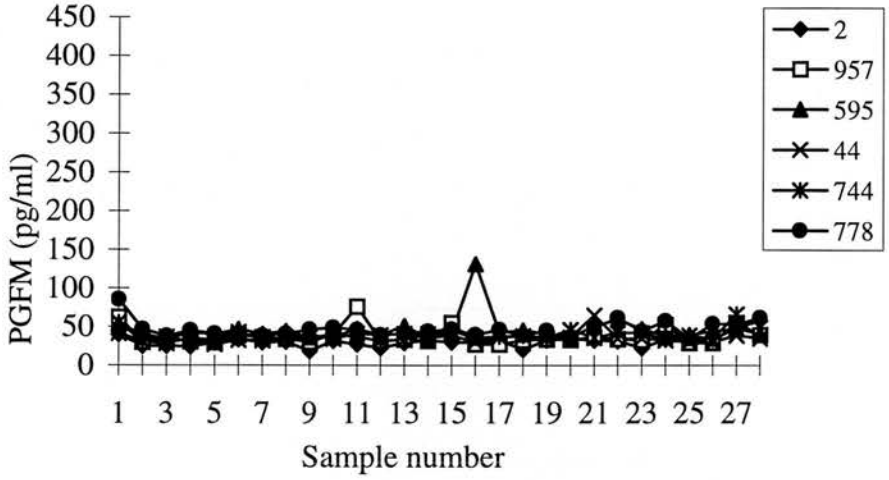
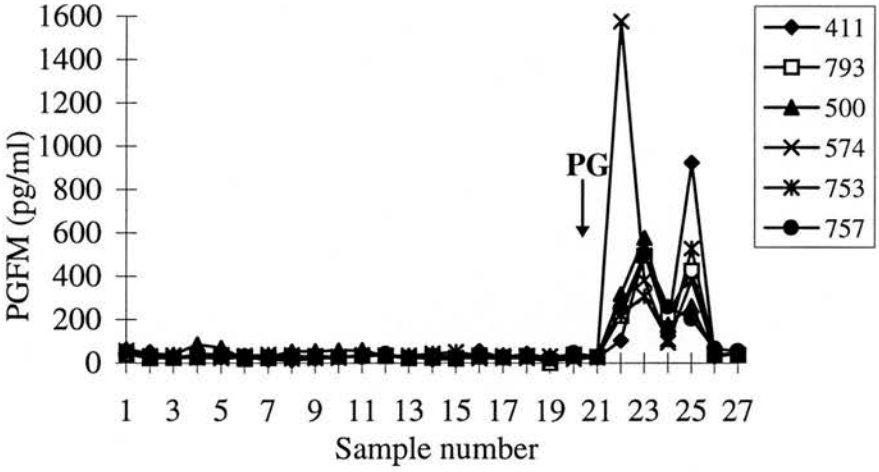


FIGURE 7.4c. Plasma PGFM concentrations for flunixin treated animals where $PGF_{2\alpha}$ was replaced for 12 hours prior to ovariectomy.



7.3.4. Immunohistochemistry

7.3.4.1. Lymphocyte populations

Four lymphocyte subsets were investigated in this study: B-lymphocytes and CD5+, CD8+ and CD4+ T-lymphocytes. B-lymphocytes were not found to be present in any of the tissue sections studied except one, which is similar to our previous studies.

There were no significant differences between numbers of CD5+, CD8+ or CD4+ T-lymphocytes counted in luteal tissue from any of the groups (Table 7.1). However, CD5+ and CD8+ T-lymphocytes were consistently lower in both groups of treated animals compared to the control group. CD4+ T-lymphocytes were present in similar numbers in each group.

7.3.4.2. Macrophages /MHC II expression

Numbers of macrophages (Table 7.1) were significantly lower in both flunixin and flunixin/ $PGF_{2\alpha}$-treated cows ($p<0.05$). There were no significant differences in MHC II expression between any of the groups (Table 7.1).

7.3.4.3. Eosinophils

Populations of eosinophils did not differ significantly between groups with a mean of 0- 2 cells counted per field. However one of the control animals had between 15-25 eosinophils/field.

Table 7.1. Populations of immune cells within CL

(mean/6 fields ± s.e.m., 6 cows per group)

	Control	Finadyne	Finadyne+PG
CD5+ T-lymphocytes	44.3 ± 11	26.44 ±3.9	35.17 ± 6.3
CD8+ T-suppressor/cytotoxic	16.53 ± 2.2	12.53 ± 1.7	10.97 ± 3.7
CD4+ T-helper/inducer	3.35 ± 1.2	3.72 ± 0.7	4.78 ± 1.6
Macrophages	11.03 ± 2.7	**4.47 ± 0.9***	**4.70 ± 0.9***
MHC II (DQ)	16.86 ± 2.1	12.19 ± 2.5	13.47 ± 2.1
MHC II (DR)	12.86 ± 2.1	11.64 ± 1.3	10.5 ± 1.8
Eosinophils	3.72 ± 2.9	0.19 ± 0.05	0.47 ± 0.3
B-cells	0	0	0

*Mean value significantly lower then control group ($p<0.05$).

7.4. Discussion

Inhibition of $PGF_{2\alpha}$ production using intensive flunixin treatment between days 16 and 18 of the oestrous cycle resulted in a significant decrease in the numbers of macrophages present in luteal tissue compared to untreated control animals. This effect was limited to macrophages and the number of T-lymphocytes and MHC II expression in the CL was not significantly different between any of the groups. B-cells were insignificant in CL from all cows and eosinophils were only present in large numbers in one cow. Both of these cell types appear to be unimportant in bovine CL at this stage of the oestrous cycle.

The decrease in the numbers of macrophages during flunixin treatment was unexpected as T-lymphocytes, not macrophages, were previously associated with the most dramatic changes in numbers at this stage of the oestrous cycle (Chapter 5). However, macrophages were observed in CL collected throughout the oestrous cycle in previous studies. Using visually-staged luteal tissue (chapter 4) macrophages were found to be present in significantly higher numbers at Stages I (days 1-4), III (days 11-17) and IV (days 18-20) of the oestrous cycle compared to Stage II (days 5-10). There were more macrophages in Stage IV CL than Stage III. The numbers of macrophages increase in Stage IV CL as a result of their role in structural luteolysis (Paavola, 1979; Bagavandoss *et al.*, 1988) and high numbers of macrophages (in comparison to Stage II) in CL in the days prior to luteolysis (Stage III) are likely to be present in preparation for this role. However it is also possible that macrophages, which are potent sources of a range of cytokines such as tumour necrosis factor-α (TNF-α), may have other, as yet unknown, roles locally within the CL at this time.

The results of this study demonstrate that $PGF_{2\alpha}$ is a key substance in controlling macrophage populations within the CL around the time of luteolysis. It is possible that $PGF_{2\alpha}$ may act directly as a chemoattractant for monocytes/macrophages. $PGF_{2\alpha}$ is a central substance in any inflammatory reaction and, when present in sufficient concentrations within the ovary and CL, may itself act as a direct stimulus for the influx of immune cells. A variety of cyclo-oxygenase products of the arachidonic acid pathway have been shown to have chemotaxic effects on different types of immune cells *in vitro* (Lees *et al.*, 1986). However there is a significant degree of variation between species and there are no specific descriptions of the effects of $PGF_{2\alpha}$ on bovine leukocytes or macrophages.

$PGF_{2\alpha}$ could also act indirectly, stimulating other cell types that are then involved in attracting macrophages into luteal tissue. If this is the case than the potential mechanisms of action may be very complex. Activation of existing immune cell populations and cytokine production by cells including lymphocytes and macrophages, as well as luteal

cells, endothelial cells or fibroblasts could be involved (Adashi, 1992a; Pate, 1995). Cytokines such as TNF-α, interleukin 1β (IL-1β) and interferon-γ (IFN-γ) have been shown to increase production of $PGF_{2\alpha}$ by luteal cells themselves (Pate and Townson, 1994). Therefore it is possible that positive feedback on local $PGF_{2\alpha}$ release may occur. Recently there has also been interest in the role of a specific cytokine, monocyte-chemoattractant protein-1 (MCP-1), in the CL. This substance is a specific chemoattractant for monocytes and expression of MCP-1 in the rat occurs before the increase in macrophages which is associated with structural regression (Townson *et al.*, 1996). It is produced by a variety of cell types including lymphocytes, macrophages and endothelial cells (Leonard and Yoshimura, 1990). MCP-1 may therefore also have a role in the cow CL and this will be discussed in more detail in Chapter 8.

Although this study describes the suppression of systemic production of $PGF_{2\alpha}$ during luteolysis, local production of $PGF_{2\alpha}$ will also be reduced by intensive treatment with flunixin. Within the CL luteal cells as well as non-luteal cells, such as endothelial cells, produce $PGF_{2\alpha}$ (Milvae and Hansel, 1983; Milvae, 1986) although the highest concentrations are seen early in the cycle (Rodgers *et al.*, 1988). Therefore it should not be assumed that uterine $PGF_{2\alpha}$ alone is involved in macrophage attraction to the CL. In fact, when the results of the previous studies are considered, it would seem likely that locally-produced $PGF_{2\alpha}$ may be significant. Increased numbers of macrophages were observed in our previous study (Chapter 4) from Stage III (days 11-17) onwards. As systemic concentrations of $PGF_{2\alpha}$ do not increase until around day 15-16 of the oestrous cycle (Basu and Kindahl,1987; Parkinson and Lamming, 1990) it appears that the increase in macrophages may occur before any increase in systemic concentrations of $PGF_{2\alpha}$. Similarly, in Chapter 5, using accurately staged luteal tissue, macrophage numbers were similar in CL collected on day 13-14 of the oestrous cycle and in CL collected around the time of luteolysis (days 15-20). Therefore, local production of $PGF_{2\alpha}$ may be important, at least initially, in the days before increased uterine production of $PGF_{2\alpha}$.

The results of this study indicate that $PGF_{2\alpha}$ is not directly involved in the influx of T-lymphocytes which occurs around day 16 of the oestrous cycle and that some other substance(s) must be involved. However, as discussed above, it is also possible that, although $PGF_{2\alpha}$ is not involved in attracting T-lymphocytes to the CL, it may be involved in activation of existing populations of T-lymphocytes. Therefore the reduction in the numbers of macrophages after flunixin treatment may be caused, either completely or in part, by a lack of stimulation of T-lymphocytes at this stage.

In this study there was no reduction in MHC II expression which might have been expected in the presence of reduced numbers of macrophages after flunixin treatment. However MHC II expression within the CL is not confined to cells of the immune system and is also found on other cells, including luteal cells, particularly in older CL and after $PGF_{2\alpha}$ induced luteolysis (Fairchild Benyo *et al.*, 1991a; Kenny *et al.*, 1991). It may be therefore that, although there were fewer macrophages in CL from animals treated with flunixin, their contribution to MHC II expression overall in the CL was of limited significance in comparison to the contribution of other cell populations present.

Although there was a significant decrease in populations of macrophages in all flunixin-treated groups there was no significant increase in populations of these cells in flunixin-treated animals which were also infused with $PGF_{2\alpha}$ prior to ovariectomy. If $PGF_{2\alpha}$ is involved in immune cell recruitment within the CL then we might expect that replacing it artificially would reverse the effect of flunixin on immune cell numbers within the CL. However it is very difficult to reproduce *in vivo* concentrations of $PGF_{2\alpha}$ artificially. In our study peripheral concentrations of $PGF_{2\alpha}$ metabolite during treatment were higher than those reported at luteolysis in various *in vivo* studies but we do not know what concentrations of $PGF_{2\alpha}$ actually reached the CL. $PGF_{2\alpha}$ has a relatively short half-life within the body ($t_{1/2}$<1 min). Therefore it may be that in our attempts to avoid inducing luteolysis we have produced concentrations of $PGF_{2\alpha}$ at the level of the ovary which were below physiological concentrations for this stage of the oestrous cycle. It is also possible that the $PGF_{2\alpha}$ concentration was adequate but that a pulsatile release pattern would have

been more effective or that there are other factors involved of which we are as yet unaware.

$PGF_{2\alpha}$ is not the only prostaglandin whose production is inhibited by treatment with flunixin although it is the most important relative to luteolysis. Other prostaglandin products of the arachidonic acid pathway known to have some effects on luteal function are prostaglandin E-2 (PGE-2) and prostacyclin (PGI_2). Both these compounds have been shown to have luteotrophic, rather than luteolytic effects in vivo (Gimenez and Henricks, 1983; Milvae, 1986) on luteal cells. However we did not observe any significant alteration in progesterone concentrations during flunixin treatment.

In conclusion, we have shown that inhibition of $PGF_{2\alpha}$ production in the days leading up to luteolysis causes a reduction in the number of macrophages within the CL at this time. It is not clear whether systemic or local $PGF_{2\alpha}$ production is most significant in this process; indeed both may be involved. $PGF_{2\alpha}$ is not only the luteolytic substance in the cow but is also involved in events occurring at the cellular level within luteal tissue prior to luteolysis. Further studies are required to investigate the mechanisms through which $PGF_{2\alpha}$ exerts its effects at this stage of the oestrous cycle.

Chapter 8

Cytokine production in the bovine corpus luteum

8.1. Introduction

In addition to studies of immune cell types in the CL, there is an increasing amount of literature investigating production of cytokines within luteal tissue as well as the effects of various cytokines on luteal cells *in vitro* (Adashi, 1992a; Brannstrom and Norman, 1993). This interest has been stimulated by the rapid expansion of information available in recent years relating to the cytokine products of immune cells and their range of effects on cells outwith the immune system.

Cytokines are soluble protein molecules which act as intercellular messengers. They are secreted by immune cells and also by some other cell types (Roitt, 1992). A large number of secreted proteins come under the broad term 'cytokines' including interleukins, colony stimulating factors, tumor necrosis factors and interferons. These substances are extremely potent, act locally at very low concentrations (10^{-15} M) and have a short half-life. The known sources and effects of some common cytokines which, for reasons discussed below, are of interest in luteal function are shown in Table 8.1. However, there is a significant degree of interaction and overlap of effect between cytokines; therefore their effects *in vivo* are unlikely to be as clear cut as described in luteal cell studies *in vitro* (Townson and Pate, 1994).

Various techniques have been used to look at cytokine production in CL (Park-Sarge and Mayo, 1993). These include bioassays and immunohistochemistry which detect the protein, as well as molecular techniques such as the polymerase chain reaction (PCR) and *in situ* hybridisation which detect messenger RNA (mRNA). Results vary considerably, not only depending on the technique used, but also depending on the particular species studied (see Vinatier *et al.*, 1995, for a review). There has been

particular interest in tumour necrosis factor-α (TNF-α) because of its potential role during luteal regression (Terranova *et al.*, 1991; Payne *et al.*, 1991; Hunt, 1993). This cytokine is produced by macrophages which are present in significant numbers in luteal tissue after luteolysis. It is also cytotoxic to tumour cells and the cellular processes involved in this have been compared to luteal regression (Ji *et al.*, 1991).

TABLE 8.1. Cell sources and effects of three cytokines which may be involved in luteal cell function.

Cytokine	**Source**	**Effect**
Tumour Necrosis Factor-alpha (TNF-α)	Macrophages T-lymphocytes	Mediator of inflammatory response Cytotoxic effect on some tumours
Interleukin-1-beta (Il-1β)	Macrophages Fibroblasts	Proliferation of activated B- and T-cells Induction of cytokine production by macrophages.
Interferon gamma (IFN-γ)	Leucocytes	Macrophage activation Stimulates expression of MHC class I and II on macrophages and other cells

TNF-α has been detected in cow CL by immunohistochemistry (Roby and Terranova, 1989) and radioimmunoassay of dialysate collected directly from the CL *in vivo* (Shaw and Britt, 1995). In addition, a considerable number of *in vitro* studies have been published relating to the effects of a variety of cytokines, either individually or in combination, on bovine luteal cells in culture (Townson and Pate, 1994, Pate, 1995, 1996). The three main cytokines studied are TNF-α, interleukin-1 beta (Il-1β) and interferon-gamma (IFN-γ). The findings from this group of studies are summarised in Table 8.2.

Table 8.2. The effects of TNF-α, IL-1β and IFN-γ, individually or in combination, on luteal cells in culture.

Cytokine(s)	Effect on mid-cycle (days 9-12) luteal cells in culture
TNF-α	Inhibition of LH-stimulated progesterone production and enhanced production of prostaglandins, including $PGF_{2\alpha}$
Il-1β	Inhibition of LH-stimulated steroidogenesis Stimulation of prostaglandin ($PGF_{2\alpha}$, PGE_2 and prostacyclin) synthesis
IFN-γ	Initial inhibition of prostaglandin synthesis for 24 hours followed by inhibition of LH-stimulated progesterone production and stimulation of prostaglandin production. Some loss of cell viability.
TNF-α+Il-1β	Positive synergistic effect on luteal production of prostaglandins
TNF-α+IFN-γ	Cytotoxic effects on luteal cells

More recently the potential role of another cytokine, monocyte chemoattractant protein-1 (MCP-1), within the CL has been of interest, particularly in relation to the influx of macrophages which occurs during structural luteolysis. MCP-1, which is also known as monocyte chemotactic and activating factor (MCAF), is a member of the intercrine β family of cytokines. The cytokines in this group are involved in inflammation and tissue repair and there are 2 subfamilies, α and β, based on their amino acid sequence and chromosomal position (see Oppenheim *et al.*, 1991 for a review).

MCP-1 is produced by a variety of cell types including fibroblasts, endothelial cells, lymphocytes and macrophages and is involved in the cellular immune response as well as response to tissue damage (Leonard and Yoshimura, 1990). MCP-1 is a specific chemoattractant for monocytes when injected into the ears of rats (Zachariae *et al.*, 1990) and also acts as a chemoattractant for monocytes *in vitro* (Yoshimura *et al.*, 1989). During tissue injury *in vivo*, platelet-derived growth factor (PDGF) stimulates release of MCP-1 from fibroblasts inducing an influx of monocytes/ macrophages which act as a first line of defence against bacteria as well as being involved in tissue remodelling. In addition to PDGF released by fibroblasts, some

other growth factors and cytokines such as TNF-α and IL-1 also act as stimulants of MCP-1 release (Larsen *et al.*, 1989; Strieter *et al.*, 1989).

These properties (specific monocyte chemoattraction / tissue remodelling) first led workers to consider a role for MCP-1 in the CL, particularly in the influx of macrophages which is observed in late stage CL. The macrophages are involved in the rapid destruction of luteal tissue which occurs after functional luteolysis (Paavola *et al.*, 1979). Both MCP-1 and MCP-2 have been described in pig luteal cells (Hosang *et al.*, 1994a, 1994b) and increased expression is seen in late stage CL in the rat, a finding that supports the theory of MCP-1 involvement around luteolysis (Townson *et al.*, 1996).

Populations of immune cells vary throughout the oestrous cycle and, in particular, there is a significant increase in numbers of T-lymphocytes (CD5+, CD8+) present within the bovine CL prior to functional luteolysis (Chapters 4 and 5). These results suggest that immune cells may have an active role in altering luteal function, particularly around the time of natural luteolysis. One way in which immune cell activity can be assessed is by measurement of cytokine gene expression.

The aim of this study was to measure cytokine mRNA expression (TNF-α, Il-1β, IFN-γ and MCP-1), using the reverse transcription-polymerase chain reaction (RT-PCR), in cow CL throughout the oestrous cycle, as well as following induced luteolysis and inhibition of $PGF_{2\alpha}$ release between days 15 and 18 of the oestrous cycle.

8.2. Materials and methods

The molecular techniques used in this study are summarised briefly here and described in more detail in Appendix 1.

8.2.1. Collection of luteal tissue

CL obtained from four groups of cows were used in this study. The synchronisation techniques, methods of CL collection and tissue treatment are described in detail elsewhere (Chapters 4, 5, 6 and 7) and can be summarised as follows;

Group A: Natural luteolysis.

The oestrous cycles of a group of 18 normally-cycling heifers were synchronised and the CL were then collected at slaughter between days 16 and 20 of the resulting oestrous cycle (standing oestrus = day 0). A portion of luteal tissue was snap frozen in dry ice / isopentane and stored at -70°C until RNA extraction was performed. Blood samples, collected for 1-2 days prior to and at the time of slaughter, were analysed for progesterone concentrations to allow assessment of which animals had undergone functional luteolysis (P4<1ng/ml). The results showed that five animals had undergone luteolysis at the time of slaughter, all other animals had progesterone concentrations consistent with a functional CL.

Group B: Days 2-14 of the oestrous cycle.

This group of 14 CL was collected from various groups of animals. Two CL were collected at the abattoir and assessed as being stage I (days 1-4) of the oestrous cycle using visual assessment (Ireland *et al.*,1980, see Chapter 2, Section 2.2.2.). Six CL were collected from animals in which oestrous synchronisation had been unsuccessful and the CL were again visually staged at the time of slaughter as Stage I (days 1-4, n=3) and Stage II (days 5-10, n=3). Six CL were collected from cows whose oestrous cycles were successfully synchronised as described in Chapter 5. The CL from these animals were collected on day 10 (n=2) or days 13-14 (n=4).

Group C: Induced luteolysis

The oestrous cycles of 16 mature dairy cows were synchronised and the animals were divided into four groups. Three groups were given 25mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse, Upjohn Ltd, Crawley, UK) either 6 (Group 1), 12 (Group 2) or 24 (Group 3) hours before surgical collection of the CL on day 10 of the oestrous cycle. Group 4

were control animals and did not receive any $PGF_{2\alpha}$. A portion of luteal tissue was snap frozen in dry ice /isopentane and stored at -70°C.

Group D: Inhibition of $PGF_{2\alpha}$ release around the time of luteolysis.

The oestrous cycles of 18 mature dairy cows were synchronised and the animals were allocated to one of three groups. In order to suppress release of endogenous $PGF_{2\alpha}$ Group 1 were treated with flunixin meglumine (Finadyne, Schering Plough, U.K.) intravenously via an indwelling cannula every 6 hours (2.2mg/kg) between days 15 and 18 of the oestrous cycle and the CL was surgically removed on day 18. Group 2 were also treated with flunixin but in addition these animals were infused with exogenous $PGF_{2\alpha}$ (Lutalyse, 170µg/hr) through the cannula for 24 hours prior to surgery. Group 3 were control cows and were treated with saline for 3 days before surgery. Blood samples were collected from all animals every 3 hours during treatment for measurement of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM), the stable metabolite of $PGF_{2\alpha}$, and progesterone concentrations. Luteal tissue was snap frozen in dry ice/isopentane and stored at -70°C.

8.2.2. Collection and storage of tissue

All luteal tissue obtained was handled in such a way as to minimise RNase contamination; gloves were used at all times and sterile petri dishes and scalpels used for cutting of tissue. The ovaries were initially placed on petri dishes on ice and then CL were carefully removed by blunt dissection from the surrounding tissue. Small portions were foil-wrapped and snap frozen in a slurry of dry ice and isopentane as soon as possible after collection (within 10 minutes after surgical collection and within 20-30 minutes after death when tissue was collected at an abattoir). Frozen samples were then stored at -70°C prior to RNA extraction.

8.2.3. RNA Extraction

Good quality RNA is a prerequisite for successful use of the RT-PCR. There are a variety of techniques available and in recent years new methods have been developed

to allow rapid, uncomplicated RNA extraction. TRI Reagent™ (Molecular Research Centre, Cincinnati, USA) and Qiagen RNeasy kits (Qiagen Ltd., Crawley, UK) were used initially for extractions but the yield and quality of the RNA produced from luteal tissue was not sufficient for RT-PCR. Finally a guanidine thiocyanate extraction protocol (Chomczynski and Sacchi, 1987) was used which gave a reasonable yield of good quality RNA. The extraction techniques are described in Appendix 1 (Section 1.4.).

8.2.4. Testing the quality of RNA

Extracted RNA was examined by spectrophotometry and electrophoresis to assess the quality and quantity of RNA yielded prior to reverse transcription.

8.2.4.1. Spectrophotometry

Samples of extracted RNA were diluted 1:100 in diethylpyrocarbonate (DEPC)-treated water and the absorbance measured in a spectrophotometer at wavelengths of 260nm and 280nm. The A_{260}:A_{280} ratio was then calculated which gave an indication of the quality of the RNA. Ratios in the region of 1.6-2 were taken to indicate RNA of good enough quality to proceed with the RT PCR. The absorbance at a wavelength of 260nm was also used to calculate the yield of RNA using the formula;

RNA (μg/ml) = A_{260} x 40 x dilution factor (100)

8.2.4.2. Formaldehyde Gel electrophoresis

A formaldehyde gel was prepared and run using 1μg RNA from each sample (Appendix 1, Section 1.5.). The gel was examined under ultraviolet light looking for 2 well-defined bands which correspond to 28S and 18S ribosomal RNA. RNA degradation was indicated where well-defined bands were not visible.

8.2.5. Reverse-Transcription PCR (RT-PCR)

RT-PCR was performed on the extracted RNA as soon as possible after preparation using standard methods which are described in detail in Appendix 1 (Section 1.6-1.7.).

8.2.5.1. Reverse transcription - Control samples

For every RNA sample that was reverse transcribed a control sample, containing no reverse transcriptase enzyme, was prepared and used in the PCR.

For every set of samples a control tube was prepared which was identical to the reverse transcription reaction but contained no RNA.

8.2.5.2. Selection of primers

The primer sequences for TNF-α, IL-1β and IFN-γ used in this study were provided by Dr R. Collins from The Institute for Animal Health, Compton, UK. The bovine MCP-1 gene, which has been characterised by Wempe *et al.* (1994), was used to design primers for MCP-1. The primer sequences and PCR product sizes are shown in Table 8.3.

Table 8.3. Primer sequences and PCR product sizes for TNF-α, IL-1β, IFN-γ, MCP-1 and ATPase.

Cytokine	Primer number	Primer Sequences	PCR Product(b.p.)
TNF-α	1	5'-ACTCAGGTCATCTTCTCAAGCC-3'	
	2	5'-ATGATCCCAAAGTAGACCTGCC-3'	464
IL-1β	1	5'-TGACGCACCCGTTCAGTCAAT-3'	
	2	5'-AGTGAAGTTCAGGCTGCAGCT-3'	564
IFN-γ	1	5'-GCAAGTAGCCCAGATGTAGC-3'	
	2	5'-AGTGAAGTTCAGGCTGCAGCT-3'	316
MCP-1	1	5'-AACAGCTTCCCGCTGAAAC-3'	
	2	5'-TCTGCACATAACTCCTTGCC-3'	270
ATPase	1	5'-ACGAACACCACTCCTGGATGAGC-3'	
	2	5'-CACGGACGTCTCCAGGCTGTGTA-3'	193

8.2.5.3. Optimisation of PCR

The volume of RTase reaction used in the PCR was varied for each cytokine studied to give a distinct PCR product. The number of cycles and annealing temperature used in the thermal cycler was also varied between 25 and 35 cycles and 55°-65°C for each cytokine to find optimal conditions for PCR.

8.2.5.4. PCR Control samples

Internal control

As ATPase is present in all living cells, a preliminary PCR was performed using primers for ATPase on a limited number of samples from each set of RTase reactions to ensure that reverse transcription had been successful and that comparable amounts of RNA had been transcribed from different samples. ATPase primers (Table 8.3) were designed from the bovine ATPase gene (Brandt *et al.*, 1988) by Dr C. Woodall, Department of Veterinary Pathology, University of Edinburgh.

Negative controls

For each sample two separate reactions were prepared. One contained the product of the RTase reaction (RTase +) and the other contained the RTase reaction which had no enzyme (RTase -). In addition, for each set of reactions prepared, a negative control was included which had been reverse transcribed but contained no RNA.

Positive controls

Samples of plasmid for TNF-α, Il-1β and IFN-γ were generously supplied by Dr R. Collins from The Institute for Animal Health, Compton, U.K (Table 8.4). The plasmids were used as positive controls for the RT-PCR. The PCR mix for plasmid is described in Appendix 1 (Section 1.7.3.). To avoid contamination of other PCR samples the plasmid was added last to the reaction in a separate location after all other reaction mixtures had been prepared.

Table 8.4. cDNA clones

cDNA	GCG Accession no.	Length and bases
TNF-α	ovine is X55152	465bp (387-852)
IL-1β	M35589	564bp (380-994)
IFN-γ	M29867	430bp (165-595)

8.2.5.5. Visualisation of PCR product

The PCR products were visualised on a 3% agarose gel prepared using a standard method (Appendix 1, Section 1.8.), stained with ethidium bromide, destained in water and visualised and photographed under UV light.

Where visible differences in intensity of bands between animals were observed the negative images were analysed by image analysis using NIH-Image 1.6 (NIH, Bethesda, MD). Repeated sample ANOVA on log-transformed data was performed and individual comparisons between bands were made using an unpaired t-test. All statistical analysis was performed using Minitab Software (Pennsylvania State University, USA). A significant difference in PCR product intensity was taken to be present where $p<0.05$.

8.2.6. Confirmation of PCR product- enzyme digests

In addition to the use of individual cytokine plasmids as positive controls, the identification of individual PCR products using primers for TNF-α, Il-1β and IFN-γ was confirmed by digestion with specific restriction enzymes (Table 8.5) as described in Appendix 1 (Section 1.9.)

Table 8.5. Restriction enzymes and digested product sizes for TNF-α, Il-1β and IFN-γ

Cytokine	Restriction enzyme	Digested product sizes (bp)
TNF-α	BglI	355, 109
Il-1β	PvuII	317, 247
IFN-γ	EcoRV	164, 152

8.2.7. Amplification and sequencing of MCP-1

In order to confirm the identity of the PCR product for MCP-1 the product was amplified and sequenced using standard techniques which are described in detail in Appendix 1 (Section 1.10.).

8.3. RESULTS

8.3.1. RNA extraction

The A_{260}/A_{280} ratios produced by RNA extracted using the three different techniques varied between 1.5 and 1.9. Extractions using TRI reagent and Qiagen RNeasy kits yielded RNA with an A_{260}/A_{280} ratio between 1.5 and 1.6. Chloroform extraction improved the A_{260}/A_{280} ratios but did not alter the appearance of the RNA on a gel. The extraction of RNA using guanidine thiocyanate phenol (Chomczynski and Sacchi, 1987) yielded good quality RNA (Fig. 8.1). The quantity of RNA extracted using this technique ranged between 150-200μg from 100mg of luteal tissue.

8.3.2. RNA loading

Amplified bands for ATPase were of similar intensity between samples which confirmed that the amounts of RNA added to the initial RTase reactions were comparable (Figure 8.2)

Figure 8.1. RNA extracted from luteal tissue (10 samples) and fractionated by formaldehyde gel electrophoresis to show 2 distinct bands which correspond to 28S and 18S ribosomal RNA.

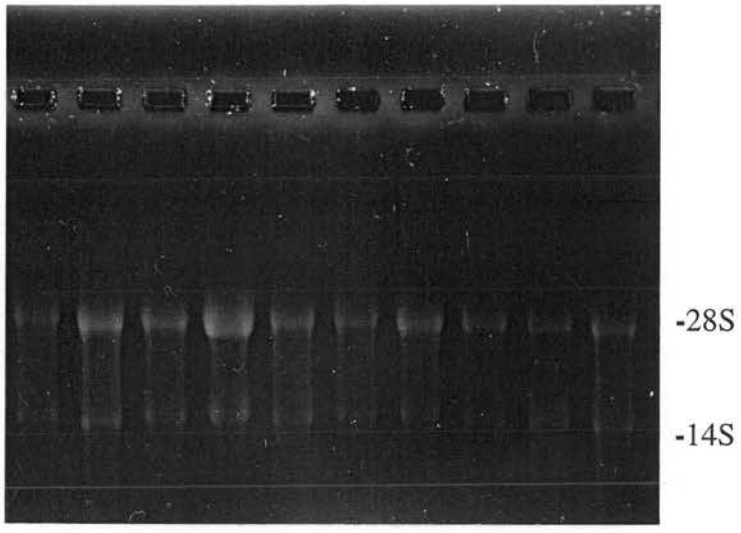
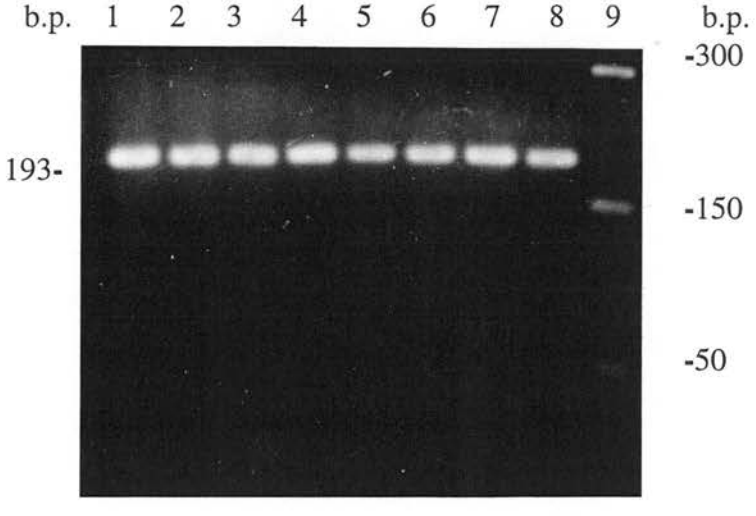


Figure 8.2. Examples of the products from RT-PCR using primers for ATPase to demonstrate comparable RNA loading between samples.
Lanes 1-8 - PCR products, Lane 9 - molecular weight marker



8.3.3. Optimisation of PCR conditions

PCR reactions for the individual cytokines were optimised. The amount of RTase reaction added to the PCR, annealing temperature and number of cycles varied (Table 8.6). For PCR the thermocycler was programmed as follows; 5 min at 95°C; 30 sec at 95°C, 30 sec at 60-65°C, 30 sec at 72°C for 30-35 cycles and finally 5 min at 72°C.

Table 8.6. Optimal PCR conditions for individual cytokines.

Cytokine	Volume of RTase reaction (μl)	Annealing temperature (°C)	Number of cycles
TNF-α	10	60	30
Il-1β	8	60	30
IFN-γ	10	60	35
MCP-1	6	60	30
ATPase	6	60	30

8.3.4. Validation of RT-PCR

8.3.4.1. Restriction enzyme analysis

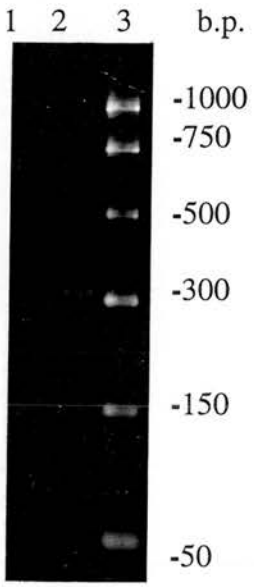
Digestion of PCR products for TNF-α, Il-1β or IFN-γ with specific restriction enzymes gave products of the expected sizes confirming that the RT-PCR products did result from amplification of the mRNA for the different cytokines within luteal tissue (Fig. 8.3).

8.3.4.2. Amplification and Sequencing of MCP-1

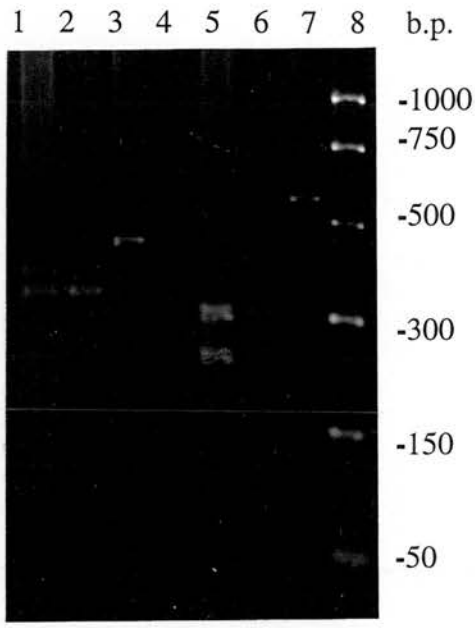
The MCP-1 PCR product was successfully amplified and sequenced. The DNA sequence was 100% homologous with the MCP-1 gene.

Figure 8.3. IFN-γ, TNF-α and IL-1β - products of enzyme digests.

a) IFN-γ



b) TNF-α and IL-1β



a) IFN-γ
- Lane 1 - IFN-γ, cut (digest product sizes; 152, 164 b.p.)
- Lane 2 - IFN-γ, uncut (316 b.p.)
- Lane 3 - molecular weight marker

b) TNF-α and Il-1β
- Lane 1 - TNF-α, cut (digest product sizes; 109, 355 b.p.)
- Lane 2 - TNF-α, cut
- Lane 3 - TNF-α, uncut (464 b.p.)
- Lane 4 - blank
- Lane 5 - Il-1β, cut (digest product sizes; 247, 317 b.p.)
- Lane 6 - Il-1β, cut
- Lane 7 - Il-1β, uncut (564 b.p.)
- Lane 8 - molecular weight marker

8.3.5. PCR for cytokines TNF-α, Il-1β, IFN-γ and MCP-1

8.3.5.1. Group A - Natural luteolysis

Amplified bands corresponding with the expected PCR product size for TNF-α, Il-1β, IFN-γ and MCP-1 were visible in all animals. Although the band intensity varied between animals, overall there were no visible differences in band intensity for TNF-α, Il-1β or IFN-γ between animals before or after luteolysis. However, the amplified bands for MCP-1 from luteal tissue after functional luteolysis were more intense than those seen before functional luteolysis (Fig. 8.4). The intensity of the bands were measured by image analysis of two sets of PCR. The intensity of the bands produced by RT-PCR of mRNA from luteal tissue after functional luteolysis was significantly higher ($p<0.01$) in both sets of reactions, than that seen in luteal tissue before luteolysis.

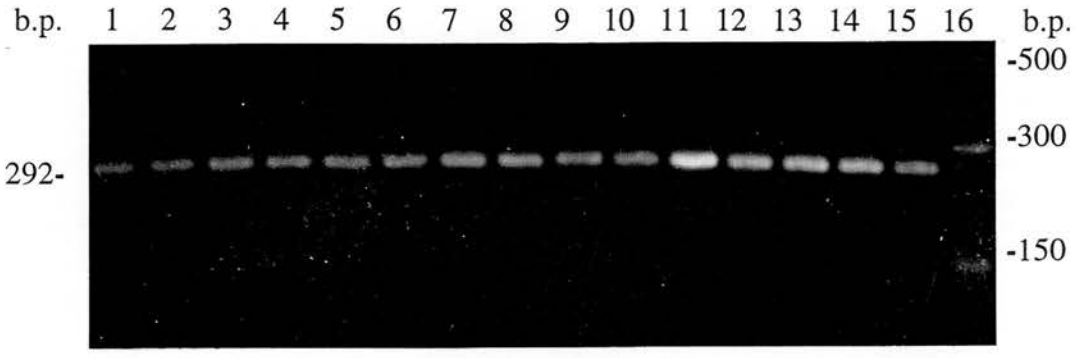
8.3.5.2. Group B - Days 2-14 of the oestrous cycle

Amplified bands corresponding with TNF-α were present in CL from all animals but the intensity of staining was faint. Bands corresponding to the expected product sizes for Il-1β and IFN-γ were also present at all stages. The intensity of staining was variable but was not obviously different between early and mid-cycle CL. MCP-1 was present in all CL collected between days 2-14 of the oestrous cycle.

8.3.5.3. Group C - Induced luteolysis

Positive PCR products for TNF-α were faintly present in CL from all cows. Positive PCR products for Il-1β, IFN-γ and MCP-1 were present in luteal samples from all animals. The band intensity varied between individual animals but there were no distinct differences between groups (for example, IFN-γ, Fig. 8.5).

Figure 8.4. Luteal tissue around the time of natural luteolysis: RT-PCR products using primers for MCP-1.

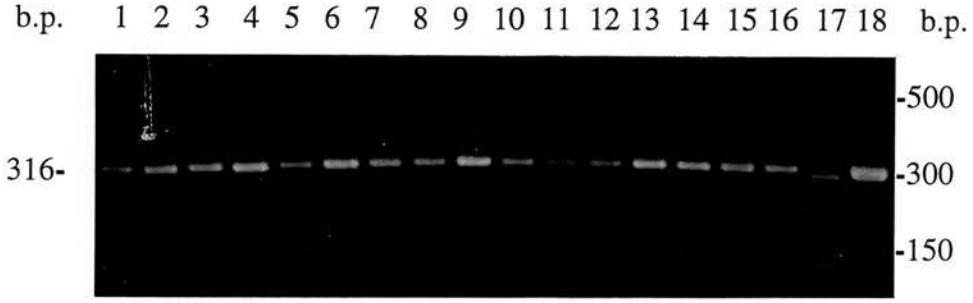


Lanes 1-10 - Pre-luteolysis, RT-PCR of luteal tissue collected between days 16 and 20 of the oestrous cycle where the CL was still functional at the time of slaughter (plasma progesterone concentration > 1ng/ml).

Lanes 11-15 - Post-luteolysis, RT-PCR of luteal tissue collected between days 16 and 20 of the oestrous cycle where the CL was not functional at the time of slaughter (plasma progesterone concentrations < 1ng/ml).

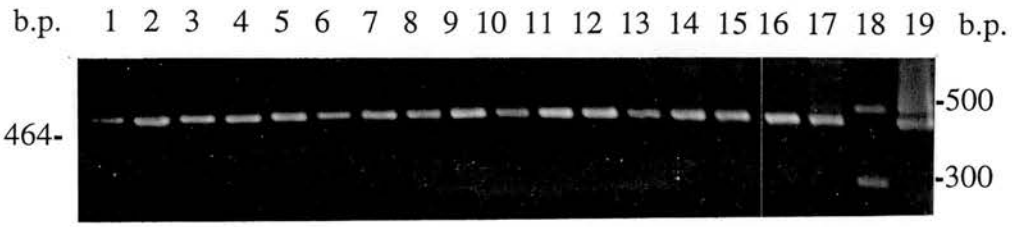
Lane 16 - molecular weight marker.

Figure 8.5. Luteal tissue 0, 6, 12 or 24 hours after luteolysis artificially induced using $PGF_{2\alpha}$ on day 10 of the oestrous cycle: RT-PCR products using primers for IFN-γ.



Lanes 1-4 - control cows (no $PGF_{2\alpha}$)
Lanes 5-8 - 6 hours after $PGF_{2\alpha}$
Lanes 9-12 - 12 hours after $PGF_{2\alpha}$
Lanes 13-16 - 24 hours after $PGF_{2\alpha}$
Lane 17 - molecular weight marker
Lane 18 - IFN-γ plasmid control

Figure 8.6. Luteal tissue collected after inhibition of $PGF_{2\alpha}$ release between days 16 and 18 of the oestrous cycle: RT-PCR products using primers for TNF-α.



Lanes 1-6 - control cows
Lanes 7-12 - Flunixin only
Lanes 13-17 - Flunixin+$PGF_{2\alpha}$
Lane 18 - molecular weight marker
Lane 19 - TNF-α plasmid control

8.3.5.4. Group D - Inhibition of $PGF_{2\alpha}$ release between days 15 and 18 of the oestrous cycle.

PCR product corresponding to TNF-α was found in CL samples from all animals studied. The bands varied in intensity between animals but not between groups (Fig. 8.6). Cow 595, from the group of cows treated with flunixin only, showed the brightest band. Il-1β was also present in luteal tissue from all cows, the most intensive bands were seen in luteal tissue from cows 185 (Control group) and cow 595. There were positive PCR products for IFN-γ in all animals studied. The most intense staining was again observed from cow 595. The PCR products for MCP-1 varied in intensity between animals but this occurred randomly and was not associated with any specific treatment group. The most intense band for MCP-1 was associated with cow 185, a control cow that underwent luteolysis during the trial.

8.4. Discussion

All four cytokines investigated, TNF-α, Il-1β, IFN-γ and MCP-1, were detectable by RT-PCR using luteal tissue from animals throughout the oestrous cycle, after induced luteolysis and during suppression of $PGF_{2\alpha}$ production between days 15 and 18 of the oestrous cycle. The most significant finding was the increased amount of MCP-1 in luteal samples from cows in group A which had undergone functional luteolysis compared to those in the same group that still had functional CL.

Apart from this distinct variation in MCP-1 mRNA in CL from Group A there was also some variation between individual animals within groups. The RT-PCR in its basic form is not designed for accurate quantification and comparison of PCR products from different samples. However, as the same amount of RNA was used throughout preparation of PCR mixtures for each individual cytokine (as confirmed by similar intensity of bands for ATPase in samples), visible differences in the intensity of bands may represent significant differences in cytokine content between samples from different animals. It may be that variations between groups do not exist but it is also possible that any changes are outwith the sensitivity of a PCR and that

another more quantitative technique, such as an RNase protection assay, should be used to study this in detail.

When investigating TNF-α, Il-1β and IFN-γ the most consistently intense bands were from a cow (595) in the group treated with flunixin to inhibit production of $PGF_{2\alpha}$. This animal had the most intense band for all three cytokines studied in a total of 18 animals. Previous results obtained using immunohistochemistry and hormone assays (Chapter 7) provide no obvious explanation for this difference. The numbers of immune cells or MHC II expression were not significantly different from the rest of the group and the concentrations of progesterone and PGFM were within the range of the other cows being treated with flunixin.

Cytokine production, in particular the presence of TNF-α, has been studied in CL from various species. One of the earliest studies using bio-assays produced inconclusive results about TNF-α production in the rabbit CL. Although TNF-α bioactivity was present in culture media from rabbit luteal cells treated with lipopolysaccharide, none was detected in unstimulated culture media (Bagavandoss *et al.*, 1988). However, immunohistochemistry has been used successfully to detect the presence of TNF-α within CL from the rat and cow (Roby and Terranova, 1989), human (Roby *et al.*, 1990) and mouse (Chen *et al.*, 1993). Il-1β has also been located in the mouse CL (Simon *et al.*, 1994). The majority of these studies have not compared cytokine production at different stages of CL lifespan, although increased staining for TNF-α and IL-1β has been described in regressing CL, in comparison to younger CL, collected from the mouse (Chen *et al.*, 1993; Simon *et al.*, 1994). There is also a detailed study in the pig which demonstrates that, in contrast to our findings in the cow, TNF-α is detectable in pig CL at various stages of pregnancy and during the oestrous cycle but cannot be detected in CL after luteolysis (Hehnke-Vagnoni *et al.*, 1995).

Immunohistochemistry has been used most frequently to detect cytokines in the CL. However a recent novel study in the cow collected samples directly from luteal tissue

during luteolysis using a microdialysis unit which had been pre-placed in the CL. Radioimmunoassay was used to measure TNF-α in the dialysate and an increase in concentration, which followed the decline in serum progesterone, was observed following both induced and natural luteolysis (Shaw and Britt, 1995). A similar finding has been recorded following induced luteolysis in the sheep where TNF-α bioactivity gradually increased in luteal tissue collected at 5 time periods between 0 and 16 hours after luteolysis induced by a single injection of $PGF_{2\alpha}$ (Ji *et al.*, 1991).

It appears therefore that, using a sensitive enough system, quantitative changes in the concentrations of cytokines can be successfully detected in tissue collected at different stages of the CL lifespan. However in addition to this, the production site of these cytokines is also important. Although TNF-α, Il-1β and IFN-γ were detected in cow CL from all groups in the present study, the specific cell sources are not known. Cytokines are not only produced by cells of the immune system but may also be produced by cell types such as fibroblasts, endothelial cells or even luteal cells themselves. Various techniques are available which may be used to identify the site of cytokine production and future studies should include *in situ* hyridisation to locate the cell sites of cytokine production within the CL.

In CL the cell types which are associated with cytokine production vary considerably between species. In the human, TNF-α has been detected in both small and large luteal cells using immunohistochemistry (Roby *et al.*, 1990), but in the cow and the rat TNF-α expression was found in thecal cords within luteal tissue and was associated with cells which also stained positively as macrophages and not luteal cells (Roby and Terranova, 1989). Similarly, in the mouse, immunoreactive TNF-α was found in macrophage-like cells and not luteal cells although luteal cells did express mRNA for TNF-α (Chen *et al.*, 1993). In contrast, Il-1β is expressed in large amounts by mouse luteal cells and in comparatively small amounts by macrophages (Simon *et al.*, 1994). Finally, in the pig CL the endothelial cell is the source of TNF-α. Macrophages were not associated with TNF-α staining in the pig CL even when present in increased numbers after luteolysis (Hehnke-Vagnoni *et al.*, 1995). These

findings are consistent with the presence of TNF-α receptors on small cells (small luteal cells and endothelial cells) in the pig (Richards and Almond, 1994)

Most of the above studies are based on immunohistochemistry. This technique will only detect protein that has actually been formed and is present at that particular moment of time. It does not assess other cells which might be potential sources of the cytokine which may be important in assessing the significance of cytokines within the CL. For example, Ji *et al.* (1991) found increasing TNF-α bioactivity (a function of the protein) in the sheep CL following induced luteolysis. However, somewhat surprisingly, TNF-α mRNA was not detectable by Northern blots or *in situ* hybridisation in any sample of luteal tissue and the authors suggested that this was because the TNF-α had been pre-formed in an external source, most probably immune cells.

The expression of MCP-1 in CL at all stages studied is not unexpected when the cellular components of luteal tissue are considered. MCP-1 is produced by a wide variety of cells including endothelial cells, fibroblasts, lymphocytes and macrophages (Leonard and Yoshimura, 1990). All of these cell types are present, sometimes in significant numbers, in luteal tissue although their proportions relative to large and small luteal cells vary at different stages of the oestrous cycle (O'Shea *et al.*, 1989; Lei *et al.*, 1991). It is possible that the presence of MCP-1 is mainly passive throughout most of the CL lifespan until after functional luteolysis. However, MCP-1 is known to have a role in tissue remodelling, in common with many of the intercrine family (Leonard and Yoshimura, 1990). Therefore, in addition to its potential role as a chemoattractant for monocytes/ macrophages in late-stage CL, MCP-1 might also be involved in the growth and remodelling of luteal tissue throughout its lifespan.

This study did not find any difference in MCP-1 expression, detectable by PCR, between very early CL and those collected between days 5-14. However there was only a limited amount of visually staged tissue available from between days 1-4 of the oestrous cycle and it would be interesting to study more CL collected at a known

time soon after ovulation, particularly as Townson *et al.* (1996) have described increased expression of MCP-1 in the early post-ovulatory rat CL using immunohistochemistry. Increased concentrations of MCP-1 in rat CL after ovulation could be associated with the rapid vascular development (angiogenesis) that takes place at this time (Zheng *et al.*, 1993) which is not dissimilar to events occurring during healing of damaged tissue (Redmer and Reynolds, 1996).

The most significant finding was the increase in MCP-1 mRNA expression within luteal tissue from animals that had undergone functional luteolysis in the 48 hours preceding slaughter. The increase in MCP-1 at this stage is consistent with a role for this substance after functional luteolysis but before structural luteolysis, probably in activating existing populations of macrophages as well as attracting other macrophages into luteal tissue.

Declining progesterone concentrations and/or increasing concentrations of $PGF_{2\alpha}$ could be the stimulus for enhanced MCP-1 expression in the cow after luteolysis. In the rat enhanced MCP-1 expression in the CL of late pregnancy is associated with declining progesterone concentrations (Townson *et al.*, 1996). It is also possible that immune cell populations present within luteal tissue could have a role in affecting MCP-1 production. T-lymphocytes are themselves a potent source of MCP-1 (Kaczmarek *et al.*, 1985; Yoshimura *et al.*, 1989) but also produce cytokines such as TNF-α and IL-1 which are known to promote expression of MCP-1 (Larsen *et al.*, 1989; Strieter *et al.*, 1989). There are significant numbers of T-lymphocytes within the cow CL from day 16 of the oestrous cycle onwards (Chapter 5). It is possible therefore that cytokine production by resident populations of T-lymphocytes, and also macrophages, may have a role in up-regulating MCP-1 mRNA expression causing an influx of macrophages into the CL.

There was no detectable variation in luteal MCP-1 expression between any of the groups treated with flunixin between days 15 and 18 of the oestrous cycle, although the CL from one control cow (185) did appear to contain higher concentrations of

MCP-1 mRNA compared to any of the other animals. This cow had undergone functional luteolysis during the trial, approximately 24 hours before CL collection, and so this was a similar result to that seen in the group around natural luteolysis. From these results it appears that MCP-1 production is not significantly affected by inhibition of $PGF_{2\alpha}$ production around luteolysis. However, as the increase in MCP-1 production in Group A was not observed until after functional luteolysis, this experiment would be more significant if repeated in the days around and immediately after luteolysis than in the days before.

Similarly, induced luteolysis did not result in any detectable increase in MCP-1 expression within luteal tissue. This was surprising as it might be expected that the rapid structural regression taking place after induced luteolysis would involve significant numbers of macrophages. However, this finding did fit in with results from tissue morphology and immunohistochemistry from these cows (Chapter 6) as the cellular events occurring after induced luteolysis were considerably different from those seen after natural luteolysis and no increase in the number of macrophages was observed at any time period after injection.

It is not clear from the results of the present study whether MCP-1 is actually produced by luteal cells themselves. Further studies involving *in situ* hybridisation or immunohistochemistry, where expression of MCP-1 can be localised to individual cell types, are required to clarify this point. Hosang *et al.* (1994a) described expression of MCP-1 by luteal cells from the pig but this study used dispersed luteal tissue which would contain large numbers of endothelial cells and fibroblasts which could themselves act as the source of MCP-1.

In conclusion, we have shown that there is message for TNF-α, Il-1β, IFN-γ and MCP-1 in cow CL collected from animals at different stages of the oestrous cycle as well as after induced luteolysis and during suppression of $PGF_{2\alpha}$ around the time of luteolysis. There is enhanced expression of MCP-1 after functional luteolysis, possibly influenced by hormonal changes or cytokine release from resident

populations of immune cells. This finding, in conjunction with immunohistochemical findings suggests a role for MCP-1 in attracting monocytes/macrophages to the CL for structural regression.

The large variance in results, both within and between species, suggests that it is not possible to draw conclusions using one technique alone. Further studies should involve techniques that detect both the secreted form of the protein (immunohistochemistry, bioassay) as well as the messenger RNA within the CL at different stages of the oestrous cycle. The transient nature of cytokine release as well as the large number of potential cellular sources involved should also be taken into account.

Chapter 9

GENERAL DISCUSSION

The hormonal changes and ovarian events which occur during the oestrous cycle of the cow have been known for many years. However, our understanding of events which take place at the cellular level within the ovary itself is limited. It appears that there are many factors yet to be clearly identified, that affect the bovine reproductive cycle. In particular there is very little information about local factors within the CL which may influence luteal function *in vivo*.

In recent years the fields of reproduction, particularly endocrinology, and immunology have become less clearly differentiated and there appears to be a significant overlap in their functions from the level of the hypothalamus to the ovary (Seamark *et al.*, 1992; Tabibzadeh, 1994). Cells of the immune system have been identified as one of the factors which could affect ovarian function at all stages of the oestrous cycle in the cow as well as other species (Brannstrom and Norman 1993; Pate, 1995). Immune cells have been described in luteal and follicular structures throughout the oestrous cycle and they were first recorded in the cow CL by Lobel and Levey (1968). The presence of these cells was originally thought to be passive, resulting from the high blood flow to the ovary. Their only known function was in destruction of the CL after luteolysis (Paavola, 1979). However, the rapid expansion in our understanding of immune cells and their potential to affect cells outwith the immune system led researchers to consider other roles for these cells within the CL.

If immune cells do have a significant role in luteal tissue then it is likely that their populations will vary during the lifespan of the CL. The aim of this study was to investigate the immune cells present in the cow CL at different stages of the oestrous cycle, particularly around luteolysis. Preliminary studies established the most appropriate technique to use. The changes in immune cell populations and MHC II expression throughout the oestrous cycle and during natural luteolysis were

investigated first. Immune cell types and numbers present after induced luteolysis were then compared with those seen during natural luteolysis to assess whether induced luteolysis could be a useful experimental model for studies around luteolysis in the cow. The effect of inhibiting $PGF_{2\alpha}$ release around the time of luteolysis on immune cells and MHC II expression was studied and finally, the presence of mRNA for four cytokines in luteal tissue was investigated using the reverse transcriptase polymerase chain reaction (RT-PCR).

For the purposes of the discussion I will first summarise the results from the different chapters. I will then discuss the significance of the findings in relation to current thinking on the immune system in CL function, discussing future studies where relevant.

The original intention was to study immune cell populations and MHC II expression using both immunohistochemistry and flow cytometry. To optimise the cell populations used for flow cytometry, collagenase dissociation, using a range of collagenase enzymes and mechanical dispersion of luteal tissue were compared (Chapter 3). There was considerable variation in the cell dispersions produced using the different techniques. Most significantly, the numbers of large cells varied markedly between dispersions. This was not unexpected as selective loss of large cells has been connected to collagenase dissociation of luteal tissue (Rodgers *et al.*, 1984; O'Shea *et al.*, 1989). However, in addition to these finding there was also variation in the LH binding between cell populations produced by different collagenase enzymes and mechanical dissociation. This was a novel use of the ubiquitous LH receptor as an indicator of luteal cell membrane damage. It was concluded that not only were luteal cells visibly damaged during tissue dissociation but cell membranes were also affected to varying degrees, depending on the type of collagenase used.

At this stage in the study a collagenase enzyme was selected which 1) produced luteal cell populations which were most closely representative of the cell populations in the

intact CL, 2) resulted in minimal reduction in LH-receptor numbers and 3) gave a well dispersed population of luteal cells. This collagenase was then employed when preparing cell dispersions for flow cytometry.

Immunohistochemistry was first used to identify immune cells and MHC II expression in CL collected at an abattoir from animals of unknown reproductive history (Chapter 4). The CL were visually staged using a widely used technique (Ireland *et al.*, 1980). The numbers of T-lymphocytes, macrophages, eosinophils and cells expressing MHC II varied at different stages of the oestrous cycle. Immune cell populations and MHC II expression were then studied in more detail around the time of luteolysis. CL were collected from animals between days 16-20 of the oestrous cycle. There was a significant increase in the numbers of T-lymphocytes (CD5+, CD8+) from day 16 of the oestrous cycle onwards which occurred before the fall in progesterone concentration associated with functional luteolysis (Chapter 5). This finding is of great significance. It implies a specific role for T-lymphocytes prior to luteolysis and is further evidence that the immune system may be important in controlling ovarian function in the cow.

This study (Chapter 5) also revealed a potential danger in the use of visually staged luteal tissue for CL studies, particularly around luteolysis. Comparing the results obtained using luteal tissue of known age to visually staged tissue, it became apparent that visually staged tissue described as Stage IV (post-luteolysis days 18-20) was often further advanced in terms of structural regression than that seen immediately after natural luteolysis. It was not possible to categorise pre- and post-luteolytic CL in the 48 hour period around luteolysis by gross or morphological appearance. It was concluded that visually staged tissue is not appropriate for detailed studies around luteolysis (although a single blood sample for progesterone analysis, taken at time of CL collection, would allow more accurate identification of the stage of cycle relative to luteolysis).

Visually staged tissue has been widely used in studies investigating CL function and the properties of luteal cells. The appearance of CL from Stage I (days 1-4), Stage II (days 5-10) and Stage IV (days 18-20, i.e. 24-48 hours after functional luteolysis) is distinct enough to allow accurate staging. However, it is not possible to differentiate late Stage III CL immediately before and immediately after luteolysis. There is a danger therefore that some studies employing Stage III CL will inadvertently have included CL that have undergone functional luteolysis but are not obviously different in structure. This is likely to affect results, as luteal cell function and responsiveness to different luteotrophic and luteolytic hormones will be altered after luteolysis.

Collection of luteal tissue around the time of natural luteolysis requires large groups of cows and repeated blood sampling to ensure sufficient animals at appropriate stages of luteolysis when CL are collected. Induced luteolysis could be a useful technique for studying cell changes around luteolysis as it can be much more easily controlled. In Chapter 6 the cellular events associated with induced luteolysis were investigated. The immune cell populations present at 6, 12 and 24 hours after induced luteolysis bore little resemblance to those seen in the 24 hours after natural luteolysis. MHC II expression was increased in all samples but there were few macrophages or lymphocytes. Morphologically, the tissue was dramatically altered. There was rapid loss of luteal cell structure at all time periods after treatment. The changes in structure were more comparable with a CL several days after natural luteolysis. These results indicated that induced luteolysis is of limited use for studies relating to cellular events during natural luteolysis.

However, the above statement applies only to this particular method of inducing luteolysis. A single bolus injection of high dose of $PGF_{2\alpha}$ is the most widely used technique for inducing luteolysis in the cow. The increase in $PGF_{2\alpha}$ concentrations and resulting changes in concentrations of other hormones are far more dramatic than those seen during natural luteolysis (Basu and Kindahl, 1987; Schallenberger *et al.*, 1984; Parkinson and Lamming, 1990). It is possible therefore that gradual infusion of $PGF_{2\alpha}$ in physiological concentrations would be a better approach. This method has

been used in the sheep. Stacy *et al.* (1976) reported that the morphological changes seen in CL after infusing $PGF_{2\alpha}$ directly through a uterine vein were similar to those during natural luteolysis. The uterine vein would be the route of choice for $PGF_{2\alpha}$ infusion but this technique is considerably more complicated than using the jugular route. Whatever route of administration is used, in addition to using a pulsatile, infusion of $PGF_{2\alpha}$, it would also be appropriate to induce luteolysis as close to the timing of natural luteolysis as possible. As the CL ages the proportions of luteal cells alter and the number of receptors for $PGF_{2\alpha}$ as well as other hormones changes (Chegini *et al.*, 1991; Sakamoto *et al.*, 1995). This is likely to affect the responsiveness of the CL *in vivo*.

Any alteration in the numbers of immune cells or MHC II expression could be caused by a range of local or systemic factors. The increase in the numbers of T-lymphocytes (CD8+, CD5+) in the days before luteolysis occurs whilst progesterone concentrations are still high but in the presence of a rising concentration of $PGF_{2\alpha}$, released from the uterus (Parkinson and Lamming, 1990). In Chapter 7 the possible relationship between the increase in T-lymphocytes and pre-luteolytic concentrations of $PGF_{2\alpha}$ was investigated. Flunixin meglumine, a cyclo-oxygenase inhibitor, was used to suppress $PGF_{2\alpha}$ release in 12 cows between days 15 and 18 of the oestrous cycle. $PGF_{2\alpha}$ was replaced artificially in 6 out of 12 cows for 12 hours prior to collection of the CL. Numbers of macrophages, but not T-lymphocytes, were significantly lower in all flunixin -treated animals compared to untreated controls. Immune cell populations and MHC II expression did not vary between cows treated with flunixin and cows treated with flunixin and $PGF_{2\alpha}$. However, this may have been due to an underestimation of the amount of exogenous $PGF_{2\alpha}$ required to give physiological concentrations within the CL. The results of the flunixin trial indicate a specific role for $PGF_{2\alpha}$ in controlling populations of macrophages within luteal tissue.

In Chapter 8 the results from RT-PCR of luteal tissue are presented. Three cytokines, whose effects on luteal cells *in vitro* have been studied in some detail (Pate, 1995,

1996), were investigated. mRNAs for tumour necrosis factor-α (TNF-α) interleukin-1β (Il-1β) and interferon-γ (IFN-γ) were found in luteal tissue from all stages of the oestrous cycle, after induced luteolysis and when $PGF_{2\alpha}$ production was inhibited by flunixin. Although cytokine production, particularly TNF-α has been studied to some degree in the cow, this is the first description of mRNA for these cytokines in CL from such a range of animals.

Monocyte chemoattractant protein-1 (MCP-1) was found in all groups of luteal tissue. However, unlike the three other cytokines, there was an obvious increase in mRNA for MCP-1 in CL from animals which had undergone natural luteolysis compared to those that were pre-luteolytic. This finding was similar to a recent study in the rat (Townson *et al.*, 1996) and suggests a role for MCP-1 in the attraction of macrophages to the CL in preparation for luteal regression. The cell sources of MCP-1 are many but include T-lymphocytes as well as other cells present in the CL. This is the first report of MCP-1 mRNA expression in the cow CL.

During the course of these studies flow cytometric analysis of luteal cell populations, stained with a range of fluorescent-linked monoclonal antibodies, was unsuccessful. This was primarily because of the difficulties in identifying separate cell types within the whole population. An attempt was made to use flow cytometry to assess MHC II expression on luteal cells after induced luteolysis (Chapter 6). This technique had been used successfully by other workers looking at MHC II expression. However, there were major problems in identification of separate cell types and flow cytometry failed to detect changes in MHC II expression that were detected using immunohistochemistry.

Flow cytometry had appeared potentially to be a very useful and accurate technique for identification of immune cells and MHC II expression in dispersed luteal tissue. It has not been widely used in studies of luteal tissue however. Kenny *et al.* (1991) recognised the damage to sheep luteal cells that occurs during collagenase dissociation and attempted to overcome this by allowing dispersed cells a recovery

period in culture for 18-24 hours prior to flow cytometry. However, whether the levels of MHC I or MHC II expression found after dissociation and culture in this study is comparable with that *in vivo* is open to question. Fairchild Benyo *et al.* (1991) studied MHC II expression at different stages of the oestrous cycle and after induced luteolysis, observing variations in MHC II expression at different stages. The potential influence of collagenase dissociation on MHC II expression itself are not discussed but it seems likely that both collagenase dispersion and preparation of dispersed cells for flow cytometry may alter MHC expression.

Immune cells- further evidence of a role in luteolysis and future studies

Our results, particularly the variation in T-lymphocytes and MHC II expression at different stages of the oestrous cycle, indicate that the immune system does have a significant role within the CL, particularly around the time of luteolysis. The exact mechanisms through which immune cells influence the CL, and the factors controlling the changes in immune cell populations and MHC II expression, are not clearly established. However from these results and other relevant studies it is possible to formulate some potential modes of action and suggest appropriate further investigations.

Any attempt to clarify the specific roles of different cell types and cytokines will be difficult. It is likely that immune cells/cytokines are involved in a number of events occurring simultaneously. Recently various workers have attempted to summarise these processes (Vinatier *et al.*, 1995; Terranova and Montgomery Rice 1997). However, results from different species vary considerably and it cannot be assumed that the effect of one cell type or cytokine within the CL in one species will be similar in another. Therefore it is necessary to consider the cow CL alone. The majority of studies relating to the immune system and the CL involve investigating the effect of immune cells and cytokines on luteal cells *in vitro* (Pate, 1995, 1996). These studies are described either in the Literature review (Chapter 1) or in the relevant chapters (Chapters 4-8).

Within the cow CL, immune cells can be expected to function by the same processes that occur throughout the body. Cell-mediated immunity rather then humoral immunity appears to be significant in the cow CL. It is likely that the main role of the immune system is to prepare for, and co-ordinate, destruction of luteal tissue after luteolysis. Therefore the primary function of increased numbers of CD5+/CD8+ T-lymphocytes may be attraction of macrophages and activation of existing macrophage populations within the CL. However in immunological terms this would be an unusual role for CD8+ T-lymphocytes. Traditionally CD4+ helper/ inducer T-lymphocytes are more central to initiation of a cell-mediated immune response. CD4+ T-lymphocytes release cytokines which will activate both CD8+ T-lymphocytes and macrophages. CD8+ T-lymphocytes are associated with the cytotoxic response to antigen presented in combination with MHC I molecules and produce fewer cytokines than CD4+ T-lymphocytes.

However, in this study, no alteration in numbers of CD4+ lymphocytes was observed at any stage of the oestrous cycle. Further studies are required to investigate whether the function of, and relationships between immune cells, are similar in luteal tissue to those in the rest of the body. A starting point would be to investigate whether, as occurs during a cell-mediated immune response, there is evidence that CD8+ activation is taking place within the CL. This is associated with expression of the interleukin-2 receptor in response to IL-2 released by CD4+ T-lymphocytes. The IL-2 receptor may be identified by immunohistochemistry.

The increases in CD8+ T-lymphocyte numbers but not CD4+ T-lymphocytes within the CL, as discussed above, could be due to unusual immune cell interactions and functions in this tissue compared to other body tissues. Alternatively, CD4+ T-lymphocytes may still play a central role. Cytokines acting locally are extremely potent and it is possible that existing populations of CD4+ T-lymphocytes, when activated, release sufficient cytokines to initiate the required immune response. However, the field of immunology is rapidly advancing and it is also possible that our understanding of all the processes and cellular reactions involved between

populations of T-lymphocytes are oversimplified. Recent studies have suggested that there are subpopulations of CD8+ T-lymphocytes which are potent sources of a variety of cytokines, including IFN-γ which is involved in activation of macrophages. In addition there is evidence that certain subpopulations of CD8+ T cells may react with antigen presented in combination with MHC II, not MHC I (Kemeney *et al.*, 1994).

Activation of T-lymphocytes (as well as macrophages and possibly luteal cells) will result in increased production of cytokines. In the present study mRNA for a variety of cytokines has been described within the CL. This proves only that there is the potential for these cytokines to be produced within the CL. It is important that future studies include identification of the active protein form of the cytokines. The use of *in situ* hybridisation or immunohistochemistry would also allow identification of the specific cell sources of different cytokines. At present, cytokine production (TNF-α has been identified in the most detail) is frequently associated with macrophages (see Terranova, 1997 for a review article) although other cell types, including luteal cells, may also be sources.

The role of MHC expression will also be more clearly understood when precise interactions between immune cells and MHC expression is established. Initially MHC II expression was associated with immune cells only but now it is accepted that non-immune cells may also express MHC II. There is a detailed study describing MHC II expression in the cow CL throughout the oestrous cycle and during pregnancy. This study involved FACS analysis of collagenase-dissociated CL (Fairchild Benyo *et al.*, 1991). MHC II expression varied between cells of different sizes but, to summarise briefly, increased MHC II expression was seen with increasing age of CL and after induced luteolysis. MHC II expression was significantly lower in CL on day 18 of early pregnancy compared to day 18 cyclic CL. These findings in CL from non-pregnant cows are similar to our own but, as discussed above, caution is required in assessing MHC II expression in cells that

have been collagenase-dispersed. There is also the problem of identifying specific cell types by flow cytometry.

In our own study MHC II-expressing cells were difficult to identify specifically because of the diffuse nature of immunohistochemical staining. However macrophage-like cells and endothelial cells in blood vessels were the most obvious cell types to express MHC II in CL from Stages I-II (days 1-10) of the oestrous cycle. MHC II expression was increased in Stage III compared to Stage II CL but was most obvious in Stage IV CL and after induced luteolysis. In these CL large numbers of cells were expressing MHC II (including luteal cells) although staining was most intense around blood vessels. MHC II expression has been described on luteal cells in culture (Fairchild and Pate, 1989).

Alteration in MHC I or MHC II expression provides an important mechanism through which immune cell responses can be controlled within the CL. Increased expression of MHC I and II on immune and non-immune cells is associated with a range of cytokines (Ohmann *et al.*,1988). Specifically, IFN-γ has been shown to increase MHC I (+25%) and MHC II (+370%) expression on luteal cells *in vitro* (Fairchild and Pate, 1989). Recent evidence also suggests that large luteal cells themselves may be able to activate T-cells in combination with MHC II expression (Greene *et al.*, 1996). Therefore a variety of immune processes may be involved with altered MHC II expression. The apparent increase in expression of MHC II by endothelial cells might also indicate that blood vessels are an important early target for destruction by immune cells during luteolysis. A reduction in blood flow within the CL is one of the first events to occur during luteolysis.

In addition to the role of immune cells in communicating with each other and initiating an immune response, these cells (and in particular their cytokine products) may be able to affect luteal cells directly. The results of a large number of *in vitro* studies (reviewed by Pate, 1995) suggest that cytokines influence bovine luteal function through alteration of progesterone release and $PGF_{2\alpha}$ production. TNF-α, Il-

1β and IFN-γ and different combinations of these cytokines have a variety of effects on luteal cells in culture: however the majority of responses are luteolytic *i.e.* progesterone production is suppressed and $PGF_{2\alpha}$ production stimulated. Although uterine $PGF_{2\alpha}$ is the luteolytic substance in the cow, it is not clear exactly how luteolysis takes place within the CL itself. Increased numbers of CD8+ T-lymphocytes, possible activation of CD4+ T-lymphocytes and activation of macrophages during an immune response will result in increased production of a range of cytokines, including TNF-α, Il-1β and IFN-γ. This suggests another possible mechanism of action for immune cells acting through their cytokine products to promote luteolysis at the cellular level within the bovine CL. This effect could be direct on specific luteal cells or could function through positive feedback mechanisms involving luteal substances including $PGF_{2\alpha}$, oxytocin and cytokines released not only from immune cells but also possibly from other cells within the CL.

The possibility that cytokines may be produced by, or exert their effects on, cells outwith the immune system provides evidence of other potential actions for these substances within the CL. In the pig CL, TNF-α has been localised to endothelial cells (Hehnke-Vagnoni *et al.*, 1995) and it is suggested that the endothelial cell would be a prime target for the action of TNF-α as damage to endothelial cells and resultant collapse of vascular components of the CL would significantly reduce the luteal blood supply. It is not clear however whether a reduction in blood supply is a mechanism for, or a consequence of, luteolysis (Nett *et al.*, 1976; Niswender *et al.*, 1976). Further evidence of a specific role for TNF-α in the pig CL is the presence of specific TNF-α receptors on the small cell population which includes small luteal cells and endothelial cells (Richards and Almond, 1994). A recently published study in the sheep has localised TNF-α to thecal endothelial cells in preovulatory follicles (Murdoch *et al.*, 1997). It seems likely that endothelial cells may also be a prime target for TNF-α action in the cow CL and further studies should examine endothelial cells in the cow CL for evidence of cytokine production and/or the presence of receptors.

Confirming the presence of cytokine receptors not only on endothelial cells but also on other cells in luteal tissue would be significant evidence of 'non-immune' roles for these substances in the CL. Adashi (1995) stated that substances assessed to be important in intraovarian regulation must fulfil certain criteria. These are evidence of: 1) local production, 2) local reception and 3) local action. In addition it is suggested that evidence of a vital role is also established through for example, neutralisation studies using specific antibodies. There is evidence of local production of cytokines within the cow CL. However, the other criteria of local reception and local action are as yet unfulfilled.

There have been no investigations into the presence of specific cytokine receptors within the cow CL although they have been described in other species (see Terranova and Montgomery Rice, 1997 for a recent review article). Specific cell sites of action for cytokines are also unclear in the cow CL. A recent *in vitro* study using human granulosa-luteal cells found that TNF-α and IL-2 stimulated proliferation of lymphocytes and monocytes in culture with luteal cells but had no effect on luteal cells themselves. The authors urged caution in attributing effects of cytokines on luteal cells specifically (Wang *et al.*, 1995). The localisation of specific receptors on cells of the CL is therefore an important step in defining their role. Cytokine receptors are located on many types of cell and have similar structural features (Kuby, 1994). There are specific receptors for individual cytokines although some cytokines, for example TNF-α, have more than one receptor (Tabibzadeh, 1994; Davies and Wlodawer, 1995).

Immune cells and their cytokine products have the potential to induce apoptosis within the CL. It is not yet known how significant apoptosis is in the process of luteal regression but apoptotic cells have been observed in luteal tissue during structural regression (Juengel *et al.*, 1993; Rueda *et al.*, 1995). Macrophages, eosinophils and neutrophils have also been shown to generate reactive oxygen species (ROS) which are involved in apoptosis. Activation of various immune cells by cytokines, including TNF-α, can stimulate ROS release (Riley and Behrman, 1991) and TNF-α has been

shown to induce apoptosis directly in combination with specific receptors (Wertz and Hanley, 1996). It is possible therefore that immune cells and cytokines may exert some of their effects within the CL through the induction of apoptosis (Pate, 1995; Tilly, 1996). In addition, recent studies have revealed that ROS may also play significant roles in affecting luteal function, in particular having a luteotrophic effect (Kato *et al.*, 1997). Therefore, immune cells and /or cytokines could also be involved in luteotrophic support of the CL at different stages of the oestrous cycle through generation of ROS. Future investigations should consider the significance of apoptosis within the CL around the time of luteolysis relative to immune cell numbers or cytokine production. It would be of some interest to investigate the numbers of apoptotic cells in CL where immune cells and cytokine production have been suppressed.

The factors that control the presence of immune cell populations within luteal tissue are unclear. Changes in the concentrations of peripheral and/or local hormones such as progesterone, oxytocin, oestradiol and $PGF_{2\alpha}$ could be involved. Various hormones have effects on immune cells at different levels from the hypothalamus to the ovary (Tabibzadeh, 1994). For example, oestrogen inhibits CD8+ T-lymphocyte activity, and during pregnancy high progesterone concentrations suppress cell-mediated immunity (Grossman, 1985). Some hormones may act directly or indirectly as chemoattractant substances such as hCG in the human which attracts neutrophils, monocytes and lymphocytes *in vitro* (Reinisch *et al.*, 1994) and $PGF_{2\alpha}$ which stimulates production of a chemoattractant for eosinophils in the sheep CL (Murdoch, 1987). It is also possible that the signals originate from existing populations of immune cells through production of cytokines.

In the course of the present study the role of $PGF_{2\alpha}$ on immune cell populations in the cow CL at the time of luteolysis was investigated. The increase in the number of T-lymphocytes (CD5+/CD8+) cells observed earlier in this study was not affected by inhibition of $PGF_{2\alpha}$ in the days leading up to luteolysis but the numbers of macrophages present were significantly reduced. It appears that $PGF_{2\alpha}$ is not the

stimulus for an influx of T-lymphocytes but does influence macrophage numbers in the CL. However there are many other factors that must be investigated before it is possible to construct a specific order of events.

There is evidence also that a changing hormonal environment alters expression of various cytokines by peripheral immune cells as well as within the reproductive tract. High concentrations of progesterone and oestrogen reduce TNF-α in blood monocytes and Il-1β is three-fold higher in blood monocytes during the luteal phase compared to the follicular phase in women (Loy *et al.*, 1992; Polan *et al.*, 1994). TNF-α is under hormonal regulation throughout the reproductive tract in women (see Hunt, 1993 for a review article). There are no studies describing alterations in immune cell populations or cytokine production in the cow CL, in relation to changes in the hormonal environment, apart from an increase in TNF-α production in the CL after luteolysis in the cow (Shaw and Britt, 1995). However, it is likely that a changing hormonal environment, either locally or peripherally, is important in altering immune cells and cytokine production within the cow CL.

If immune cells play a significant role in luteolysis then their presence and/or activity must be suppressed for successful maintenance of pregnancy. The populations of T-lymphocytes in the cow CL during pregnancy have not been described in detail, although reduced numbers of macrophages and lymphocytes in pregnant cow CL compared to CL from a non-pregnant cycle were observed in an early study (Lobel and Levey, 1968). Interferon-τ released by the trophoblast has been shown to have a range of anti-luteolytic effects, including inhibition of release of luteolytic pulses of $PGF_{2\alpha}$ from the uterus (Bazer and Johnson, 1991; Bazer *et al.*, 1991; Thatcher *et al.*, 1995). However, basal concentrations of $PGF_{2\alpha}$ are not lower in the pregnant cycle and low level pulses of $PGF_{2\alpha}$ have been recorded between days 14 and 16 after oestrus in pregnant animals (Kindahl *et al.*, 1976; Parkinson and Lamming, 1990). In addition, $PGF_{2\alpha}$ receptors in the CL are not reduced in early pregnancy (Sakamoto *et al.*, 1995). Therefore, other mechanisms, taking effect after $PGF_{2\alpha}$ binding, may also be involved in inhibition of luteolysis (Wiltbank *et al.*, 1992b).

Culture media from day 18 embryos were immunosuppressive as were uterine flushings from day 18 pregnant and non-pregnant cows (French and Northey, 1983; Fisher *et al.*, 1985). The anastamoses between the uterine vein and artery have been shown to transport uterine $PGF_{2\alpha}$ to the ovary. It is conceivable that immunosuppressive factors (possibly interferon-τ) could also be transported to the ovary by this route. Fisher *et al.* (1985) failed to find any evidence of immmunosuppressive substances in the uterine vein blood. However the authors indicated that the assay may not have been sufficiently sensitive to detect low concentrations. Lymphocyte proliferation is however inhibited by interferon-τ (Low *et al.*, 1991; Skopets *et al.*, 1992). There is also a decline in the number of intra-epithelial lymphocytes in the bovine endometrium at the site of attachment of the conceptus (Hansen, 1995).

Future studies should involve investigations into immune cell populations within CL from pregnant cows around day 16-18 of pregnancy. In addition more detailed studies into the immunosuppressive effects of blood from the uterine vein / ovarian artery (specifically interferon-τ) at this stage of the oestrous cycle would be of interest.

Another more invasive approach to investigate the altered T-lymphocyte population would be to depress the numbers of T-lymphocytes present within the ovary and CL. There are already various studies that confirm that artificially altering the immune status affects reproduction in general. For example, ovulation was inhibited in rats treated with anti-thymocyte serum (Bukovsky *et al.*, 1977) and, in the cow, reducing the total numbers of circulating lymphocytes altered CL function (Alila and Hansel, 1984). However, in order to investigate immune cells in the CL more specifically a more subtle approach will be necessary whereby only the ovary is affected. This could prove somewhat difficult, although the successful use of a microdialysis system placed within the cow CL *in vivo* might encourage the development of a technique to allow the supply of immunosuppressive substances directly to the CL (Shaw and Britt, 1995).

From the above observations it can be seen that there are many possible routes of further investigation, all of which would add to our understanding of how the immune system functions within the CL, particularly around luteolysis. T-lymphocytes, macrophages and TNF-α, Il-1β and IFN-γ are the primary cell sources and cytokines of interest. However the presence of MCP-1 in cow CL was a significant finding in this study, particularly as MCP-1 expression was increased prior to structural luteolysis.

MCP-1 has never been described in bovine luteal tissue previously and its presence could explain the role of increased numbers of CD5+/CD8+ T-lymphocytes in cow CL before functional luteolysis. Further investigations in our laboratory have already indicated that T-lymphocytes are the source of MCP-1 in the cow CL prior to structural luteolysis (unpublished observations). The regions of tissue where MCP-1 is strongly expressed on *in situ* hybridisation are associated with populations of CD5+ T-lymphocytes stained by immunohistochemistry of parallel sections. Therefore T-lymphocytes may be central to the attraction and activation of macrophages into the CL during structural luteolysis. Future studies relating to MCP-1 will investigate its expression in animals where $PGF_{2\alpha}$ production was inhibited by flunixin as this resulted in a decrease in the numbers of luteal macrophages. $PGF_{2\alpha}$ could be the substance that signals the release of MCP-1 from T-lymphocyte populations (and possibly other cell types) within the CL.

Conclusion

To conclude; based on the results of this study, and our present understanding of basic immunological and reproductive processes, the following order of events within the CL around luteolysis are proposed;

Between days 5 and 10-14 of a non-pregnant cycle, populations of immune cells and MHC II expression stay reasonably constant. Around day 16 of the oestrous cycle, increased numbers of CD8+ T-lymphocytes enter luteal tissue, possibly under the

influence of a changing hormonal environment, although specific chemoattractant substances could be involved. The populations of T-lymphocytes then act, either directly or indirectly through their cytokine products, in the processes which occur during luteolysis, *i.e.* decreased steroidogenesis and/or luteal production of $PGF_{2\alpha}$. Luteolytic concentrations of $PGF_{2\alpha}$ at this time stimulate release of MCP-1 from cells of the CL, including T-lymphocytes, causing activation of existing populations of macrophages and also acting as a chemoattractant to other macrophages, bringing them into the CL at the time of structural luteolysis. During luteolysis MHC II expression is also upregulated (possibly by $PGF_{2\alpha}$ or cytokines) and the destruction of luteal tissue takes place rapidly as a result of a co-ordinated immune response against the cells of the CL. In addition, destruction of the CL may be accelerated by an increase in the rate of apoptosis induced by cytokines produced by immune cells or even luteal cells. The rapid loss of CL function and progesterone production during luteolysis may be a direct effect of cytokines, in particular TNF-α, on endothelial cells, causing a collapse of the blood supply within the CL.

These findings demonstrate that the cells of the immune system are significant in affecting CL function around luteolysis in the cow, although the exact nature of their role has yet to be clearly defined. They also highlight specific areas of future research relating to the immune system and reproductive function in the cow. Further studies should concentrate initially on investigating immune cell populations and cytokine production within the CL from the early-pregnant cow. In addition, specific cytokine cell sources and cytokine receptors within the CL must also be located in order to clarify the significance of cytokines within luteal tissue.

Appendix 1

Molecular Techniques

1.1. Materials

Sources of specific chemicals and molecular kits are described in the relevant protocol. All other chemicals were purchased from Sigma Chemical Co., Poole, Dorset, UK, Pharmacia Biotech, St Albans, Herts, UK or Gibco BRL, Life Technologies Ltd., Paisley, Scotland, UK.

1.2. Buffers / culture media

1.2.1. TAE buffer (50x)

242g Tris base

57.1ml glacial acetic acid

100ml EDTA (0.5M, pH 8.0).

1.2.2. MOPS (10x)

3-(N-morpholino) propanesulfonic acid [MOPS, 0.2M]

sodium acetate (0.05M, pH 7.0)

Na_2EDTA (0.01M).

1.2.3. TE buffer

Tris-HCl (10mM, pH 7.5)

EDTA (1mM)

1.2.4. TBE buffer (10x)

108g Tris base

55g boric acid

9.3g $Na_2EDTA.2H_2O$

Add H_2O to a volume of 1 litre, filter and autoclave.

1.2.5. L-broth

950ml deionized H_2O

10g bacto-tryptone

5g bacto-yeast extract

10g NaCl

Adjust pH to 7.0 with 5N NaOH and sterilise.

1.3. Diethylpyrocarbonate (DEPC)-treatment

All solutions and utensils used when working with RNA were DEPC-treated to minimise RNase contamination as follows;

Solutions

Treated with 0.1% DEPC overnight and then sterilised.

Glassware/utensils

Treated with 1% DEPC for 2 hours at 37°C, rinsed with sterile water and then autoclaved prior to use.

1.4. Extraction of RNA

1.4.1. Method 1

TRI Reagent™ (Molecular Research Centre, Cincinnati, USA), a ready-to-use reagent containing phenol and guanidine thiocyanate, was used to extract RNA following the method described by the manufacturers which is summarised below. Sterile glassware and plugged sterile pipette tips were used throughout;

1. 50-100mg of frozen luteal tissue was added to 1 ml of TRI reagent in a sterile glass homogeniser on ice and homogenised rapidly.
2. The homogenised samples were then decanted into 1.5ml screw top Eppendorf tubes and stored on ice for 5 min.

3. Chloroform (0.2ml) was added and the tubes were shaken vigorously for 15 sec then stored for 2-15 min prior to centrifugation at 12000 *g* for 15 min at 4°C.
4. The upper aqueous phase was transferred to a 1.5ml Eppendorf, 0.5ml of isopropanol was added and the tubes were then stored at room temperature for 5-10 min. This was followed by centrifugation at 12000 *g* for 10 min at 4°C after which an RNA precipitate was visible on the side of the tube.
5. The supernatant was then removed and the RNA pellet washed twice in 75% ethanol before air drying for 5-10 min at room temperature.
6. The RNA was stored at -20°C after dissolving the pellet in 40µl sterile distilled water.

1.4.2. Method 2

RNA was extracted using Qiagen RNeasy kits (Qiagen Ltd., Crawley, UK) following the recommended protocol. Sterile plugged pipette tips or positive placement pipettes were used throughout.

1. 30-40mg of luteal tissue was homogenised in 600µl lysis buffer RLT (containing guanidinium isothiocyanate and β-mercaptoethanol)
2. 600µl of 70% ethanol was added, mixed by pipetting and loaded onto an RNeasy spin column which was then centrifuged at 8000 *g* for 15 sec at room temperature.
3. The flow through was discarded and the column was then washed with 700µl wash buffer RW1 (containing guanidinium isothiocyanate).
4. The column was washed twice more with 500µl of wash buffer RPE (concentrate diluted with 4 volumes of 100% ethanol) with a final centrifugation at maximum speed for 2 min to dry the spin column membrane.
5. 30µl of DEPC-treated water was added and the column was centrifuged at 8000 *g* for 60 sec to elute the RNA. This step was repeated in order to collect as much RNA as possible.

The quality of RNA produced from luteal tissue using this technique was poor. In order to assess whether this could have been due to significant RNA degradation

after extraction, the method was modified by extracting the RNA initially produced with phenol:chloroform as follows;

1.4.2.1. Additional chloroform extraction

1. An equal volume of phenol:chloroform was added to the RNA sample produced using the Qiagen RNeasy kit and mixed to form an emulsion.
2. The sample was centrifuged at 8000 *g* for 15 sec at room temperature and the upper aqueous phase removed to a fresh tube.
3. An equal volume of chloroform was added and steps 1-2 repeated.
4. 0.1 volumes of sodium acetate (3M, pH 5) and 2.5 volumes of ethanol (100%) were added and the tubes were stored at -20°C for 30 min and then centrifuged at 8000 *g* for 20 min at 4°C.
5. The cell pellet was washed twice in ethanol (70%), briefly air dried and then resuspended in the original volume of DEPC-treated water.

1.4.3. Method 3

RNA extraction was performed using a basic guanidine thiocyanate technique described by Chomczynski and Sacchi (1987).

1. 100µl of β-mercaptoethanol was added to 14ml of denaturing solution (4M guanidine thiocyanate in 42mM sodium citrate).
2. 200-400mg of luteal tissue was minced on ice and added to 5ml of denaturing solution in a 50ml polypropylene tube also on ice. The tissue was homogenised using a polytron homogeniser.
3. 50µl of N-Lauryl sarcosine (20%), 0.5ml of sodium acetate (2M), 5ml of chloroform and 1ml of chloroform/isoamyl alcohol (24:1) were added to the homogenate. The tubes were then shaken vigorously for 10 sec and stored on ice for 15 min.

4. The samples were then centrifuged at 10000 *g* for 20 min at 4°C and the aqueous phase then transferred to a fresh tube and mixed with an equal volume of isopropanol. The tubes were stored overnight at -20°C to precipitate the RNA.
5. After precipitation the tubes were centrifuged at 10000 *g* for 20 min at 4°C. The supernatant was discarded and the pellet then dissolved in 1ml of denaturing solution (containing 0.2mM of β-mercaptoethanol and 0.83% of N-lauryl sarcosine).
6. 1ml of isopropanol was added and the samples were stored at -20°C for 1 hour and then centrifuged at 10000 *g* for 20 min at 4°C.
7. The RNA pellet was washed in ethanol (75%), air-dried and then resuspended in 100µl of DEPC-treated water. A 1/100 dilution was prepared for spectrophotometry and, after calculating the amount of RNA, each sample was diluted in dH_2O to give a concentration of approximately 1µg RNA/µl. For long term storage, 3 volumes of ethanol (100%) were added to each tube and the samples stored at -70°C.

1.5. Formaldehyde Gel electrophoresis

An agarose gel was prepared;

1g agarose (Appligene Oncor., Co. Durham, UK)
10ml MOPS buffer (10X)
75ml DEPC-treated water

The solution was heated in a microwave until the agarose dissolved and then cooled to 50°C and 40% volume of formaldehyde (17ml) added immediately before pouring. The gel was then allowed to set at room temperature for 1 hour before submersion in running buffer (1xMOPS).

In preparation for gel electrophoresis an RNA mix was prepared for each sample as follows; 1µg RNA

12.5µl formamide
2.5ul MOPS (10x)
4µl formaldehyde

The RNA mixtures were heated in the thermal cycler at 65°C for 10 min, 2.5µl of 6x gel loading buffer (50% glycerol, 0.1mg/ml bromophenol blue) added and then 25µl was loaded onto the gel. The samples were run at 150mA for around 45 min, stained with ethidium bromide for 20 min and then destained for 30-40 min in dH_2O.

1.6. Reverse transcription of RNA

The RTase reaction was prepared as follows

1. RTase reaction; 1µg of RNA sample in10µl DEPC-treated water

   0.5µl of $d(NTP)_6$ (125pmol, Pharmacia Biotech, UK)

   0.5µl of RNasin (Promega UK Ltd., Southampton, UK)

   The RNA was denatured by heating to 70°C for 10 min in a thermocycler. The reactions were then stored at 4°C prior to addition of the enzyme mix.

2. Reverse transcriptase (RTase) mix (enough for 20 reactions)

   84µl of $MgCl_2$ (25mM)

   42µl of RTase buffer (10x, Gibco BRL)

   [250mM Tris-HCL (pH 8.3), 375mM Kcl, 15mM $MgCL_2$]

   42µl of dNTP mix (10mM, Pharmacia Biotech.)

   12µl of RTase [Superscript™ II, Gibco BRL, (25U/µl)]

   20µl of DEPC-treated H_2O

   9ul of RTase mix was added to each sample and the tubes were then placed in the thermocycler which was programmed to run for 20°C/10 min, 42°C/ 60 min, 95°C/ 5 min and then hold at 4°C.

3. All samples were diluted to 100µl by adding;

   4µl of $MgCl_2$ (25mM),

   8µl of RTase buffer (10x)

   68µl of dH_2O

4. The samples were stored at -70°C prior to PCR.

1.7. PCR

1.7.1. Method 1 - Hot Start PCR

1. A PCR mix was prepared as follows for each sample;

   6µl-10µl of RTase reaction

   12µl of dH_2O

   1µl of PCR buffer (10x, Gibco BRL)

   [200mM Tris-HCl (pH 8.4), 500mM KCl]

2. The tubes were placed in a thermocycler at 90°C for 60 sec.
3. A *Taq* / primer mix was prepared; 5µl of *Taq* DNA polymerase, (5U/µl)

   [Gibco BRL]

   10µl of primer mix (100-200pmol)

   10µl of dH_2O

   1µl of *Taq* /primer mix was added to each tube using separate plugged tips. The *Taq* DNA polymerase was kept on ice at all times to avoid reduction of enzyme activity.
4. The tubes were then centrifuged briefly and placed in the thermocycler and run at; 93°C/5 min, then 30-35 amplification cycles of 93°C/30 sec, 60-65°C/30 sec, 72°C/30 sec and finally 72°C/5 min. The annealing temperature and number of cycles used varied between different primers.

1.7.2. Method 2 - Using TaqStart Antibody

TaqStart™ antibody (Clontech Laboratories, California, USA), is a neutralising monoclonal antibody which blocks the activity of the *Taq* DNA polymerase enzyme during the setting up of a PCR reaction, reducing the production of non-specific amplification products. Antibody is inactivated when the temperature is raised above 70°C. Cost prohibited its use throughout, and Taq Start was only used where non-specific products were a problem.

1. A basic PCR mix was prepared for each sample as described above.
2. Taq polymerase/primer mix; 5µl of *Taq* DNA polymerase
   5µl of TaqStart antibody (1.1µg/µl)
   10µl of primer mix (100-200pmol)
   5µl of dH_2O

   1µl of *Taq* polymerase/primer mix was added to each PCR mix using separate plugged tips for each tube.
3. The thermocycler was then activated to cycle mode as above.

1.7.3. PCR of cytokine plasmid

A PCR reaction mix was prepared;

14.4µl of dH_2O
1µl of cytokine plasmid (approx. 1.3µg)
0.6µl of $MgCl_2$ (25mM)
2µl of PCR buffer (10x)
1µl of dNTP mix (4mM)
1µl of *Taq* polymerase / primer mix (see section 1.7.2.)

The tubes were then included in the thermocycler as described above.

1.8. Preparation of an agarose gel

1. A 3% gel was prepared containing; 1.5g of NuSieve® GTG® Agarose
   (FMC BioProducts, Rockland, USA)
   0.75g of Appligene agarose
   75ml of 1xTAE buffer

   The solution was mixed well, heated in a microwave until the agarose had dissolved and then cooled to 50°C.
2. The solution was then poured into a gel tray and allowed to set for 1 hour at 4°C. The combs were then carefully removed and the gel submerged in approximately 800ml of running buffer (1xTAE).

3. 1µl of 6x blue/orange loading buffer (Promega Ltd., Southampton, UK) was added to 5µl of each PCR product and mixed gently before being loaded carefully onto the gel using separate pipette tips for each sample.
4. Molecular weight marker (300ng/5µl, Promega Ltd., Southampton, UK) was diluted 50:50 in dH_2O. For each lane required, 5µl was then added to 1µl of loading buffer, mixed gently and then loaded onto the gel.
5. The gel was then run at 150mA for around 50 min and then submerged in a solution of ethidium bromide (0.5µg/ml) for 30 min.
6. The gel was destained for 30 min in dH_2O, examined under ultraviolet light and photographed.

1.9. Confirmation of PCR products - enzyme digests

TNF-α, Il-1β and IFN-γ PCR products were digested with specific restriction enzymes as follows;

Restriction enzyme digests were performed in a final volume of 30µl containing;

15µl of PCR product,

30 U of Bgl I, Pvu II (Boehringer Mannheim UK) or EcoRV (New England Biolabs)

1x reaction buffer (as provided with the individual enzymes)

The reactions were incubated at 37°C for 90 min and were fractionated on an agarose gel.

1.10. Sequencing of MCP-1

The PCR product for MCP-1 was amplified and sequenced as follows;

1.10.1. RT-PCR

Bovine luteal RNA was reverse transcribed as described above (Appendix 1, Section 1.6) followed by PCR using MCP-1 primers (Appendix 1, Section 1.7).

1.10.2. PCR Purification

PCR purification was carried out using the QIA quick PCR kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions;

1. 15µl of PCR product was added to 75µl of PB buffer, mixed and added to a spin column for centrifugation at maximum speed in a bench-top centrifuge for 60 sec.
2. The flow through was discarded and the column washed with 750µl of PE buffer.
3. The column was then centrifuged at maximum speed for 2 periods of 60 sec to allow drying of the membrane.
3. The PCR product/DNA was eluted using 50µl of dH_20.

1.10.3. Ligation

Ligation was performed using Promega T4 DNA ligase (Promega Ltd., Southampton, UK).

1. Ligation mix; 1µl of T4 DNA ligase buffer
   1µl of pGEM-T vector
   0.5, 1.5 or 4.5µl of purified PCR product
   6.5, 5.5 or 2.5ml of dH_2O
   1µl of T4 DNA ligase

2. The reactions were incubated at 15°C for 3 hours.

1.10.4. Transformation

1. Promega high efficiency JM109 cells were removed from storage at -70°C and placed on ice for 5 min until just thawed.
2. The cells were mixed gently and 50µl aliquots placed in 1.5ml tubes on ice.
3. 2µl from each of the ligation reactions, which contained different volumes of DNA, was added to each tube, mixed gently and placed on ice for 15 min.
4. The cells were heat-shocked in a water bath at 42°C for 45 sec and then returned to ice.
5. 900µl of room temperature L-broth was added to each tube and the samples were then shaken for 1 hour at 37°C.
6. 100µl of transformation mix from each tube was plated onto agar plates (400ml agar, 30mg ampicillin, 16µl 5-bromo-4-chloro-3-indolyl-β-galactopyranoside [X-Gal] and 4µl isopropyl-β-thiogalactopyranoside [IPTG]) and kept at 37°C overnight.
7. Colonies were examined and numbers of white (containing insert) and blue (no insert) colonies counted. Five separate white colonies and 1 blue colony were added to 6 Universals containing 10ml of L-broth / ampicillin (100µg/ml) and incubated overnight with shaking at 37°C.

1.10.5. DNA purification and isolation.

1.10.5.1. Minipreps

Wizard minipreps DNA purification systems (Promega UK Ltd., Southampton, UK) were used according to the manufacturer's instructions;

1. 3ml of cells from each of the overnight incubations were pelleted by centrifugation at maximum speed in a bench-top centrifuge for 1 min and then resuspended in 200µl of cell resuspension solution.
2. 200µl of cell lysis solution was added and the tube inverted repeatedly until the cell suspension became clear.

3. 200µl of neutralisation solution was added and mixed by inversion.
4. The tubes were then centrifuged at maximum speed for 5 min and the supernatant removed to a fresh tube.
5. 1ml of DNA purification resin was added to each tube and mixed by inversion.
6. The resin/DNA mix was pipetted into a syringe barrel attached to a Wizard minicolumn (Promega Ltd.) and a vacuum applied to pull the mix into the minicolumn.
7. 2ml of column wash solution was added to the syringe barrel and the vacuum reapplied to draw the wash solution through the column and then for a further 2 min to dry the resin.
8. The minicolumn was then transferred to a microcentrifuge tube and spun at maximum speed for 20 sec to remove any remaining column wash.
9. 50µl of dH_2O (65°C) was then applied to the column and after 1 min the column was spun at maximum speed for 20 sec and the plasmid DNA collected in a new 1.5ml tube.
10. For each tube the A_{260}/A_{280} ratio was measured after dilution of the plasmid 1/100 with dH_2O.

1.10.5.2. Miniprep digests

The miniprep products were digested using specific restriction enzymes to confirm that ligation had been successful.

1. Reactions were prepared;

    15µl from each miniprep,

    5µl of 10x buffer 3 (New England Biolabs)

    10µl of dH_2O

    2.5µl of NCO1 (New England Biolabs)

    2.5µl of NOT1 (New England Biolabs)

2. The samples were then incubated at 37°C for 1 hour.

3. Each sample was prepared for a PCR

1µl of sample

0.6µl of $MgCl_2$ (25mM)

1µl of dNTP mix (4mM)

14.4µl of dH2O

2µl of PCR buffer (10x)

1µl of Taq polymerase /primer mix

Taq mix;

2µl of *Taq* DNA polymerase

2µl of Taqstart antibody

0.8µl of primer 1/0.8µl of primer 2

4.4µl of dH_2O

4. The PCR product was amplified for 30 cycles at 60°C and then visualised on a 3% agarose gel.

1.10.5.3. Maxiprep

Maxipreps were performed using Wizard™ Plus Maxipreps DNA purification system (Promega UK Ltd., Southampton, UK) following the manufacturer's instructions.

1. 10ml from a miniprep sample was added to 500ml of L-broth (containing 100µg/ml ampicillin) and incubated at 37°C overnight with shaking.
2. 5ml of culture media was retained for a frozen glycerol culture (Section 1.10.6.) and the remaining medium was centrifuged for 10 min (25°C, 5000 *g*).
3. The resulting cell pellet was suspended in 15ml of cell lysis solution, mixed gently and left at room temperature for 20 min.
4. 15ml of neutralisation solution was added and then centrifuged for 15 min (25°C, 14000 *g*).
5. The solution was then filtered through four layers of cheesecloth, 0.5 volumes of isopropanol added and mixed gently before centrifugation for 15 min (25°C, 14000 *g*).

6. The supernatant was carefully poured off and the centrifuge bottle allowed to drain for 5 min.
7. The cell pellet was resuspended in 2ml of TE buffer, 10ml of DNA resin was added and mixed.
8. The solution was poured into a maxicolumn (Promega Ltd.) and a vacuum applied. 13ml of column wash were used to wash out the centrifuge bottle and added to the maxicolumn.
9. The column was then washed through with 12ml of column wash and then 5ml of 80% ethanol and a vacuum applied for 1 min.
10. 1.5ml of TE buffer (65°C) was added to the column which was then centrifuged for 5 min (15°C,1300 *g*) to allow collection of the plasmid.
11. The A_{260}/A_{280} ratio was calculated and the plasmid was stored at 4°C.

1.10.6. Frozen glycerol culture

A small amount of culture medium was frozen for long term storage as follows;

1. 1.5ml of culture media from overnight incubation at 37°C were centrifuged for 15 min at maximum speed in a bench-top centrifuge (4°C).
2. The supernatant was poured off and the cells resuspended in 1ml of TM buffer [50mM Tris-Cl (pH 7.5), 10mM $MgSO_4$] and mixed well.
3. 1ml of sterile glycerol was added and mixed.
4. 0.5ml aliquots were snap-frozen in liquid nitrogen and then stored at -70°C.

1.10.7. Sequencing of the PCR product

The DNA was sequenced using the Sequenase™ Version 2.0 DNA sequencing kit (Amersham Life Science, USA).

1.10.7.1. Denaturing double-stranded DNA

1. 10µl of plasmid was mixed with 10µl of distilled water, 2µl of NaOH (2M) and 2µl of ethanol (100%) and incubated for 30 min at 37°C.

2. 2µl of sodium acetate (NaAc, 3M, pH5.5) and 60µl of ethanol (100%) were added and mixed gently. The mixture was incubated for 30 min at -20°C.
3. The tube was centrifuged for 15 min at maximum speed in a bench-top centrifuge.
4. The pellet was washed in ethanol (75%) and then air dried before being resuspended in 7µl of dH_2O.

1.10.7.2. Annealing

Annealing was performed using the DNA sequencing kit (Amersham Life Science) according to the manufacturer's instructions.

1. Annealing mixture; 5µl of DNA
   2µl of dH2O
   2µl of reaction buffer
   (200mM Tris-HCl, [pH 7.5], 100mM $MgCl_2$, 250mM NaCl)
   1µl of primer
2. The DNA was annealed by heating for 2 min at 65°C then slowly cooled to 35°C over 15-30 min. The tube was centrifuged briefly and stored on ice.
3. Labelling reaction; 10µl of annealed DNA mixture
   1µl of DTT (0.1M)
   2µl of diluted labelling mix
   0.5µl of [^{35}S]dATP
   2µl of diluted Sequenase polymerase
   The labelling reaction was mixed and incubated at room temperature for 2-5 min.
4. 3.5µl of labelling reaction was added to 4 termination tubes, each containing 2.5µl termination mixture (G, A, T, and C) and pre-warmed to 37°C. The reactions were mixed and incubated at 37°C for 5 min.
5. The reactions were stopped by adding 4µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF).
6. The samples were heated to 75°C for 2 min immediately before loading onto the sequencing gel.

1.10.7.3. Sequencing gel

A sequencing gel was prepared using SequaGel™ Sequencing System (National Diagnostics, Hull, UK) following the manufacturer's instructions as follows;

To prepare a 6% monomer Sequagel

1. Basic gel solution 24ml of SequaGel concentrate
   66ml of SequaGel diluent
   10ml of SequaGel buffer
2. Add 0.8ml of 10% (w/v) ammonium persulphate and mix.
3. Polymerise by adding 40µl N,N, N',N'tetramethyl-ethylenediamine (TEMED) and mix.
4. Pour gel into cassette.
5. Running buffers are 0.5x TBE (top) and 0.5x TBE/0.1M Na_2HPO_4 (bottom).

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