Difference in localization of eosinophils and mast cells in the bovine ovary

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The bovine ovary contains a considerable number of leucocytes which can be located with an antibody against the CD18 molecule. In the present study, subtyping and cell counting were carried out on histological sections stained with Sirius red for eosinophils and with toluidine blue for mast cells. The CD18⁺ cells were identified immunohistologically. Eosinophils and mast cells contributed considerably to the CD18⁺ pool. The number of eosinophils in the corpus luteum increased rapidly in early development to approximately 90% of the CD18⁺ cells, and decreased to 30% during secretion and to 10% during regression. Mast cells were not detectable in the follicles, the corpus luteum and the periphery of the cortex, but were observed in the interstitial cortical stroma and the medulla. The number of mast cells in these regions, which corresponded to 60–76% of the CD18⁺ cells, did not change significantly throughout the oestrous cycle. It is concluded that eosinophils are selectively recruited at the periovulatory period and that mast cells are unevenly distributed.

Introduction

Leucocytes invade specific regions of the mammalian ovary at precise times of the oestrous cycle and may be involved in regulating the life cycle of follicles and corpora lutea (Pate, 1995; Vinatier *et al.*, 1995; Norman and Brännström, 1996). Preferentially segmented leucocytes are observed in preovulatory follicles. In rats and humans, these cells are identified as neutrophils by immunohistology (Brännström *et al.*, 1993, 1994a,b; Best *et al.*, 1996), whereas in sheep and pigs, eosinophils have been observed in Giemsa-stained sections and electron microscopy (Cavender and Murdoch, 1988; Murdoch and Steadman, 1991; Standaert *et al.*, 1991). In cows, the presence of eosinophils in preovulatory follicles was described 40 years ago (Cupps *et al.*, 1959).

Eosinophils are known to be involved in parasitic infestation, asthma and allergic conditions (Bignold, 1995). The physiological significance of these cells is still unclear. Eosinophils are an important source of numerous growth factors, cytokines and chemokines associated with crystalloid secretory granules (Walsh, 1997). They are rapidly mobilized and may release inflammatory mediators that control leucocyte recruitment, degradation of the basal membrane and angiogenesis. Since these events play a role during follicular rupture, ovulation has been described as a 'physiological inflammation', and eosinophils are considered to contribute to the inflammatory nature of this event (Espey, 1994). Eosinophils are also thought to participate in luteolysis due to their occurrence in regressing corpora lutea

of pigs (Standaert *et al.*, 1991) and of sheep after treatment with PGF_{2α} (Murdoch, 1987; Murdoch *et al.*, 1988). If this is the case, eosinophils should be detected in regressing corpora lutea of cows, a species with a long oestrous cycle which is comparable to that in pigs and sheep; such a study has not been carried out.

Mast cells are widely distributed throughout many different tissues and organs and have been located in the ovaries of mice, rats, hamsters and cows (Jones *et al.*, 1980; Nakamura *et al.*, 1987; Krishna *et al.*, 1989; Krishna and Jaiswal, 1994). In species with a well developed interstitial cortical stroma and a long oestrous cycle such as cows, mast cells reside in the cortex and in the medulla (Nakamura *et al.*, 1987). In species with a short oestrous cycle, such as rats and hamsters, mast cells are found only in the medulla (Jones *et al.*, 1980; Krishna *et al.*, 1989). Mast cells appear to degranulate and to release histamine under the influence of the LH peak as described for hamsters (Krishna and Terranova, 1985). Similar to eosinophils, mast cells produce a number of inflammatory mediators which are composed and activated in such way that leucocytes are recruited (Kubes and Granger, 1996; McNeil, 1996). In view of the similar functions in leucocyte recruitment, the location of both eosinophils and mast cells in the bovine ovary is worthy of investigation.

In the bovine ovary, the accumulation and number of leucocytes depends upon the type of follicle and the different stages of the corpora lutea, as shown by immunolocalization of CD18⁺ cells (Spanel-Borowski *et al.*, 1997). The distribution of eosinophils and mast cells has not been studied in depth. Therefore, the aim of this study was to investigate leucocyte subtypes in the bovine ovary.

Materials and Methods

A total of 45 pairs of bovine ovaries were collected at a local abattoir immediately after the animals were killed and the ovaries were transported to the laboratory in 0.05 mol PBS l⁻¹. The ovaries were classified into the different stages of the oestrous cycle from appearance with the naked eye (Ireland *et al.*, 1980) and by histological and biochemical findings (Ricken *et al.*, 1995). At the follicular stage, ovaries ($n = 6$) contained dominant follicles between 1 and 2 cm in diameter and showed regressive corpora lutea of the preceding oestrous cycle. This was considered to indicate that the selected ovaries were from cyclic cows. The follicular wall of the dominant follicles consisted of folds, as verified subsequently by histology. During early development ($n = 6$), the corpus luteum was red and up to 0.5 mm in diameter. Owing to convolutions of the luteinizing thecal layer, prominent septa were found. During the stage of late development ($n = 6$), the capillary bed (detected by a factor VIIIr positive immunoresponse as reported by Ricken *et al.* (1995)), was seen clearly. In the early secretory stage of ($n = 7$), simple connective tissue septa were observed in the corpora lutea and the capillary bed was at the highest density. A decrease in the density of the capillaries was apparent in the late secretory stage ($n = 7$). The absence of capillaries and the presence of arterioles were typical during regression (early stage $n = 7$; late stage $n = 6$).

Fixation and staining

The ovaries were cut into three pieces containing either antral follicles or corpora lutea and each with a portion of the interstitial cortical stroma and the medulla. One piece was fixed in liquid nitrogen and stored at -20°C until required. The other two pieces were fixed in 4% (v/v) buffered formaldehyde with or without 5% (w/v) lead acetate for approximately 12 h and embedded in paraffin wax. Sections (7 µm) were mounted on slides precoated with the paper glue (Cementit®; Merz and Benteli AG, Niederwangen). The eosinophils were stained specifically in the formaldehyde-fixed tissue as reported by Bogomoletz (1980). Sirius red (500 mg; Bayer AB, Leverkusen) was dissolved in 45 ml aqua bidest, 50 ml absolute ethanol and 1 ml of 1% (w/v) NaOH, and approximately 4 ml of 20% (w/v) NaCl was added until slight precipitation had occurred. The solution was filtered on the following day and kept stable for 2 months at room temperature. Paraffin wax was removed from the sections and they were stained with haematoxylin, differentiated in running tap water, treated with 70% ethanol and stained with Sirius red solution at room temperature for 1 h. After complete dehydration with increasing concentrations of ethanol, the sections were mounted in Canada balsam. Mast cells were detected in tissues fixed in 4% (v/v) buffered formaldehyde together with 5% (w/v) lead acetate. The cells were stained with 1% (w/v) toluidine solution for 20 min, dehydrated and mounted as described earlier.

Indirect immunohistology

Integrins of the β_2 type are known to be expressed

selectively by mononuclear and segmented leucocytes (Bosman, 1993). Type β_2 integrins were detected in 10 µm cryostat sections using a monoclonal antibody against the CD18 chain of bovine β_2 integrins (VMRD, Inc., Pullman, WA). The avidin-biotin-horseradish peroxidase (HRP) complex technique was used (Vectostain® Elite ABC kit from Vector Laboratories, supplied by Alexis, Grünberg) and 0.05 mol PBS l⁻¹ and 0.05 mol Tris-buffered saline l⁻¹ (TBS, pH 7.6) for antibody dilution and buffer rinses. The rinses were carried out between steps (2) and (4), using TBS with 0.125% (v/v) Tween (Biorad, Munich; 2 × 5 min) followed by TBS without Tween. (1) The slides were placed in a cuvette containing 0.3% (v/v) H₂O₂ in absolute methanol for 30 min to eliminate endogenous peroxidase activity and to reduce the background staining. The slides were rinsed in TBS (3 × 5 min) and treated with 2% normal goat serum for 30 min. (2) Without a buffer rinse, the slides were incubated with the primary antibody diluted 1:100 in PBS with 0.25% bovine serum albumin (A-4378, Sigma, Deisenhofen). Incubation was continued overnight in a humidified chamber at 4°C. The controls were run with a non-specific mouse immunoglobulin of the IgG1 subtype (DAKO, Hambourg), that is, the same subtype and protein concentration used for the primary antibody. (3) The slides were placed in a cuvette containing the second goat anti-mouse antibody (BA-9200, Vector Laboratories) diluted 1:200 in TBS with 1.5% normal goat serum for 20 min at room temperature. (4) The sections were incubated with the avidin-biotin-HRP complex (40 drops of the kit solution in 50 ml TBS) for 30 min. (5) 3-Amino-9-ethylcarbazole (29 mg; AEC, Sigma) was dissolved in 100 ml of 0.1 mol sodium acetate buffer l⁻¹ at pH 5.2 and supplemented with 0.03% (v/v) H₂O₂ and 7.1 ml N,N-dimethylformamide (Sigma). The sections were treated with the AEC solution for 10 min at 37°C. (6) After rinsing with distilled water and counterstaining with Ehrlich's haematoxylin, the sections were embedded in a water-soluble medium (Crystal Mount®, Natutec GmbH, Frankfurt).

Leucocyte counting

An observer examined the same section on two occasions with an interval of 1 day. Since no significant differences in the number of CD18⁺ cells were found, the number of eosinophils, mast cells and CD18⁺ leucocytes in the stained sections was determined once. In the dominant follicles, the layers of granulosa or theca cells were considered separately. In the developing corpora lutea, areas with luteinizing theca and granulosa cells were screened, whereas in subsequent stages, septa and luteal cells were considered. Within the interstitial cortical stroma and the medulla, the number of leucocytes in ten areas, each of 0.06 mm², was counted, using the KS 100 Version 2.00 image analysis system (Kontron Elektronik, Eching, Munich). The mean values ± SEM were calculated for samples at each stage of the oestrous cycle as well as percentages of eosinophils and mast cells in respect of the total number of CD18⁺ leucocytes. Statistically significant differences among all group means were assessed by the non-parametric Kruskal-Wallis-test, followed by the Mann-Whitney U test.

Results

Eosinophils with a bilobed nucleus and distinct red staining of the cytoplasm were only observed in the dominant follicles and corpora lutea. Compared with antral follicles with negligible numbers of eosinophils, the dominant follicles contained some eosinophils that had spread irregularly throughout the theca. After follicular rupture, that is at the early developmental stage of the corpus luteum, there was a greater increase in the number of eosinophils in the theca than in the granulosa layer (Fig. 1a,b). A large number of eosinophils had accumulated within and around dilated vessels. At the late stage of development, the number of eosinophils remained greater in the luteinizing theca layer than in the granulosa layer, although the overall number was less than at the early stage. By the secretory stage, the corpus luteum had developed to its final size, and it was not possible to determine whether the large and small luteal cells

were of granulosa or thecal origin. The number of eosinophils was small (Fig. 1c) and remained small during regression. The number of eosinophils decreased, while the number of CD18⁺ cells increased (Fig. 1c,d). There was a peak in the number of eosinophils in the early stage of the developing corpus luteum, which was statistically different from the numbers observed in the subsequent stages (Fig. 2).

Unlike eosinophils, mast cells were never found in the walls of intact antral or dominant follicles (Fig. 3a). Mast cells did not invade the hypertrophied thecal layer or the fibrotic body of regressed antral follicles (Fig. 3b), nor did they invade new or old corpora lutea. Mast cells were not observed in completely regressed corpora lutea, as detected by arterioles with a thick media. Mast cells were rarely seen in the marginal zone of the interstitial cortical stroma, in which the primordial follicles were located (Fig. 3c). Mast cells appeared to be evenly spread in the major portion of the interstitial cortical stroma and were found in the vicinity of

Fig. 1. Eosinophils in the bovine corpus luteum. (a) During early development, numerous eosinophils were observed in the former thecal layer (*), whereas there were fewer eosinophils between the granulosa cells during luteinization (arrow). (b) Eosinophils were associated with postcapillary venules (arrow). (c) During the secretory stage, eosinophils (arrows) were sparsely distributed and fewer in number than CD18⁺ leucocytes (d). (a–c) Sirius red staining; (d) indirect immunohistology. Scale bars represent (a) 100 μ m and (b–d) 50 μ m.

Table 1. Absolute number of CD18⁺ cells in 0.6 mm² areas of cortex and medulla and of dominant follicles and corpora lutea in the bovine ovary during different stages of the oestrous cycle

Compartment	Follicular stage <i>n</i> = 6	Stage of the corpus luteum					
		Development		Secretion		Regression	
		Early <i>n</i> = 6	Late <i>n</i> = 6	Early <i>n</i> = 7	Late <i>n</i> = 7	Early <i>n</i> = 7	Late <i>n</i> = 6
Stroma							
Cortex	99 ± 5	164 ± 21	162 ± 24	114 ± 25	174 ± 22	163 ± 18	147 ± 16
Medulla	170 ± 16	239 ± 30	202 ± 25	140 ± 24	238 ± 13	245 ± 23	236 ± 30
Follicle/corpus luteum							
Granulosa/luteal cells	0	145 ± 21	164 ± 23	100 ± 8	130 ± 9	189 ± 32	Crowded
Theca/luteal cells	73 ± 26	335 ± 47	184 ± 19	106 ± 10	136 ± 23	205 ± 24	Crowded

Values are means ± SEM.

Fig. 2. The number of eosinophils in the bovine corpus luteum increased during early development. Histological sections were stained with Sirius red and cells were counted by examining dominant follicles during the follicular phase and corpora lutea at different stages. Values are mean ± SEM. The numbers of eosinophils in the luteinizing granulosa layer (●) and the thecal layer (○) were significantly different up to the secretory stage ($P < 0.01$ to 0.05). ^a $P < 0.05$ and ^b $P < 0.005$ versus late development.

the theca externa of antral and dominant follicles. A large number of mast cells were also observed in the medulla, often aligned within the adventitia of the shunt-like arteries (Fig. 3d). A considerable number of these mast cells were CD18⁺ cells as revealed by comparing sections stained for mast cells with those stained for all leucocytes (Fig. 3e,f). When the absolute number of mast cells was counted within the cortex or the medulla, a greater number of mast cells were found in the medulla than in the cortex. No obvious difference was detected among the different stages of the oestrous cycle, although there appeared to be smaller numbers of mast cells during the late developmental and secretory stages than during regression (Fig. 4).

The CD18⁺ leucocytes were observed in the same regions as the eosinophils and mast cells. They were also observed in

other regions such as regressing or regressed antral follicles, and in old corpora lutea of different generations (data not shown). In the cortex and the medulla, the number of CD18⁺ cells appeared to remain constant throughout the oestrous cycle (Table 1). In the corpus luteum during early development and during regression, the number of CD18⁺ positive cells showed peak values. This difference was statistically significant in the region of the theca/luteal cells during early development ($P < 0.005$) compared with the other stages. CD18⁺ leucocytes were present in very small numbers in the granulosa layer at the follicular stage; the number was significantly different compared with the other stages.

In comparison with the CD18⁺ cells, the percentage of eosinophils changed markedly: 90% during early development, 54% during late development, approximately 30% during the secretory stage, and 10% during regression. The less marked changes in the number of CD18⁺ cells (Table 1) indicate the influx of another leucocyte subtype during the later stages. The percentage of mast cells in relation to CD18⁺ cells did not change significantly either in the cortex or the medulla. The percentage remained high throughout the oestrous cycle: 64%, 76% and 60% during development, secretion and regression, respectively. Hence, a considerable proportion of the CD18⁺ cells in the cortex and in the medulla represented mast cells.

Discussion

Eosinophils, mast cells and CD18⁺ cells were located in specific regions of the bovine ovary and the numbers of these cells at different stages of the oestrous cycle were determined. Simultaneous labelling of eosinophils or mast cells together with CD18⁺ cells was not possible with paraffin wax sections, which are excellent for staining eosinophils and mast cells, but inadequate for the immunohistological location of CD18⁺ cells. Simultaneous labelling of toluidine blue-stained mast cells and nerves has been achieved successfully with cryostat sections of the rat synovium after fixation with Bouin's solution (Hukkanen *et al.*, 1991). In the

Fig. 3. Mast cells in the bovine ovary. The mast cells are observed in the interstitial cortical stroma and close to the wall of (a) a dominant follicle with a granulosa layer thrown into folds and (b) a fibrotic body which represents the final stage of follicular atresia. (c) Mast cells were not detected in the periphery of the cortex, but were present in large numbers in the adjoining interstitial cortical stroma. (d) In the medulla, mast cells are aligned within the adventitia of the muscular blood vessels with a thick media. (e) Mast cells are distributed throughout the medulla. (f) Many CD18⁺ cells represent mast cells, as is apparent by comparing (e) with (f). (a–e) Toluidine blue staining; (f) indirect immunohistology. Scale bars represent (a,b,e and f) 100 μ m and (c,d) 50 μ m.

Fig. 4. The number of mast cells is greater in the medulla (○) than in the interstitial cortical stroma (●) of the bovine ovary. Histological sections were stained with toluidine blue and cells were counted at the follicular phase, and at the subsequent luteal stages. Values are mean $\pm$ SEM.

present study, this procedure presented difficulties in discerning toluidine blue stained mast cells in the CD18⁺ cell pool due to overlapping responses. The problem was not resolved with the double-labelling technique against CD18 and against chymase or tryptase, which are mast cell-specific enzymes. The antibody, only available against the human chymase, showed no crossreactivity in the bovine ovary, whereas the antibody against the human tryptase detected just two-thirds of the mast cells stained with toluidine blue. Therefore, formaldehyde fixed cryostat sections were used, which were cut into series and stained consecutively with (i) toluidine blue, (ii) against the CD18 molecule and (iii) with Sirius red. The outcome supported the results obtained with paraffin wax sections: the major portion of CD18⁺ cells in the medulla and the interstitial cortical stroma represented mast cells.

A small number of eosinophils were observed in the theca of antral and dominant follicles in contrast to other studies of preovulatory follicles of sheep and pigs (Cavender and Murdoch, 1988; Standaert *et al.*, 1991). These studies failed to detect a sudden increase in the number of eosinophils in the developing corpus luteum. This finding is in contrast to those of the present study in which the time-frame for the selective influx of eosinophils was restricted to only 1 or 2 days. The mechanism appears to be under the control of specific adhesion molecules such as very late activation antigen 4 (VLA-4), a β_1 integrin expressed on eosinophils, and the vascular cell adhesion molecule (VCAM-1), a member of the immunoglobulin superfamily that is upregulated on endothelial cells (Wardlaw *et al.*, 1994; Bochner and Schleimer, 1994). In addition, interleukin 3 and 5 promote the adhesion of eosinophils to microvascular endothelial cells (Walsh *et al.*, 1991) as well as RANTES (regulated on activation, normal T-cell expressed and secreted) and eotaxin, chemoattractants of the C-C-chemokine family (Okada *et al.*, 1997; Walsh, 1997). Experiments on RANTES and eotaxin expression in the

bovine ovary are being carried out with RT-PCR and *in situ* hybridization. The preliminary results indicate that ovarian macrophages are involved in the precisely timed recruitment of eosinophils (Aust *et al.*, 1999).

The signal for the recruitment of eosinophils and other leucocytes is probably related to the LH peak, inducing granulosa cells to suddenly release inflammatory mediators (Norman and Brännström, 1996). Slight alterations in the amount and composition of the mediators may be responsible for the variation in the number of eosinophils observed among the samples in the present study. The occurrence of eosinophils runs in parallel with the period of angiogenesis and luteinization, supporting the proposal that eosinophils may influence both events (Murdoch and Steadman, 1991). During the secretory stage, a small number of eosinophils was observed throughout the corpus luteum, which probably reflects cessation in the mechanism for recruiting eosinophils together with an increase in the size of the fully grown corpus luteum. A second eosinophil influx in the bovine corpus luteum at the onset of luteolysis was not observed in the present study, which is in contrast to reports for sheep and pigs (Cavender and Murdoch, 1988; Murdoch and Steadman, 1991; Standaert *et al.*, 1991). This discrepancy may be explained by species differences or differences in fixation, staining, and in pretreatment with or without prostaglandin.

In the present study, mast cells were not detected on the inside of the follicles and corpora lutea, and were present in high density in the medulla and the interstitial cortical stroma throughout the oestrous cycle. This uneven distribution was also observed in human ovaries (U. Heider and K. Spänel-Borowski, unpublished). Nakamura *et al.* (1987) counted these cells in the theca externa of dominant follicles and in the capsule of corpora lutea and did not observe mast cells in the interstitial cortical stroma of the bovine ovary. However, the theca externa of a dominant follicle consists of a few cell layers in a circular arrangement around the basal membrane in the absence of a distinct border towards the cortical stroma. Therefore, the theca externa may be confused with the cortical stroma. The capsule of a corpus luteum represents the interstitial cortical stroma because of the complete transition of the theca into the corpus luteum.

The function of intraovarian mast cells requires clarification. Currently, they are thought to be involved in the increased vascular permeability after degranulation and histamine release during the preovulatory period (Jones *et al.*, 1980; Krishna *et al.*, 1989). Degranulation is reflected by a decrease in mast cells in the bovine cortex surrounding the developing corpus luteum (Nakamura *et al.*, 1987). In the present study, a decrease in the number of mast cells was observed during the late stage of corpus luteum development, which was not statistically significant due to the limited amount of material at this stage. Mast cells tended to accumulate in the bovine ovary within the adventitia of thick muscular arteries, indicating that these cells may be influential in smooth muscle tone. It is known that mast cells form synaptic-like contacts with nerve fibres in the skin and gastrointestinal tract (McKay and Bienenstock, 1994; Botchkarev *et al.*, 1997). If these contacts are present in the ovary, membrane depolarization at the synaptic-like

contact is followed by release of cytokines and neuropeptides from mast cells. Their factors are able to bind to corresponding receptors of nerve fibres. Hence, mast cells, together with nerve fibres, may form a bidirectional interaction and be responsible for the local neuroimmune connection. Mediators of mast cells act on endothelial cells and eosinophils in the recruitment of eosinophils (Kubes and Granger 1996; Oskéritzian *et al.*, 1996). It is apparent from these observations that the intraovarian relationships among eosinophils, mast cells and nerves require further investigation.

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