Immune cell populations in the equine corpus luteum throughout the oestrous cycle and early pregnancy: an immunohistochemical and flow cytometric study

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Recent evidence indicates that the cells of the immune system and their large network of secretory products, or cytokines, play an active role in the ovary throughout the oestrous cycle. In the present study, immune cell populations (T and B lymphocytes, macrophages, granulocytes and eosinophils) and expression of major histocompatibility complex (MHC) class II were investigated in corpora lutea from mares in early (days 2–4), mid- (days 7–10) and late (days 12–14) dioestrous, the post-luteolytic phase (days 16–17) and early pregnancy. The number of T lymphocytes within the corpus luteum increased in the late luteal phase. CD4+ cells did not increase until day 16, whereas the number of CD8+ cells increased before functional luteolysis; an apparently selective luteal infiltration of CD8+ cells was observed. MHC class II expression by non-steroidogenic cells was increased in samples from days 16–17, as was the number of infiltrating macrophages. Flow cytometry revealed very low expression of MHC class II by large luteal cells at all stages of the oestrous cycle. In early pregnancy, the number of CD4+ and CD8+ cells and macrophages decreased, as did MHC class II expression, compared with mid-dioestrous samples. B cells were present in very small numbers in all samples examined. Eosinophils were similarly sparsely distributed and numbers decreased further in pregnancy. After exogenous PGF₂α administration, populations of CD4+ cells and non-specific esterase staining cells were significantly smaller than after natural luteolysis, whereas eosinophil numbers were increased compared with samples from days 16–17. However, the number of CD8+ and CD5+ cells and MHC class II expression were not significantly different from those observed after natural luteolysis. These findings indicate that populations of immune cells in the equine corpus luteum vary during its lifespan. The selective increase in CD8+ cells before functional luteolysis indicates that they have a physiological role in the regression of the corpus luteum.

Introduction
A number of studies in different animal species indicate that immune cells play an important role in cyclic ovarian activity (Bukovsky and Presl, 1979; Murdoch et al., 1988; Brannstrom and Norman, 1993). The observed similarities between inflammation and ovulation, such as vascular changes and influx of various leucocytes including macrophages, prompted initial investigation of the possibility that the immune system had a role in controlling ovarian function (Bukovsky and Presl, 1979; Espey, 1980).

The corpus luteum is a heterogeneous structure, consisting of a number of different cell populations. The morphology of the equine corpus luteum is unlike that of ruminants, in that there is marked trabeculation of the tissue. This results from the collapse of the large equine pre-ovulatory follicle (mean diameter 45–50 mm) at ovulation. The trabeculae consist of extracellular matrix, fibroblasts and small cells which are thought to be of thecal origin (Harrison, 1946), and contain much of the vasculature of the corpus luteum. The steroidogenic large luteal cells of the equine corpus luteum are thought to arise solely from the granulosa layer of the follicle (Van Niekerk et al., 1975).

The functional corpus luteum is a very vascular structure which means that there are leucocytes circulating through the luteal tissue throughout its lifespan. There is also a resident leucocyte population within the ovarian interstitial tissue. In a number of species, different inflammatory cell populations such as mast cells (Jones and Hsueh, 1981; Mori, 1990), macrophages (Paavola, 1979; Brannstrom and Norman, 1993), lymphocytes (Brannstrom and Norman,
et al., 1993) and granulocytes (Pepperell et al., 1992; Brannstrom et al., 1993) have been observed in the ovary throughout the oestrous cycle (Bagavandoss et al. 1991; Standaert et al., 1991; Brannstrom et al., 1994a). The primary function of macrophages observed within the corpus luteum was presumed to be removal of debris by phagocytosis during regression (Paavola, 1979). Similarly, the other leucocyte populations were initially thought to be involved in removing cells during structural regression. However, recent research has focused on the possibility that these cells play a more active role in the function of the corpus luteum (Bukovsky and Presl, 1979; Murdoch et al., 1988; Bagavandoss et al., 1991). White blood cells, especially macrophages and granulocytes, are a rich source of an array of cytokines with pleiotropic functions, a number of which have been implicated in altering cell function in the ovary (Adashi et al., 1990; Adashi, 1990; Pate and Townson, 1994). If cytokines play a physiological role in the ovary, leucocytes must be present in sufficient numbers to produce concentrations of cytokines that could influence the function of the corpus luteum. In addition, changes in immune cell populations throughout the oestrous cycle would be expected, especially at key events such as luteolysis and ovulation. Studies in women (Petrovska et al., 1992; Brannstrom et al., 1994a), pigs (Standaert et al., 1991), rats (Brannstrom et al., 1993, 1994b) and rabbits (Bagavandoss et al., 1990, 1991) found variations in populations of immune cells in corpora lutea during the oestrous cycle. The number of macrophages has been reported to increase before the onset of luteolysis in some species (Bagavandoss et al., 1988; Lei et al., 1991), and a study in cows showed the number of immune cells that infiltrate the corpus luteum is much smaller in early pregnancy than in the non-fertile cycle (Lobel and Levy, 1968).

If immune cells and their secreted products are involved in luteal regression, there must be a trigger that activates the immune cells. Alteration in, or aberrant expression of, major histocompatibility complex (MHC) glycoproteins on luteal cell surfaces has been proposed as such a trigger. The expression of MHC class II is central to regulation of the immune response, and is limited primarily to lymphocytes and antigen-presenting cells such as macrophages and B cells. MHC class II can also be expressed by cells of non-lymphoid origin, and this aberrant expression usually indicates that the tissue is involved in an inflammatory or immune response and is thought to be an underlying cause in certain autoimmune endocrine disorders (Kuby, 1994; Roitt, 1996). Manifestation of class II molecules by non-lymphoid cells may enable these cells to become antigen-presenting, allowing them to present previously unrecognisable autoantigens to lymphocytes for the initiation or enhancement of immune responses against the target tissue. MHC class II antigens have been detected on human luteal cells (Khoury and Marshall, 1990), and increased expression has been observed on ruminant luteal cells towards the end of the luteal phase and also after PGF2α-induced luteolysis (Fairchild Benyo et al., 1991; Kenny et al., 1991).

PGF2α produced by the endometrium and transported to the ovary by a counter-current mechanism of blood vessels is accepted to be the hormone responsible for the initiation of luteolysis and regression of the corpus luteum in ruminants (Inskipp and Murdoch, 1980; Niswender et al., 1985; Knickerbocker et al., 1988). The close apposition of ovarian and uterine blood vessels in ruminants allows transport of prostaglandin by diffusion from the uterine vein to the ovarian artery, by-passing the systemic circulation. In mares, the role of PGF2α in luteolysis is not as clearly defined. There is no close apposition of ovarian and uterine blood vessels in mares, and any prostaglandin released by the uterus must enter the systemic circulation to reach the ovary. However, in other species most (approximately 90%) of the prostaglandin is cleared from the circulation in one passage through the lungs. Therefore, it must be assumed that lung clearance is less effective in equines, or that only extremely small quantities of PGF2α are required at the ovary. Mares are known to be many times (approximately 10–18 times) more sensitive than cows, pigs or ewes to the luteolytic effects of PGF2α when administered by systemic routes (Douglas and Ginter, 1973, 1975; Oxender et al., 1975). This may be explained, in part, by the high affinity of the equine PGF2α receptor, which is about ten times greater than that of the receptors on the bovine corpus luteum (Kimball and Wnygarden, 1977), and would thus reduce the need for a local utero-ovarian PGF2α-concentrating mechanism.

Although PGF2α is the primary luteolytic agent in ruminants, exposure of cultured bovine luteal cells to PGF2α alone is not sufficient to inhibit progesterone production completely (Pate and Condon, 1984). Therefore, although PGF2α has a central role in luteolysis, other mechanisms must be essential for complete luteal regression. A number of studies indicate that in mares, as in many other species, a number of agents, including other hormones such as oxytocin, oestrone and prostaglone, may be involved in triggering the release of PGF2α. In addition, the mechanism by which prostaglandin might initiate luteolysis is not clear, although it has been the subject of studies in several species giving a variety of results (Nett et al., 1976; Niswender et al., 1976; Hatcher and Niswender, 1982; Silvia et al., 1984; Hoyer and Marion, 1989; Willbanks et al., 1989a, b; Behrman et al., 1993).

If luteolysis involves an immune response, it would have to be suppressed during maternal recognition of pregnancy. Infiltration of lymphocytes and macrophages into the bovine (Lobel and Levy, 1968) and rabbit (Bagavandoss et al., 1988; Bagavandoss et al., 1990) corpus luteum of pregnancy does not occur to the same extent as in the corpus luteum of the normal oestrous cycle. In addition, MHC class II expression is significantly decreased on all cell populations comprising the corpus luteum of pregnant compared with non-pregnant cows (Fairchild Benyo et al., 1991) and sheep (Kenny et al., 1991).

The purpose of this study was to investigate immune cell populations and expression of MHC class II in the equine corpus luteum throughout the oestrous cycle, in early pregnancy and after administration of exogenous PGF2α.

Materials and Methods

Animals

Genetally normal healthy pony mares weighing 250–350 kg and aged between 3 and 15 years were used in this study.
The mares showed normal patterns of oestrus and ovulation throughout the spring and summer. They were examined by transrectal ultrasonography throughout the oestrous cycle to monitor follicular growth. During oestrus, the mares were scanned once a day until ovulation occurred (day of ovulation = day 0). The mares subsequently underwent unilateral ovariotomy by colpotomy (Colbern and Reagan, 1987) at different stages of dioestrus: early (days 2–4), mid- (days 7–10) and late dioestrus. The last group of mares was further divided into pre-luteolytic (days 12–14) and post-luteolytic (day 16+). Another group of mares was ovarioectomized during the mid-luteal phase, 24 h after administration of exogenous PGF
\textsubscript{2\alpha} analogue (1 ml Estrumate i.m. (equivalent to 263 μg cloprostenol); Mallinckrodt Veterinary Ltd, Uxbridge). The last group of mares in the study were mated by artificial insemination, diagnosed as pregnant by ultrasound scan and subsequently monitored to confirm continuing pregnancy up to and at the time of surgery. The ovary containing the corpus luteum was removed between day 20 and day 50 of pregnancy. Each group consisted of five to eleven mares.

All surgery was performed under standing sedation and analgesia using acepromazine (0.05 mg kg\textsuperscript{-1} i.m.; C-Vet Ltd, Bury St Edmunds), romifidine (0.05 mg kg\textsuperscript{-1} i.v.; Sedivet, Boehringer Ingelheim Ltd, Bracknell), butorphanol (0.05 mg kg\textsuperscript{-1} i.v.; Torbugesic, Willow Francis, Crawley) and flunixin meglumine (1.1 mg kg\textsuperscript{-1} i.v.; Finadine, Schering-Plough Animal Health, Welwyn Garden City). Tetanus prophylaxis was administered before surgery and all mares received in-feed trimethoprim and sulphadiazine (30 mg kg\textsuperscript{-1} day\textsuperscript{-1}; Uniprim, Cheminex Laboratories Ltd, Corby) for 4 days after surgery. Blood samples were collected from all mares by jugular venepuncture into an evacuated heparinized tube (Becton Dickinson UK Ltd, Cowley, Oxford) for progesterone analysis for 2–3 days before and immediately before surgery, to assess corpus luteum function. Blood samples were centrifuged at 2000 g for 15 min at 4°C. Plasma was stored at −20°C before the assays were performed.

Processing of samples

Immediately after surgery, the corpus luteum was dissected out of the ovarian tissue. Samples for immunohistochemistry were placed in OCT compound (Miles Inc., Elkhart, IN), snap frozen in dry ice and isopentane, and stored at −70°C. Fresh tissue was processed immediately for analysis by flow cytometry.

One piece of corpus luteum was fixed for 24 h in 4% (w/v) paraformaldehyde. These samples were used for histochemical staining.

Progesterone assay

Progesterone concentrations were determined in unextracted plasma using a validated technique (Corrie et al., 1981) modified by Law et al. (1992). Standards were prepared in plasma from an ovarioctomized mare. The main cross-reactivities of the antisera were with 5-pregnane-3,20 dione (9.5%), 11-deoxycorticosterone (6.2%), and 17-hydroxy-progesterone (3.4%). The limit of detection of the standard curve was 0.5 ng ml\textsuperscript{−1}, and the intra- and interassay coefficients of variation were 9 and 12.6%, respectively (Watson et al., 1995).

Immunohistochemistry

Cryostat sections (6 μm thick) were cut from each sample, placed on gelatin-coated (Biobond; British Biocell International, Cardiff) slides and air-dried for 2 h before they were fixed for 5 min in acetone at 4°C. Sections were washed in PBS (pH 7.3) and stained according to a modified avidin–biotin complex (ABC) staining method (Hsu et al., 1981) using a Vectastain Elite ABC Kit (Vector Laboratories, Peterborough) and primary monoclonal antibodies (mAbs) against immune cell specific antigens. mAbs to CD4 (HB 61A), CD8 (HT 14A) and CD5 (HB 19A) were purchased from VMRD (Washington State University) and a mAb to B cells (MAC 292) was obtained from the Animal Health Trust (Newmarket). The specificities of these antibodies were reported by Kydd et al. (1994). Three anti-MHC class II mAbs were used in the study. VPM 54 (Department of Veterinary Pathology, University of Edinburgh) and TH14B (VMRD, Washington State University) are specific for the ruminant MHC class II DR locus, and VPM 36 (Department of Veterinary Pathology, University of Edinburgh) is DQ specific (Dutia et al., 1995). All these mAbs crossreact with the equine antigen as there is strong interspecies conservation of the MHC antigen (Hopkins et al., 1986). After initial investigations in which all three antibodies produced almost identical staining patterns, VPM 36 and 54 were used throughout the rest of the study.

Endogenous peroxidase staining was blocked by incubating the sections with 5 U glucose oxidase ml\textsuperscript{-1} in 10 mmol B-D-glucose l\textsuperscript{-1}, 1 mmol sodium azide l\textsuperscript{-1} and 0.1 mol PBS 1\textsuperscript{-1} (pH 7.3) for 50 min at 37°C. The sections were treated with 1.5% normal horse serum for 15 min at room temperature, followed by overnight incubation at 4°C with the primary antibodies. The sections were washed for 10 min with PBS before they were incubated with the secondary biotinylated antibody for 30 min at room temperature. The avidin–biotin complex reagent was added after further washing, and the sections again incubated for 30 min at room temperature. Chromagen (3-amino-9-ethylcarbazole, AEC) was added as the final substrate and a red reaction product was visible after 7–8 min. Sections were then washed in tap water, counter-stained with Meyer’s haematoxylin and mounted using an aqueous mounting agent (Immumount; Shandon, Pittsburgh, PA). Negative control sections in which serum was substituted for the primary antibody were included, while sections of equine lymph node were used as positive controls throughout.

Histochemistry

Frozen sections were also stained for non-specific esterases (Hudson and Hay, 1989) to aid identification of...
macrophages. Carbol-chromotrope staining (Lendrum, 1944) was carried out on sections from samples fixed in paraffin for detection of eosinophils. Fixed sections were also stained with haematoxylin and eosin to confirm that cell morphology had been adequately preserved.

Cell counts

Preliminary studies indicated that the immune cells were more numerous in the trabeculae of the corpus luteum than among the luteal cells. Immune cells were also found in greater numbers in the capsule of the corpus luteum. For this reason, the capsule and associated fibrous tissue were avoided when examining the samples. Only extravascular positively stained cells present in the viewing field were counted. Five randomly selected fields were examined at low power: representative fields were selected from the outer, middle and inner areas of each corpus luteum. The mean of the five fields was used as the final figure.

Flow cytometry

Samples were divided into three groups: mid- (days 6–8), late pre-luteolytic (days 12–14) and late post-luteolytic (days 16–17) luteal phase. Luteal tissue was finely minced and resuspended in 30 ml Hank's balanced salt solution (Ca2+ and Mg2+ free) buffered with 20 mmol Hepes (buffered HBSS; Flow Laboratories, Irvine). Cells were mechanically dissociated from the tissue by gentle inversion 10–15 times. The suspension was passed through a 100 μm stainless steel mesh and the cells were collected by centrifugation (400 g, 10 min) and resuspended in buffered HBSS. The cells were washed twice in fresh ice-cold FACS buffer (1% bovine serum albumin and 0.1% (w/v) azide in PBS) and counted in a haemocytometer. A cytospin preparation of each tissue sample was stained with Diff-QuickTM (Baxter, Thetford) to evaluate the cell population obtained by the dissociation process. Cells from individual corpora lutea were processed for indirect immunofluorescence.

Approximately 1 x 105 viable luteal cells per tube were incubated for 30 min at 4°C with optimal dilutions of each of three primary mAbs for MHC class II antigen: TH14B (VMRD Inc., Pullman, WA) and VPM 36 and 54 tissue culture supernatants (Department of Veterinary Pathology, University of Edinburgh). Controls included cells incubated with 1:500 normal mouse serum substituted for the primary antibody to monitor possible non-specific binding of antibody, and cells incubated with PBS instead of antibody. Cells were washed twice with FACS buffer and visualized with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, High Wycombe) diluted 1:250. Cells were again washed twice in FACS buffer and fixed in 1% (w/v) paraformaldehyde (BDH, Poole) and maintained at 4°C in the dark until the analysis was performed within 6 days of labelling.

A minimum of 5000 cells from each sample was analysed using a fluorescence-activated cell analyser (FACScan; Becton Dickinson, Los Angeles, CA). Cells with different physical characteristics (that is, size and complexity) were analysed separately by gating using forward scatter (FSC: cell size) and side scatter (SSC: cell complexity) parameters. Gating of the scatter plots after flow cytometry permitted isolation of cell populations of interest and exclusion of debris and red blood cells. Equine luteal cells are relatively large, measuring 26–36 μm depending on the stage of the oestrous cycle. As MHC class II expression by these cells was of interest, a gate was set that encompassed the largest cells on the plot (Fig. 1, area A) and excluded debris, red blood cells and inflammatory cells (Fig. 1, area B). Early corpora lutea (days 2–4) were excluded from flow cytometry due to the large proportion of red blood cells present in these samples. The percentage of positively labelled cells was calculated by subtracting signals from non-specifically labelled cells.

Statistical analysis

Immunohistochemistry results were analysed using the Kruskal–Wallis test for non-parametric data. When differences between samples were present they were then analysed using the Mann–Whitney U test. Flow cytometry results were analysed using one-way ANOVA. A P value < 0.05 was considered to be significant. All statistical analyses were performed using Minitab software (Pennsylvania State University, PA).

Results

Progesterone assay

Progesterone concentrations were used to determine corpus luteum activity in the mares at the time of surgery. Progesterone concentrations decrease rapidly at functional luteolysis before structural luteolysis and physical regression, and are therefore a very sensitive indicator of alterations in luteal performance. Luteolysis was considered to have occurred when plasma progesterone concentrations were < 2 ng ml−1. All the animals in the days 2–4, days 7–10 and days 12–14 groups had progesterone concentrations consistent with the presence of a functional corpus luteum before and at surgery. Mares in the days 16–17 group had progesterone concentrations that indicated that functional luteolysis had occurred, as did those animals that had been administered PGF2α.

Immune cell detection

At all stages of dioestrus, lymphocytes were more numerous in the trabeculae of the corpus luteum than among the luteal cells (Fig. 2). CD4+ and CD8+ cells showed similar distribution patterns and were present in approximately equal numbers. CD4+ cells were present in significantly greater numbers in the samples from days 16–17 than in the tissues from days 2–4 (P < 0.01) or days 7–10 (P < 0.05) (Fig. 3). Visual examination revealed an apparent increase in the
number of cells in the trabeculae during the late luteal phase along with a migration of cells among the luteal cells (Fig. 4). CD8+ cells were present in significantly greater numbers at days 12–14 than at days 2–4 ($P < 0.05$). CD5+ cells were present in small numbers throughout the cycle and there were no significant differences among the groups.

Populations of CD4+ cells were significantly smaller in early pregnancy than at any stage of the oestrous cycle.
(P < 0.01, except days 2–4: P < 0.05) (Fig. 3). Corpora lutea from pregnant mares also contained significantly smaller numbers of CD8+ cells than at days 12–14 or days 16–17 of the oestrous cycle (P < 0.01) (Fig. 3). There was no significant difference in the number of CD5+ cells in early pregnancy compared with any stage of the oestrous cycle.

B cells were present in very small numbers at all stages of the oestrous cycle; the average number per field examined was between zero and three cells. However, the number of cells was significantly greater in corpora lutea from the early (days 2–4; P < 0.05) and late (days 16–17; P < 0.01) phases than the mid-luteal phase (days 7–10). The number of B cells was significantly lower in pregnancy compared with early luteal (days 2–4) samples (P < 0.05).

Cells positive for non-specific esterase staining (macrophages) were present in significantly greater numbers in post-luteolytic samples (days 16–17) than at any other stage of the oestrous cycle or early pregnancy (P < 0.05) (Fig. 5). In addition, there was a significant increase in positive cells between samples from days 7–10 and those from days 12–14 (P < 0.05).

The number of MHC class II-positive cells was significantly increased in samples from days 16–17 compared with tissues from days 7–10 (Fig. 6). One of the mAbs (VPM 54) also showed a significant increase in expression between samples from days 2–4 and days 16–17 (P < 0.05). MHC class II expression decreased in early pregnancy. The number of cells expressing MHC class II was significantly smaller in pregnancy than at days 12–14 (P < 0.05) and days 16–17 (P < 0.01) of the oestrous cycle.

Eosinophils were present in very small numbers throughout the oestrous cycle and showed no significant differences in numbers during the period studied. A small number of eosinophils was also present in early pregnancy; this number was significantly smaller than at days 12–14 of the oestrous cycle (P < 0.05).

After PGF

administration, the number of CD4+ cells was significantly smaller than after natural luteolysis (P < 0.01), whereas the number of CD8+ cells was not significantly altered (Fig.3). The number of CD5+ cells was also unchanged, whereas the number of B cells was significantly smaller than in samples from days 16–17 (P < 0.05). There was no difference in MHC class II expression after PGF

administration compared with after natural luteolysis for both mAbs used (Fig. 6) but there was significantly greater expression (P < 0.05) than in samples from days 7–10 and pregnant mares. Cells positive for non-specific esterase staining were present in significantly smaller numbers (P < 0.05) compared with samples from days 16–17, but in significantly greater numbers than in samples from days 7–10 (Fig. 5).

Flow cytometry

Negligible MHC class II expression by equine luteal cells was detected with all three mAbs at all stages of the oestrous cycle.
cycle examined. In addition, there were no significant differences in expression among any of the time periods analysed (Fig. 1).

**Discussion**

Different inflammatory cell populations have been observed in the ovaries of a number of species throughout the oestrous cycle. Studies on the local interactions occurring between immune and reproductive cells indicate a key role for leucocytes and their products in ovarian physiology. To date, there have been no reports on immune cell populations in the equine corpus luteum. The results of this study show that the immune cell populations in the equine corpus luteum vary during the oestrous cycle and after administration of PGE$_{2o}$.

The macrophage is the best documented ovarian cell of lymphohaematopoietic origin and has been localized in the ovary of several species (Paavola, 1979; Lei et al., 1991; Wang et al., 1992; Brannstrom and Norman, 1993). Initially, macrophages and other leucocytes were thought to be involved in removing luteal cells by phagocytosis during structural regression. However, cells of the mononuclear phagocyte system possess considerable functional heterogeneity in tissues and body cavities. In addition to their phagocytic role, macrophages secrete a wide variety of products that are involved in connective tissue breakdown and vascular changes and are ovulatory mediators (Brannstrom et al., 1994). They are also a rich source of multifunctional cytokines such as tumour necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin 1 (IL-1), both of which are capable of influencing various ovarian cell functions (Roby and Terranova, 1989; Nakamura et al., 1990; Roby et al., 1990; Zolli et al., 1990; Hurwitz et al., 1991; Veldhuis et al., 1991).

In the present study, identification of macrophages was problematic. A number of antibodies were tested with highly variable results and marked differences among antibodies. Therefore, non-specific esterase staining was used for detection of macrophages in this study. Cellular esterases are ubiquitous and appear to represent a series of different enzymes acting upon select substrates. Under defined reaction conditions, it is possible to determine haematopoietic types of cell using specific esterase substrates. Under the conditions used in this study, the enzyme detected is found primarily in monocytes, macrophages and histiocytes, and is virtually undetectable in granulocytes. Significantly increased numbers of positive cells were present in post-luteolytic samples compared with every other stage of the cycle. However, there was also a significant increase in the number of positive cells between samples from days 7–10 and days 12–14. This may indicate an influx of macrophages into the equine corpus luteum before functional luteolysis, implying a possible role for macrophages and their array of secretory products in functional as well as structural luteolysis.

CD4+ and CD8+ cells were present in approximately equal numbers during dioestrous. The number of CD4+ cells increased significantly in the corpus luteum after luteolysis compared with early and mid-luteal samples. As this increase occurred after functional luteolysis, the influx may not be relevant to the regression of the corpus luteum. However, CD8+ cytotoxic T cells increased significantly before functional luteolysis (days 12–14), which indicates a role for these cells and their products in luteolysis. Furthermore, in the peripheral blood of horses, the ratio of CD4+:CD8+ T cells is > 2:1 (Lunn et al., 1991), indicating that there is selective infiltration of CD8+ lymphocytes into the equine corpus luteum. A similar observation has been noted in the rat ovary during the periovulatory period (Brannstrom et al., 1993), while in cows, CD8+ cells appear to be involved in functional luteolysis (Nakamura-Moffor et al., 1994). Thus, it seems that these cells may have a physiological role in control of ovarian function. Activated T lymphocytes are a rich source of a variety of cytokines such as IL-2 and interferon $\gamma$ (IFN-$\gamma$), both of which affect steroidogenesis of human granulosa-lutein cells (Wang et al., 1991, 1992). IFN-$\gamma$ also alters bovine luteal cell prostaglandin production and progesterone synthesis (Fairchild and Pate, 1991), which indicates a role in luteolysis, and can also have direct cytotoxic effects on cells especially when present in combination with some other cytokines (Campbell et al., 1988; Fairchild and Pate, 1991; Benyo and Pate, 1992). Such properties, when taken in conjunction with cyclic variations in the number of cells, indicate a physiological role for T cells in cyclic ovarian activity.

In the present study, three different T-lymphocyte markers were used. CD4+ helper T cells and CD8+ cytotoxic killer T cells are two mutually exclusive subpopulations of T lymphocytes in horses (Lunn et al., 1991; Kydd et al., 1994), the numbers of which, when added together, equal those of the CD5+ cells, a pan-T-cell marker (Kydd et al., 1994). This was not the case in the present study in which CD5+ cells were present only in very small numbers and were always greatly outnumbered by other T cells. Research in sheep revealed a similar discrepancy (Hopkins and Dutia, 1990), and it appears that the CD5 molecule is not present at cellular activation. This proposal would explain other reports in which CD4+ and CD8+ cells greatly outnumbered CD5+ cells (Gorrell et al., 1988, Meeusen et al., 1988). In the present study, the number of CD5+ cells in the equine corpus luteum did not vary significantly throughout the oestrous cycle or in early pregnancy.

B lymphocytes were present in very small numbers throughout the oestrous cycle. Studies of the human corpus luteum failed to detect any B cells during the menstrual cycle (Wang et al., 1992; Brannstrom et al., 1994). Reports in a number of other species also fail to report the presence of B cells (Standaert et al., 1991; Petrovskia et al., 1992; Brannstrom et al., 1994). B cells are antibody-producing cells that are active in humoral immune responses, a role which is probably not relevant to the function of the corpus luteum.

MHC class II expressing cells detected by immunohistochemistry were present in significantly greater numbers in post-luteolytic samples (days 16–17) than in mid-luteal tissue. An increase in number was noted in samples from days 12–14, although the difference was not significant. This increase probably reflects the increased number of immune cells present as the corpus luteum undergoes structural luteolysis and the phagocytic cells move in. Expression of MHC class II by equine large steroidogenenic
luteal cells was not detected in this study, either by immunohistochemistry or flow cytometry. This finding is in contrast to studies in cows (Fairchild Benyo et al., 1991) and sheep (Kenny et al., 1991) which reported MHC class II expression by luteal cells towards the end of the luteal cycle and after PGF\textsubscript{2\alpha}-induced luteolysis (Fairchild Benyo et al., 1991). However, Anderson (1997) also failed to detect MHC class II expression in the bovine corpus luteum.

Eosinophil infiltration into the corpus luteum during luteolysis has been observed in a number of species, particularly sheep (Nett et al., 1976; Murdoch et al., 1988) and pigs (Standaert et al., 1991). A role for these cells in initiation of luteal regression has been indicated in pigs (Standaert et al., 1991). In the present study, eosinophils were present in the equine corpus luteum in very small numbers throughout the oestrous cycle, and showed no significant increase at luteolysis.

If immune cells are involved in luteolysis, the immune response would have to be suppressed during maternal recognition of pregnancy. The findings in the present study are in agreement with studies in cows (Lobel and Levy, 1968) and rabbits (Bagavandoss et al., 1988) in which a decreased infiltration of lymphocytes and macrophages into the corpus luteum of pregnancy was observed. Significantly smaller numbers of both CD4+ and CD8+ cells were observed in early pregnancy than during the oestrous cycle. The number of macrophages also decreased significantly in early pregnancy. MHC class II expression was reduced in the equine corpus luteum in early pregnancy, but this may simply reflect the decreased number of T lymphocytes and macrophages present in the tissue. In contrast, MHC class II expression is significantly decreased in both luteal and non-luteal cell populations comprising the corpus luteum of pregnant compared with non-pregnant cows (Fairchild Benyo et al., 1991) and sheep (Kenny et al., 1991). The signal for suppression of MHC class II expression in these species is not known, but may be related to the trophoblast protein IFN-\(\tau\) (Pate, 1995). No equivalent conceptus-derived protein has been identified in mares.

In veterinary practice, the exogenous administration of PGF\textsubscript{2\alpha} is commonly used to induce luteolysis artificially in mares. The dose used is the same as that used in the present study, which caused some interesting changes in cell populations in the corpus luteum. Plasma progesterone concentrations indicated that the mares had undergone functional luteolysis by the time of ovariecotmy. The number of CD4+ cells was significantly smaller than after naturally occurring functional luteolysis, whereas there was no significant difference in populations of CD8+ cells. MHC class II expression was also not significantly different from that after natural luteolysis.

The non-specific esterase staining results showed significantly fewer positive cells, considered to represent macrophages, after prostaglandin treatment compared with samples from days 16–17. It is possible that the more rapid regression of the corpus luteum after artificially induced luteolysis did not allow sufficient time for the macrophages and CD4+ cells to infiltrate the tissue to the same extent as observed after natural luteolysis. In the present study, samples were taken from 1 to 3 days after natural functional luteolysis, as determined by progesterone concentrations, compared with 24 h after prostaglandin administration. Alternatively, induced luteolysis may not follow the same series of events observed in natural luteolysis, and substances that attract macrophages may be missing from the process. The pharmacological dose of prostaglandin used to induce luteolysis artificially is many orders of magnitude greater than normal physiological concentrations of endogenous prostaglandin in the corpus luteum at luteolysis, and this may also alter the normal order of events. PGF\textsubscript{2\alpha} has marked vasoconstrictive actions that are thought to be responsible for some of the morphological changes in the corpus luteum at luteolysis (Nett et al., 1976; Stacy et al., 1976). The excessive dose of prostaglandin used to induce luteolysis may also exaggerate this effect. In addition, the vasoconstrictive effects of PGF\textsubscript{2\alpha} may affect the infiltration of inflammatory cells into the corpus luteum.

In the present study, eosinophils were present in the equine corpus luteum in very small numbers throughout the oestrous cycle, and showed no significant increase at luteolysis. In contrast, after PGF\textsubscript{2\alpha} administration, the number of eosinophils increased to significantly greater amount than after natural luteolysis. This indicates that exogenous prostaglandin can attract eosinophils into the equine corpus luteum, either directly or through changes induced in the luteal tissue.

In conclusion, the results of the present study indicate that various immune cells, particularly lymphocytes, differentially migrate into and out of, the equine corpus luteum during the oestrous cycle, early pregnancy and after administration of PGF\textsubscript{2\alpha}. The temporal nature of this migration indicates involvement of certain populations of these cells in modulation of luteal activity. Further studies are required to identify specific subproducts of these cells that are present throughout the life of the corpus luteum to clarify the role of immune cells within this tissue.

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