Leucocyte proliferation in the bovine corpus luteum

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Leucocytes vary in type and number during the lifespan of a corpus luteum. The aim of this study was to determine whether there is an increase in the number of lymphocytes and macrophages as a result of local proliferation. Bovine corpora lutea were classified into stages of development, secretion and regression. A new double immunolabelling method was established for nuclear Ki-67 antigen (a marker for cell proliferation) and for leucocyte surface antigens (detection of CD2-, CD3-, CD4-, CD8-positive lymphocytes and CD14-positive monocytes). Differential cell counting was performed. Between the stages of development and regression there was an increase in the number of T-lymphocytes and macrophages.

The percentage of proliferating leucocytes in relation to the total number of proliferating cells was approximately 20% at the stage of advanced secretion and 70% at late regression. The increase in the number of proliferating leucocytes at late regression was due to CD14-positive macrophages. These macrophages migrated from small blood vessels into the septa of corpora lutea at the early stage of regression. Macrophages showed local proliferation in the late stage of regression when capillaries were no longer present. It is concluded that the physiological involution of the corpus luteum is an inflammatory-like condition, which includes local proliferation of monocytes.

Introduction

Leucocytes populate corpora lutea of several species including humans (Wang *et al.*, 1992; Brännström and Norman, 1994; Best *et al.*, 1996), cows (Lawler *et al.*, 1994; Spanel-Borowski *et al.*, 1997; Penny *et al.*, 1999) and pigs (Standaert *et al.*, 1991). Staining the leucocytes with the common leucocyte antibody against CD18 and counting the positive cells showed that the number of these cells increases between the stages of development, secretion and regression of the corpus luteum (Spanel-Borowski *et al.*, 1997). The leucocyte pool appears to contain subtypes that are independent of the stage of the oestrous cycle: eosinophils are found in preovulatory follicles (sheep), during the development of the corpus luteum (cows) and in the regressing corpus luteum after PGF_{2α}-induced luteolysis (Murdoch, 1987; Cavender and Murdoch, 1988). More lymphocytes (CD4- and CD8-positive) are present at the onset of functional luteolysis than in the other stages of the oestrous cycle (Penny *et al.*, 1999). Macrophages dominate all stages of the oestrous cycle except between day 6 and day 12 (stage II, Penny *et al.*, 1999).

Luteal leucocytes are assumed to be involved in the growth, differentiation and regression of corpora lutea by secretion of a variety of cytokines (Adashi, 1992; Brännström *et al.*, 1994; Sunderkötter *et al.*, 1994; Pate, 1995). These leucocytes may act in a direct or indirect

manner and influence the secretion of progesterone and prostaglandin (Fairchild *et al.*, 1991; Townson and Pate, 1994). The cellular interaction between leucocytes and luteal endothelial cells probably determines the time course and cyclic recruitment of leucocyte populations, or may induce proliferation of resident macrophages within a corpus luteum. This is deduced from a recent report on intratesticular proliferation of resident macrophages (Schlatt *et al.*, 1999).

The major proportion of proliferating cells in the corpora lutea of rats, cows and primates are endothelial cells (Jablonka-Shariff, 1993; Zheng *et al.*, 1994; Christenson and Stouffer, 1996). These cells represent approximately 50% of the total number of cells in a fully developed corpus luteum (Farin *et al.*, 1986; Lei *et al.*, 1991; Reynolds and Redmer, 1999). A substantial number of proliferating cells was observed in the regressing corpora lutea of pigs (but not in cows or sheep); however, the types of cell were not specified (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Reynolds and Redmer, 1999). The regressing corpus luteum contains an abundance of macrophages as demonstrated in cows (Spanel-Borowski *et al.*, 1997). Under local inflammatory stimuli bone marrow-derived monocytes may divide a few times in their target tissue before they become mature macrophages (van Furth *et al.*, 1985; van Furth, 1988); thus, immature macrophages that are proliferating may occur in the regressing bovine corpus luteum.

The aim of the present study was to determine the number of proliferating macrophages in all stages of the cyclic corpus luteum of cows. An immunohistological

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technique was established for double-labelling proliferating leucocytes with the antibody against the nuclear Ki-67 antigen and with an antibody against leucocyte surface antigens. The Ki-67 antigen, an established marker for cell proliferation, displays all active phases of the cell cycle, that is G1, S, G2 and mitosis. The Ki-67 antigen is considered a more appropriate marker than proliferating cell nuclear antigen (PCNA), which reveals non-proliferating cells under DNA repair (Hall *et al.*, 1993). Antibodies against different leucocyte surface antigens (CD2, CD3, CD4, CD8, CD14, CD11/18) were used to differentiate leucocyte subtypes.

Materials and Methods

Ovaries with corpora lutea were collected from non-pregnant cows within 30 min after the animals were killed at a local abattoir. Pregnancy was excluded by macroscopic examination of the uterine horns. The ovaries were kept in ice-cold 0.01 mol PBS l⁻¹ until preparation at the laboratory. Each corpus luteum was dissected from cortical tissue and pieces of luteal tissue were either frozen immediately in liquid nitrogen for storage at -80°C or fixed with 4% (w/v) PBS buffered formaldehyde and embedded in paraffin wax according to the standard histological technique. The corpora lutea were classified according to the stage of the oestrous cycle stage by macroscopic appearance (Ireland *et al.*, 1980) and histological criteria (Ricken *et al.*, 1995; Reibiger and Spaniel-Borowski, 2000). The stages of the oestrous cycle were estimated as follows: days 1–4 for early development, days 11–17 for the secretory stage, days 19–20 and subsequent days for regression. Eosinophils were present at a very early stage of development of the corpus luteum ($n = 3$), that is, 1–2 days after ovulation. A fully developed capillary network was apparent at the secretory stage ($n = 16$) and there was a decrease in the capillary network at the stage of regression ($n = 23$). Finally, classification of the corpora lutea was confirmed by our new immunohistological data on cell proliferation (see below).

Immunohistology

Deep-frozen tissue was cut into serial sections (10 µm). The sections were mounted on object slides that had been coated with poly L-lysine (0.1% w/v, P 8920, Sigma, Deisenhofen), air-dried for 2 h and stored at -20°C until use. A paper glue (Cementit® Merz and Benteli AG, Niederwangen) was used to adhere the paraffin wax sections to the slides.

The single-labelling technique for Ki-67 antigen followed the antigen retrieval procedure according to the manufacture's instructions (Dianova, Hamburg). Cryostat sections were fixed in 4% (w/v) PBS buffered formaldehyde, washed in PBS and transferred into 0.01 mol citrate buffer l⁻¹ at pH 6.0. Buffer was bubbled up in a microwave oven and the slides were cooked at 350 W for 15 min,

cooled at room temperature for approximately 15 min, washed once in aqua dest and placed in 0.05 mol Tris-buffered saline l⁻¹ at pH 7.6. Subsequently, endogenous peroxidase activity was quenched with 0.3% (v/v) H₂O₂ in absolute methanol for 30 min. Non-specific protein binding sites were blocked with 1.5 % (v/v) normal goat serum (Dako, Hamburg) in Tris-buffered saline for 30 min. The slides were incubated with the primary monoclonal antibody (anti Ki-67, 1:50 in 1.5% (v/v) goat serum, clone: Mib-5) for 1 h at room temperature. After the slides were washed in Tris-buffered saline, biotinylated goat anti-mouse antibody (1:200, Vector Laboratories, by Alexis, Grünberg) was applied as secondary antibody for 30 min. Immunostaining was performed using the avidin–biotin–peroxidase complex from the Vectostain kit (Vector Laboratories) and 3-amino-9-ethyl-carbazole (Sigma, Deisenhofen) for peroxidase detection as described by Spaniel-Borowski *et al.* (1997).

For single labelling of endothelial cells and smooth muscle cells, paraffin wax was removed from the sections and they were rehydrated and pre-incubated with 0.05% (w/v) protease (EC 3.4.24.31, type XIV from *Streptomyces griseus*; Sigma-Aldrich Chemie GmbH, Steinheim) in Tris-buffered saline for 30 min at room temperature. Enzyme digestion was stopped by washing extensively with Tris-buffered saline. Endogenous peroxidase activity and non-specific immunoglobulin binding sites were blocked. Either rabbit anti-factor VIII related (FVIIIr) antigen (detection of endothelial cells, 1:1000 in normal goat serum; Dako) or mouse anti ASM-1 (detection of smooth muscle cells and pericytes, 1:250 in normal goat serum; Progen, Heidelberg) were incubated for 1.5 h. The biotinylated secondary antibodies (goat anti-rabbit, 1:200 in normal goat serum, and goat anti-mouse, 1:200 in normal goat serum; Vector Laboratories) were applied for 30 min at room temperature. The final immunostaining was performed with 3'3'-diaminobenzidine (DAB) as peroxidase substrate. Between each incubation step, sections were washed twice with Tris-buffered saline containing 0.125% (v/v) Tween 20 (Bio-Rad, Krefeld) and twice with Tris-buffered saline. Negative controls were carried out with normal goat serum instead of the primary antibody.

The protocol for the double-labelling technique used on cryostat sections was according to Lan *et al.* (1995) and Tornehave *et al.* (2000). One antigen (Ki-67) was detected with microwave oven treatment and the other antigen (leucocyte adhesion molecule) was detected separately. The Ki-67 antibody can be used in conjunction with microwave oven treatment, in contrast to the PCNA antibody which may label cell nuclei non-specifically after treatment (Heenen *et al.*, 1998). Since the histochemical detection of peroxidase activity with the substrate DAB (Sigma-Aldrich, Deisenhofen) gave a brown heat-stable reaction product, the leucocyte surface antigen was detected first using the DAB substrate and subsequently the proliferation marker Ki-67 was visualized with the 3-amino-9-ethyl-carbazole substrate, which turned red by peroxidase

Table 1. Ratio of positively stained cells:total number of cells (% mean $\pm$ SEM) in bovine corpora lutea at different stages of the oestrous cycle

Antigen	Early development	Secretion		Regression	
		Early	Advanced	Early	Advanced
Ki-67	10.1 $\pm$ 1.1 (3)	23.3 $\pm$ 2.3 (9)*	4.5 $\pm$ 1.1 (7)***	0.5 $\pm$ 0.1 (12)***	3.4 $\pm$ 0.4 (9)***
CD18	47.4 $\pm$ 13.1 (3)	4.0 $\pm$ 0.4 (7)*	4.7 $\pm$ 0.7 (5)	4.9 $\pm$ 0.3 (12)	56.4 $\pm$ 8.9 (11)***
CD14	1.7 $\pm$ 1.0 (3)	1.6 $\pm$ 0.2 (9)	2.2 $\pm$ 0.3 (7)**	1.1 $\pm$ 0.4 (12)**	36.1 $\pm$ 9.3 (11)***
CD2	ND	0.7 $\pm$ 0.1 (9)	1.3 $\pm$ 0.4 (7)*	3.3 $\pm$ 0.4 (12)**	7.6 $\pm$ 0.9 (11)***
CD8	ND	0.7 $\pm$ 0.1 (7)	1.2 $\pm$ 0.4 (5)	2.4 $\pm$ 0.2 (10)**	5.5 $\pm$ 1.1 (7)**
CD4	ND	ND	0.2 $\pm$ 0.1 (3)	0.5 $\pm$ 0.1 (8)	1.2 $\pm$ 0.2 (7)**
CD3	ND	ND	ND	2.6 $\pm$ 0.5 (8)	6.8 $\pm$ 1.4 (7)**

Number of corpora lutea in parentheses.

ND: not determined.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively, compared with the preceding stage.

activity. All primary mouse monoclonal antibodies were diluted in 1.5% (v/v) normal goat serum as follows: anti-bovine CD11/18 (anti-pan leucocytes, 1:200, clone BAQ 30A, VMRD Inc. Pullman, WA), anti-ovine CD14 (anti-monocytes-macrophages, 1:80, clone VPM65, VMRD), anti-CD3 (anti-T-lymphocyte, 1:50, clone MM1A, VMRD), anti-bovine CD2 (anti-lymphocyte, 1:100, clone CC42, Serotec, Oxford) anti-bovine CD4 (anti-T-helper lymphocyte, 1:20, clone CC30, Serotec), anti-bovine CD8 (anti-T-suppressor/cytotoxic lymphocyte, 1:20, clone CC63, Serotec). For the detection of CD11/18, CD14 and CD2 antigens, cryostat sections were fixed with formaldehyde, whereas for CD4 and CD8 antigens, sections were not fixed. The detection of CD2-positive subpopulations was performed only for regressing corpora lutea.

After blocking endogenous peroxidase activity and non-specific immunoglobulin binding sites, sections were incubated with one of the leucocyte-specific primary antibodies for at least 1.5 h at room temperature. Negative controls were carried out with 2% (v/v) normal goat serum rather than the primary leucocyte-specific antibody. Additional steps were completed as described for detection of Ki-67 antigen (single labelling); however, DAB was not used as peroxidase substrate. The sections were washed, placed in Tris-buffered saline for 30 min and transferred into citrate buffer. Steps for the demonstration of the Ki-67 antigen were carried out using 3-amino-9-ethyl-carbozole as peroxidase substrate. The sections were rinsed in distilled water and counterstained with Mayer's hemalaun and mounted in glycerine gelatine (Hollborn, Leipzig), a water soluble embedding medium.

Cell counting and documentation

Proliferating and non-proliferating leucocytes in double-labelled sections were examined with a light photomicroscope (Axioskop, Zeiss, Jena). A $\times 40$ objective and a 1 cm² ocular grid were used. Leucocytes were observed as dark brown cells with or without a red nucleus regardless of whether the cells were proliferating. Positive cells were counted in at least eight fields of view in a systematic random approach, and were calculated as cells per mm². In regressing corpora lutea that contained numerous leucocytes, CD18- or CD14-positive cells were also counted, whereas leucocytes remained separate. Septa and large blood vessels were not counted. Statistically significant differences among all group means were assessed by the non-parametric Kruskal-Wallis test followed by the Mann-Whitney U test.

A light photomicroscope (Axioplan 2, Zeiss) equipped with a Progress camera and a personal computer was used to take digitalized pictures. Pictures were adjusted with appropriate software (Photoeditor, Adobe Photoshop 5).

Results

Proliferating cells

The criteria used to classify the stages of the corpora lutea were confirmed by the density of Ki-67 positive nuclei (Table 1). Dominant follicles have a large number of proliferating cells (ratio of 49.8 ± 5.2 and 47.5 ± 5.0 in the granulosa and thecal layer, respectively; Fig. 1a),

Fig. 1. Cell proliferation in a dominant bovine follicle and in bovine corpora lutea at different stages of the oestrous cycle. Double immunolabelling for the nuclear Ki-67 antigen and the leucocyte surface antigen CD18 (a–c) or CD2 (d) was carried out. (a) A dominant follicle containing numerous Ki-67-positive nuclei (red) indicating an extremely high proliferation rate in the granulosa (G) and thecal (T) layers. There are a few CD18-positive leucocytes (brown). (b) The early secretory stage is characterized by a large number of proliferating cells (red nucleus), which are either attributed to dark brown stained leucocytes or to other types of cell in the corpus luteum. (c) At the early stage of regression, there are very few proliferating cells (arrow). (d) At the late stage of regression, the number of proliferating cells (red nuclei) is greater than at the early stage of regression. Scale bars represent 25 µm.

whereas fewer proliferating cells were observed at the early stage of corpus luteum development. At the advanced stage of development (not shown) and at the early stage of secretion there was an increase in the number of proliferating cells (Fig. 1b). At the end of the secretory stage a low proliferation rate (ratio of 4.5 ± 1.1) was observed; however, the proliferation rate was greater than at the early stage of regression (Fig. 1c). Finally, at the advanced stage of regression, a new peak in proliferation was noted which corresponded to proliferating macrophages (Figs 1d and 2b). Corpora lutea that were at the final stages of regression were rich in arterioles, but lacked proliferating cells.

Leucocyte distribution

The different densities of Ki-67 positive cells during the oestrous cycle correlated with changes in leucocyte populations. The major CD18-positive population at the early stage of development represented eosinophils (ratio of 47.4 ± 13.1 , $n = 3$), as determined by the bilobulated nucleus in haematoxylin and eosin stained sections. After a marked decrease in the number of eosinophils, a few

CD18-positive leucocytes were detected at the stages of secretion and early regression. CD18-positive cells densely populated the corpora lutea at the late stage of regression. There was a moderate number of macrophages (CD14-positive) in corpora lutea at the secretory stage, very few at the early stage of regression and a large number at the late stage of regression. There was a moderate distribution of T-lymphocytes (CD2-positive) until the advanced stage of secretion and there was a considerable increase in number between early and advanced regression. Approximately 84% of all CD2-positive cells were characterized as CD3-positive T-lymphocytes. The proportion of CD8-positive T-suppressor/cytotoxic cells was 4.7 fold higher than that of CD4-positive T-helper cells.

Proliferating leucocytes were detected with the double-labelling technique (Fig. 2, Table 2). In corpora lutea at the stages of early development and early secretion, less than 1% of all proliferating cells were CD18-positive leucocytes. At the advanced secretory and advanced regression stages of the corpus luteum, approximately 20% (ratio of 0.11 ± 0.07) and 71% (ratio of 2.39 ± 0.44) of proliferating cells were CD18-positive leucocytes, respectively.

Throughout the secretory stage there were only a

Fig. 2. Percentage of proliferating leucocytes (CD18⁺:Ki-67⁺ cells) in relation to the total number of proliferating cells (Ki-67⁺ cells) at the different stages of the bovine oestrous cycle. Data are shown as box plots. * $P < 0.001$ compared with the preceding stage.

few proliferating leucocytes among the CD2- and CD14-positive leucocytes (0.8% of CD14-positive macrophages (Fig. 3a) and 1.5% of CD2-positive lymphocytes). At the advanced secretory stage of the corpus luteum, 78% of the proliferating CD2- and CD14-positive leucocytes were CD2-positive T-lymphocytes (Fig. 3c), indicating an increase of CD2-positive leucocytes over CD14-positive leucocytes. The onset of the increase in the number of leucocytes (Tab.1) at the advanced stage of regression coincided with an increase in the number of proliferating CD14-positive macrophages (ratio of 1.62 ± 0.2 , Fig. 3b). At this stage, the proliferating CD2- and CD14-positive leucocytes consisted of 95% CD14-positive macrophages. However, unlike the macrophages, no increase in proliferating CD2-positive lymphocytes was apparent at the advanced stage of regression (Fig. 3c,d; 0.9% proliferation rate).

At the early stage of regression a large number of leucocytes infiltrated the septal areas of corpora lutea, whereas fewer leucocytes appeared among the luteal cells and capillaries (Fig. 4a,b). The disruption of the capillary bed had been initiated (Fig. 4c) as indicated by the decrease in the density of the capillaries and the appearance of arterioles (Fig. 4d). At the advanced stage of regression when the corpus luteum was crowded with leucocytes, no leucocyte infiltration via blood vessels in the septa was detected (Fig. 4e,f), the capillary bed was no longer visible (Fig. 4g) and many arterioles were present (Fig. 4h). Macrophages were not present in the arteriole walls.

Comparable with regressing corpora lutea, follicles undergoing atresia were invaded by CD18-positive leucocytes (Fig. 5a). Proliferation was observed in the area of the former granulosa and thecal layers and more activity was observed in the centre than at the periphery. Some of the proliferating cells were leucocytes (Fig. 5b). Proliferating leucocytes were not observed in a hyalinized atretic follicle (not shown).

Discussion

The double-immunolabelling technique used in the present study has several advantages. Firstly, the technique is easy to carry out because the secondary biotinylated antibodies, the amplification of the antibodies using avidin-biotin-peroxidase complex and the histochemical peroxidase reaction (apart from the reaction substrate) are similar for the detection of the first (leucocyte adhesion antigen) and the second (Ki-67-nuclear) antigen. Secondly the cell surface membrane and the nucleus represent different cellular compartments, thus interference in staining for the first or second antigen is avoided. Furthermore, the two antigens are located distinctly with two specific monoclonal antibodies that both originated in mice. This aspect means that unwanted binding, such as the primary antibody of the second detection step with residual free binding sites of the secondary antibody from the first step (Negoesu *et al.*, 1994), is negligible. Finally, the DAB substrate reaction by peroxidase activity renders a water non-soluble and heat-stable brown product that represents the detection complex of the first antibody, which includes the biotin-labelled secondary antibody and the HRP-

Table 2. Ratio of positive Ki-67 stained cells:total number of cells (% mean $\pm$ SEM) in bovine corpora lutea at different stages of the oestrous cycle

Antigen	Secretion		Regression	
	Early	Advanced	Early	Advanced
CD18	0.09 ± 0.02 (9)	0.17 ± 0.03 (6)	0.11 ± 0.07 (12)	2.39 ± 0.44 (9)*
CD14	0.02 ± 0.01 (9)	0.05 ± 0.01 (7)	0.02 ± 0.00 (10)	1.62 ± 0.20 (9)*
CD2	0.01 ± 0.00 (8)	0.06 ± 0.02 (6)	0.07 ± 0.02 (11)	0.07 ± 0.01 (10)

Number of corpora lutea in parentheses.

* $P < 0.001$ compared with the preceding stage.

Fig. 3. Proliferating leucocytes in the bovine corpus luteum as revealed by double-immunolabelling. The antibody against the nuclear Ki-67 reveals the red nuclei of proliferating leucocytes, which are simultaneously detected by the brown staining response of leucocyte surface molecules. (a) Proliferating macrophages (arrows) at the early stage of secretion and (b) at the advanced stage of regression. Proliferating CD2- (c) and CD8-positive (d) lymphocytes (arrows). Scale bars represent 10 μ m.

labelled avidin–biotin complex (shielding effect by Sternberger and Joseph, 1979). The DAB product enables the microwave retrieval procedure to be used for the second antigen (Ki-67 nuclear antigen), which is subsequently verified by the substrate reaction.

Three peaks of cell proliferation are apparent throughout the oestrous cycle of the bovine ovary: (i) in the dominant follicle; (ii) in the corpus luteum at the stages of advanced development and early secretion, and (iii) at the advanced stage of regression. Each peak appears to support the development of a particular type of cell, that is, granulosa and thecal cells, endothelial cells and smooth muscle cells and, finally, macrophages. The large number of proliferating endocrine cells in dominant follicles has been described by Hirshfeld, (1991). Proliferating endothelial cells in the course of angiogenesis have been recorded for the corpus luteum at the stages of development and early secretion (Jablonka-Shariff, 1993; Zheng et al., 1994; Christenson and Stouffer, 1996) and these findings were confirmed with the double-labelling technique for FVIIIr antigen and the Ki-67 antigen. Proliferating macrophages have not been observed in the corpus luteum at the advanced stage of regression. The small number of proliferating cells observed at the early stages of development of corpora lutea, probably represents a resumption in cell

proliferation, which is reduced to zero in the preovulatory follicle (Spaniel-Borowski *et al.*, 1981).

In the present study between the stages of development and regression T-lymphocytes increased in number, and suppressor/cytotoxic (CD8-positive) lymphocytes were dominant, as also observed by Penny *et al.* (1999). As a result of a decrease in endothelial cell proliferation in the advanced secretory stage, there is a relative increase in proliferating T-lymphocytes (78%) in relation to the pool of proliferating cells. However, in relation to the total number of lymphocytes, only about 3% of proliferating CD2-positive lymphocytes were observed throughout the oestrous cycle. This value may represent a basic influx of lymphoblasts from the blood.

Regressing corpora lutea are densely populated by macrophages, which phagocytose luteal cell debris (Paavola, 1979). The luteal macrophages are attracted by the MCP-1 protein, a chemokine used for the recruitment of macrophages, which is upregulated in regressing corpora lutea (Townson *et al.*, 1996; Bowen *et al.*, 1999). In the present study it was established that at the early stage of regression, CD18-positive leucocytes preferentially populate the septa of corpora lutea, whereas at the advanced stage of regression, numerous leucocytes were observed among thick-walled arterioles. The leucocytes were identified as

Fig. 4. Leucocyte distribution in the regressing bovine corpus luteum. Single labelling for CD18 (a,e) and CD2 (b,f) on leucocytes, for FVIIIr antigen on endothelial cells (c,g) and for ASM-1 on smooth muscle cells (d,h). CD18-positive leucocytes (a) and CD2-positive lymphocytes (b) are preferentially found in the septa at the early stage of regression (a–d). At this stage, capillaries begin to disappear (c), and arterioles (arrows) are developing (d). At the advanced stage of regression (e–h) when the luteal tissue is crowded with CD18-positive leucocytes, septa contain a few CD18- (e) and CD2-positive leucocytes (f). CD2- and CD18-positive cells are not observed in the vessel wall. At this stage the capillary is no longer visible (g) and numerous thick-walled arterioles have developed (h). Scale bars represent 50 µm.

Fig. 5. A regressing bovine antral follicle heavily infiltrated by CD18-positive cells. (a) Proliferating cells (red) are apparent in the former granulosa and thecal layers. (b) A proliferating macrophage (arrow) is noted in the thecal layer. Double-immunolabelling for the Ki-67 antigen and the CD18 antigen (a) or the CD14 antigen (b). Scale bars represent (a) 50 μ m and (b) 10 μ m.

macrophages, and in consideration of the macrophage-dependent proliferation at the advanced stage of regression, the macrophages in regressing corpora lutea appear to have two sources of origin: (i) the septal blood vessels through which monocytes migrated from the blood into corpora lutea at the early stage of regression; and (ii) the emigrating monocytes which proliferate under suitable haematopoietic surroundings, such as the local production of macrophage colony stimulating factor.

Under normal steady-state conditions approximately 5% of monocytes divide once or twice after they arrive at their target organ (van Furth, 1988). Therefore it is assumed that more than 90% of tissue macrophages are renewed by an influx of monocytes. Under acute inflammation, a local increase in the number of macrophages is due to an influx of monocytes and to their local proliferation from day 2 onwards (van Furth, 1988). The kinetics of macrophages reported under pathological conditions appear to play a role in the physiological involution of the corpus luteum, which may be considered as an inflammatory-like condition and should not be compared with the proliferation of resident macrophages in the developing rat testis (Schlatt *et al.*, 1999).

In summary, a new double-immunolabelling technique for microwave-sensitive and non-sensitive antigen was used to demonstrate a steady-state number of proliferating leucocytes in bovine corpora lutea throughout the oestrous cycle. In contrast, an increase in proliferating macrophages occurs in regressing corpora lutea. This increase is initially due to an influx of monocytes (early regression), and subsequently by local proliferation (advanced regression). The local proliferation of macrophages appears to compensate for post-capillary venules which, when present, are responsible for leucocyte recruitment.

This study was financially supported by the Centre of Clinical Research (IZKF, project B6) at the University of Leipzig.

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Received 12 May 2000.

Accepted 25 August 2000.