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Role of Prolactin in the Regulation of Macrophages and in the Proliferative Activity of Vascular Cells in Newly Formed and Regressing Rat Corpora Lutea[1]

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ABSTRACT

The proliferative activity of vascular cells and the number of macrophages were studied in corpora lutea of cycling and pregnant rats after prolactin (PRL) administration or depletion with the dopaminergic agonist CB154.

Pregnant rats showed a higher proliferative activity of the vascular cells in newly formed corpora lutea than did cycling rats in metestrus. When cycling rats were treated with PRL, the proliferative activity was equivalent to that of pregnant rats. Treatment of pregnant rats with CB154 decreased the proliferative activity of vascular cells to the level in cycling rats. Otherwise, the proliferative activity was not modified in cycling rats after CB154 treatment. This indicates that the increase in the proliferative activity of vascular cells in the corpus luteum of pregnancy was due to the twice-daily PRL surges induced by mating.

Treatment of cycling rats with CB154 decreased the number of macrophages in both newly formed and regressing corpora lutea, whereas PRL treatment increased the number of macrophages in regressing corpora lutea. In pregnant rats, treatment with CB154 decreased the number of macrophages in both newly formed and regressing corpora lutea. These results suggest that both the preovulatory and the twice-daily PRL surges regulate the macrophage population in newly formed and regressing corpora lutea.

INTRODUCTION

The dual role of prolactin (PRL) as a luteotrophic and luteolytic hormone is well established [1–4]. In the rat, the cyclic preovulatory PRL surge and the twice-daily PRL surges induced by mating facilitate the development of newly formed corpora lutea while inducing regression of the corpora lutea of previous cycles [5–7].

In recent years, the potential relevance of immune cells in ovarian physiology has been widely considered [8, 9]. Macrophages are one of the most relevant immune cells in the ovary and are present in the corpus luteum of many species [10–14]. Most studies have related the presence of macrophages to the phagocytosis of dead cells in regressing corpora lutea [12–15]. Recently, the expression of monocyte chemoattractant protein 1 (MCP-1) has been reported in the corpus luteum of the rat [14], and treatment of hypophysectomized rats with PRL induces the expression of MCP-1 and the invasion of macrophages in the corpus luteum [15], suggesting that invasion of macrophages constitutes an important component of the luteolytic effect of PRL. Macrophage infiltration has also been found in porcine corpus luteum during prostaglandin $F_{2\alpha}$-induced luteolysis [13].

Stimulatory effects of macrophages in the ovary have also been reported. Macrophages enhance PRL-induced progesterone secretion from mature granulosa cells in rats [16] and mice [17]. Furthermore, macrophages are a source of mitogenic factors and paracrine regulators [18] and have been reported to stimulate the proliferative activity of granulosa cells in growing follicles [19, 20]. In the rat corpus luteum, macrophages are more abundant at early than at late pregnancy [21], which suggests a role for these cells during early growth of the corpus luteum.

During the first 2 days of pregnancy, the corpus luteum is equivalent to that of cycling rats. For instance, no differences in the size of steroidogenic cells or in progesterone secretion exist [22, 23]. However, the corpus luteum of pregnancy is exposed to the twice-daily PRL surges induced by mating, whereas the cyclic corpus luteum is not. At this early time, extensive proliferation of endothelial cells, related to neovascularization of the growing corpus luteum, takes place in both the cyclic corpus luteum and that of pregnancy [22, 24].

The objective of this study was to analyze the possible luteotrophic and luteolytic effects of the preovulatory and twice-daily PRL surges in cycling and early-pregnant rats, as well as the possible involvement of macrophages in these events.

MATERIALS AND METHODS

Animals and Treatments

Adult female Wistar rats were used. The estrous cycle was monitored by daily vaginal smears. Only rats showing at least two consecutive 4-day cycles were used. The animals were maintained under controlled light (14L:10D; lights-on at 0500 h) and temperature (21°C) and had free access to rat chow and tap water.

Ovine PRL (oPRL-18) was obtained from the NIADDK (Baltimore, MD). 2-Br-α-ergocryptine (CB154), a dopaminergic agonist that specifically inhibits PRL secretion, was purchased from Sandoz (Basel, Switzerland). 5-Bromodeoxyuridine (BrdU) was purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal antibodies against BrdU and macrophages (ED1) were purchased from Dako Diagnostica (Hamburg, Germany) and Serotec (Oxford, UK), respectively.

Experimental Design

Cycling rats. Twenty cycling rats were divided into groups as follows. Rats in one group were treated with CB154 (1 mg/rat at 1000 h on proestrus) to suppress the preovulatory PRL surge. Controls received the same volume of the vehicle (250 μl of 70% ethanol). Rats in the other group were treated with PRL (250 μg of oPRL at 1900 h on proestrus and at 1000 and 1900 h on estrus) to simulate the twice-daily PRL surges induced by mating.

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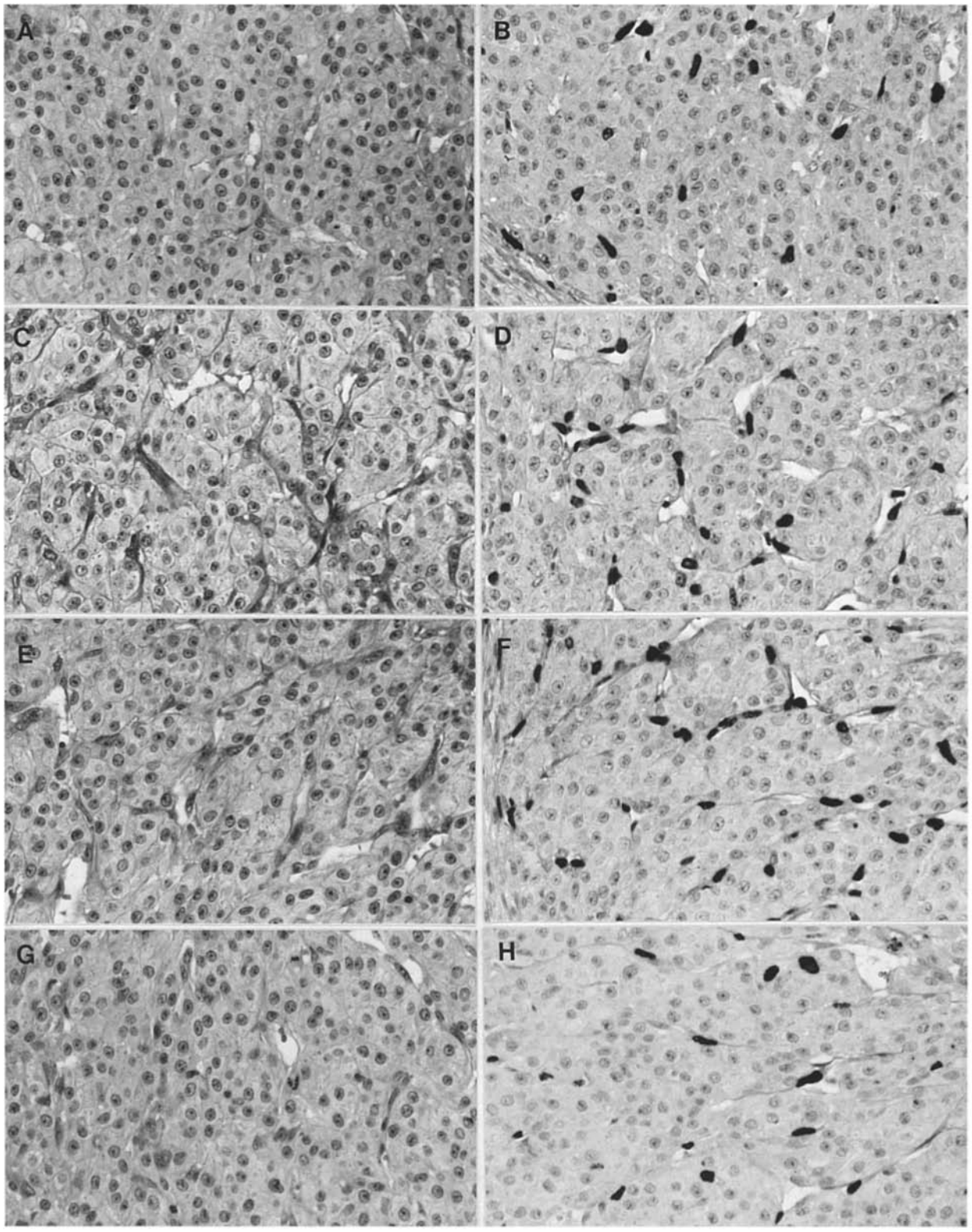

FIG. 1. Micrographs of newly formed corpora lutea in cycling (**A–D**) and pregnant (**E–H**) rats treated with vehicle (**A**, **B**, **E**, **F**), PRL (**C** ,**D**) or CB154-st (**G** ,**H**). See the legend of Figure 2 for details on the various treatment groups. Sections were stained with alcian blue-hematoxylin and eosin (**A**, **C**, **E**, **G**) to highlight the vascular pattern, evident in PRL-treated cycling (**C**) and in vehicle-treated pregnant (**E**) rats, or were immunostained (**B**, **D**, **F**, **H**) for DNA-incorporated BrdU to show proliferating cells. ×250.

Controls were injected with the same volume of the vehicle (0.03 M $NaHCO_3$, 0.15 M NaCl, 0.5% BSA, pH 9.5).

Pregnant rats. Cycling rats were left with males in the evening of proestrus, and on the following day the presence of spermatozoa in the vaginal smear was checked. The day when spermatozoa were found was considered Day 1 of pregnancy. Twenty rats were divided into the following groups: 1) rats injected with 1 mg of CB154 at 1900 h on proestrus and at 1000 and 1900 h on the first day of pregnancy (short CB154 treatment; CB154-st) in order to sup-

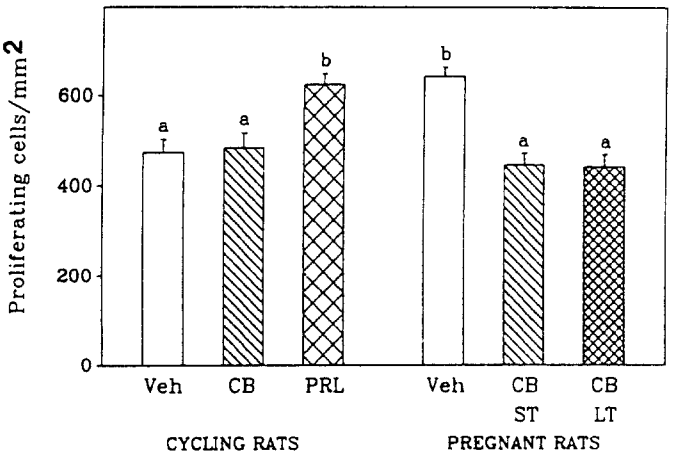

FIG. 2. Number of proliferating vascular cells in newly formed corpora lutea from cycling and pregnant rats on metestrus or Day 2 of pregnancy, respectively. Cycling rats were treated with vehicle (Veh), CB154 on proestrus (1000 h) (CB), or oPRL on proestrus (1900 h) and estrus (1000 and 1900 h) (PRL). Pregnant rats were treated with vehicle (Veh) or CB154 on proestrus (1900 h) and the first day of pregnancy (1000 and 1900 h) (short-CB154 treatment, CB-ST) or on proestrus (1000 and 1900 h) and the first day of pregnancy (1000 and 1900 h) (long-CB154 treatment, CB-LT). The data represent the mean ± SEM (n = 10 for vehicle-treated rats and n = 5 for the other groups). Different superscripts mean significant ($p < 0.01$) differences. ANOVA and Tukey's test.

press the twice-daily PRL surges induced by mating; 2) rats injected with 1 mg of CB154 at 1000 and 1900 h on proestrus and at 1000 and 1900 h on the first day of pregnancy (long CB154 treatment; CB154-lt) in order to suppress both the preovulatory and the twice-daily PRL surges. It has been reported that a dose of 1 mg of CB154 effectively inhibits PRL secretion in the rat [5]. Controls were injected with the same volume of the vehicle (250 μl of 70% ethanol) according to the same time schedule. All animals (five animals per group) were killed on metestrus (cycling rats) or on the second day of pregnancy (1100 h). One hour before death, the animals were injected i.p. with 50 mg/kg BW of BrdU in 1 M Tris-HCl buffer (pH 7.6). Trunk blood was collected and serum stored at −20°C until assayed.

Tissue Processing

The right ovaries were dissected, fixed in Bouin-Hollande's fluid (0.20 M picric acid, 0.12 M copper acetate, and 4% formaldehyde in water) for 24 h, and processed for paraffin embedding. The left ovaries were fixed in 4% paraformaldehyde in Sorensen buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 7.3) for 24 h and processed for paraffin embedding.

The ovaries were serially sectioned (4-μm-thick sections). Under a phase-contrast microscope, nonconsecutive sections showing newly formed or regressing corpora lutea were selected for immunohistochemistry. Detection of DNA-incorporated BrdU was carried out in Bouin-Hollande's fluid-fixed tissues, whereas macrophages were detected in paraformaldehyde-fixed tissues.

Immunohistochemistry

BrdU is a thymidine analogue that is incorporated into DNA during the S phase of the cell cycle. Details on the immunohistochemical demonstration of DNA-incorporated BrdU have been already published [24]. In this study, 4-μm-thick immunostained sections were counterstained with hematoxylin. Adjacent nonimmunostained sections were stained with alcian blue-hematoxylin-eosin for study of the morphological features of corpora lutea. Alcian blue staining was incorporated in order to highlight the vascular pattern.

Macrophages were detected by immunohistochemistry with the monoclonal antibody ED1. This antibody recognizes a lysosome-associated antigen in macrophages [25] and has been previously used to detect corpora lutea macrophages [14, 15]. Paraformaldehyde-fixed sections (4-μm-thick) were placed on poly-L-lysine-coated slides, and after dewaxing and inhibition of peroxidase with 2% hydrogen peroxide in methanol for 30 min, sections were rinsed in PBS, blocked with 10% normal rabbit serum for 2 h, and incubated overnight with mouse monoclonal ED1 antibody (diluted 1:400). The sections were processed according to the avidin-biotin-peroxidase complex method [26]. Briefly, sections were treated sequentially with rabbit anti-mouse IgG-biotin conjugate (Sigma, London, UK; 1:1000 1 h at room temperature) and avidin-biotin-peroxidase complex (Vector Labs., Burlingame, CA; 1 h at room temperature). Tissue-bound peroxidase was visualized by incubation in 0.03% diaminobenzidine-tetrahydrochloride (type IV; Sigma, St. Louis, MO), 0.01% hydrogen peroxide in 0.1 M Tris buffer (pH 7.6) for 1 min. Afterwards, sections were darkened in 1% copper sulfate for 5 min and counterstained with hematoxylin.

Negative controls for immunohistochemistry were run by incubating the sections with nonimmune serum instead of the primary antibody.

Cell Counting

Newly formed and regressing corpora lutea were easily distinguishable. New corpora lutea showed steroidogenic cells with small basophilic cytoplasm and exhibited scarce stromal cells. Regressing corpora lutea (i.e., those of the previous cycle) showed steroidogenic cells with large eosinophilic cytoplasm and round nuclei, and stromal cells were abundant.

The number of proliferating (BrdU labeled) cells was counted in newly formed corpora lutea. Five sections per corpus luteum from five corpora lutea per rat from five rats per group were systematically scored with the ×100 objective, and the number of BrdU-labeled cells and the area scored were recorded. The proliferative activity was scarce in regressing corpora lutea, and counts were not performed.

Macrophages were counted in newly formed and regressing corpora lutea, according to the same counting rule as in ED1-immunostained sections. The central fibrous core was excluded because it frequently contained blood remnants. All counts were performed by two independent observers, and the mean values were determined.

RIA

Serum concentrations of PRL were measured in duplicate in 10-μl samples using the double-antibody RIA method with the RIA kit supplied by NIH (Bethesda, MD) and according to the method described previously [27]. Rat PRL-I-6 was labeled with ^{125}I by the chloramine T method [28]. Concentrations were expressed in ng/ml of the reference preparation PRL-rat-RP-3. All samples were assayed in the same assay. The intraassay coefficient of variation was 8%, and the sensitivity 10 pg/tube.

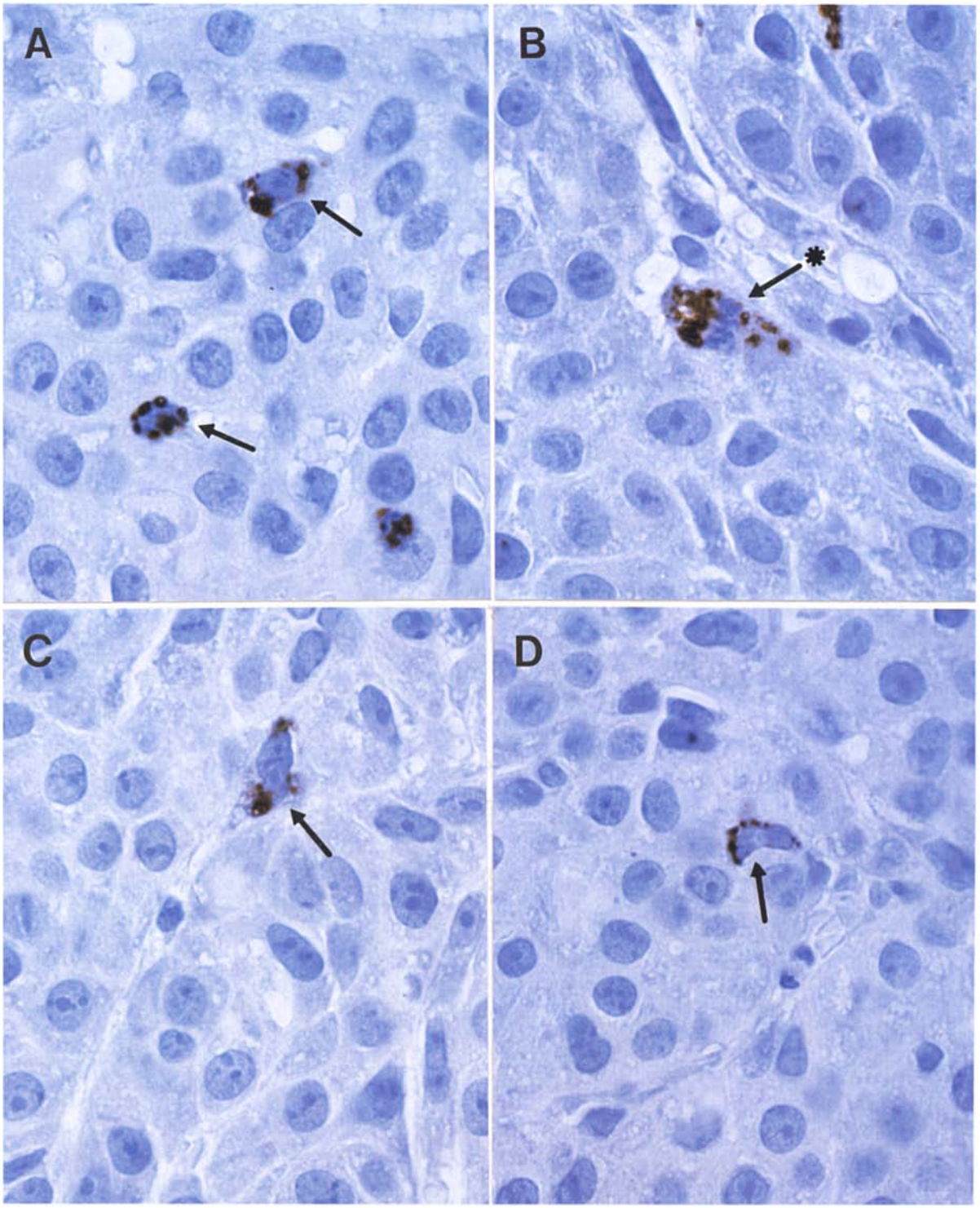

FIG. 3. Micrographs of newly formed corpora lutea from cycling (**A**, **B**) and pregnant (**C**, **D**) rats treated with vehicle (**A–C**) or CB154 from the morning of proestrus (CB154-lt) (**D**). Sections were immunostained with ED1 monoclonal antibody and counterstained with hematoxylin. Macrophages are indicated by arrows. Mitotic macrophages were occasionally found (asterisk). ×1000.

Statistical analyses were performed by ANOVA and Tukey's test for multiple comparisons among means. The 0.05 level was considered significant. Data are presented as the mean ± SEM for five animals per group. Since significant differences were not found between the different vehicle groups in cycling (70% ethanol vs. 0.03 M $NaHCO_3$, 0.15 M NaCl, 0.5% BSA) or pregnant (70% ethanol in short vs. long treatment) rats, results are shown together.

RESULTS

Proliferative Activity of Vascular Cells in Newly Formed Corpora Lutea

Newly formed corpora lutea (in both cycling and pregnant rats) showed not-fully luteinized steroidogenic cells with small basophilic cytoplasm. BrdU-labeled stromal cells, uniformly distributed through the section, were abun-

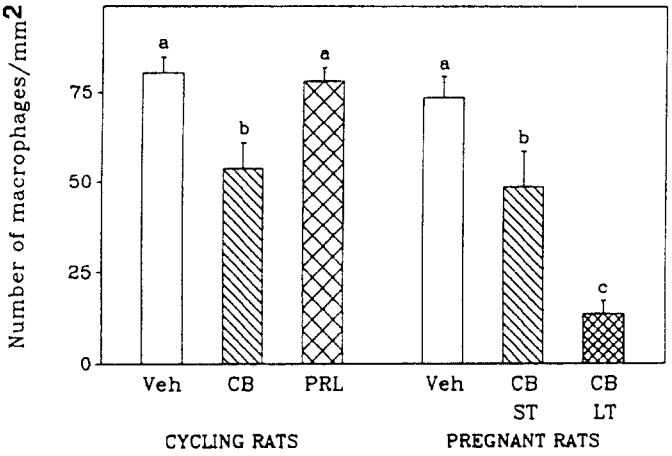

FIG. 4. Number of macrophages in newly formed corpora lutea from cycling and pregnant rats. See the legend of Figure 2 for details on treatments. The data represent the mean ± SEM (n = 10 for vehicle-treated rats and n = 5 for the other groups). Different superscripts mean significant ($p < 0.01$) differences. ANOVA and Tukey's test.

dant (Fig. 1). Most of these cells were lining blood or lymphatic vessels and corresponded to endothelial cells. In PRL-treated cycling and vehicle-treated pregnant rats, the vascular pattern of the luteal tissue was more marked and BrdU-labeled cells were more abundant (Fig. 1). The corpora lutea of CB154-treated pregnant rats showed morphological features similar to those of vehicle-treated cycling rats. Quantitative data are shown in Figure 2. Treatment of cycling rats with CB154 did not modify the number of proliferating cells. Otherwise, PRL treatment increased (by 34%) the number of proliferating cells. Vehicle-treated pregnant rats showed increased (by 35%) numbers of proliferating cells in relation to vehicle-treated cycling rats. Treatment with CB154 decreased the number of proliferating cells to the level in vehicle-treated cycling rats when treatment was started either on the morning (CB154-lt) or on the evening (CB154-st) of proestrus.

Macrophages in Newly Formed Corpora Lutea

Macrophages were scarce in newly formed corpora lutea in both cycling and pregnant rats. These cells showed irregular elongated nuclei and scarce cytoplasm (Fig. 3). Mitotic figures were occasionally observed (Fig. 3B). Quantitative data are shown in Figure 4. The number of macrophages was equivalent in cycling and pregnant rats and was not affected by PRL treatment in cycling rats. However, CB154 treatment induced a decrease in the number of macrophages in both cycling (a 32% fall) and pregnant rats. In pregnant rats, the decrease was higher when CB154 treatment was started on the morning (an 82% fall) than on the evening (a 33% fall) of proestrus.

Macrophages in Regressing Corpora Lutea

Regressing corpora lutea (i.e., those of the previous cycle) of vehicle-treated cycling rats showed the expected regressive changes, such as a high ratio of stromal to parenchymal cells, areas of vacuolization, and abundant cell debris and apoptotic bodies (Fig. 5). These regressive changes were accentuated in PRL-treated cycling and vehicle-treated pregnant rats. In contrast, regressive changes were minimal in CB154-treated rats (Fig. 5D).

In immunostained sections from vehicle-treated cycling rats, macrophages were abundant. These cells showed large nuclei and large vacuolated cytoplasm containing ingested material (Fig. 6). In CB154-treated cycling rats, macro-

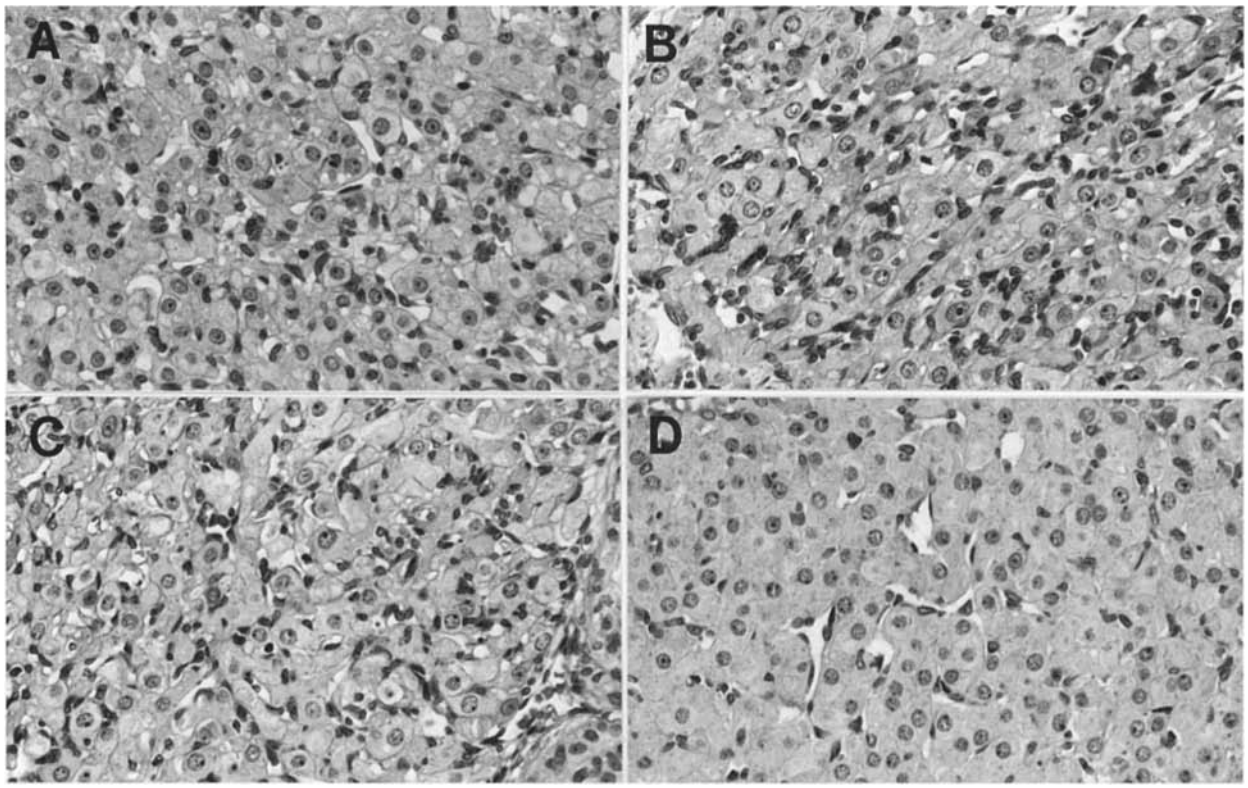

FIG. 5. Micrographs of regressing corpora lutea from cycling (**A**, **B**) and pregnant (**C**, **D**) rats treated with vehicle (**A**, **C**), oPRL (**B**), or CB154-lt (**D**). See the legend of Figure 2 for details on the various treatment groups. Alcian blue-hematoxylin and eosin. ×250.

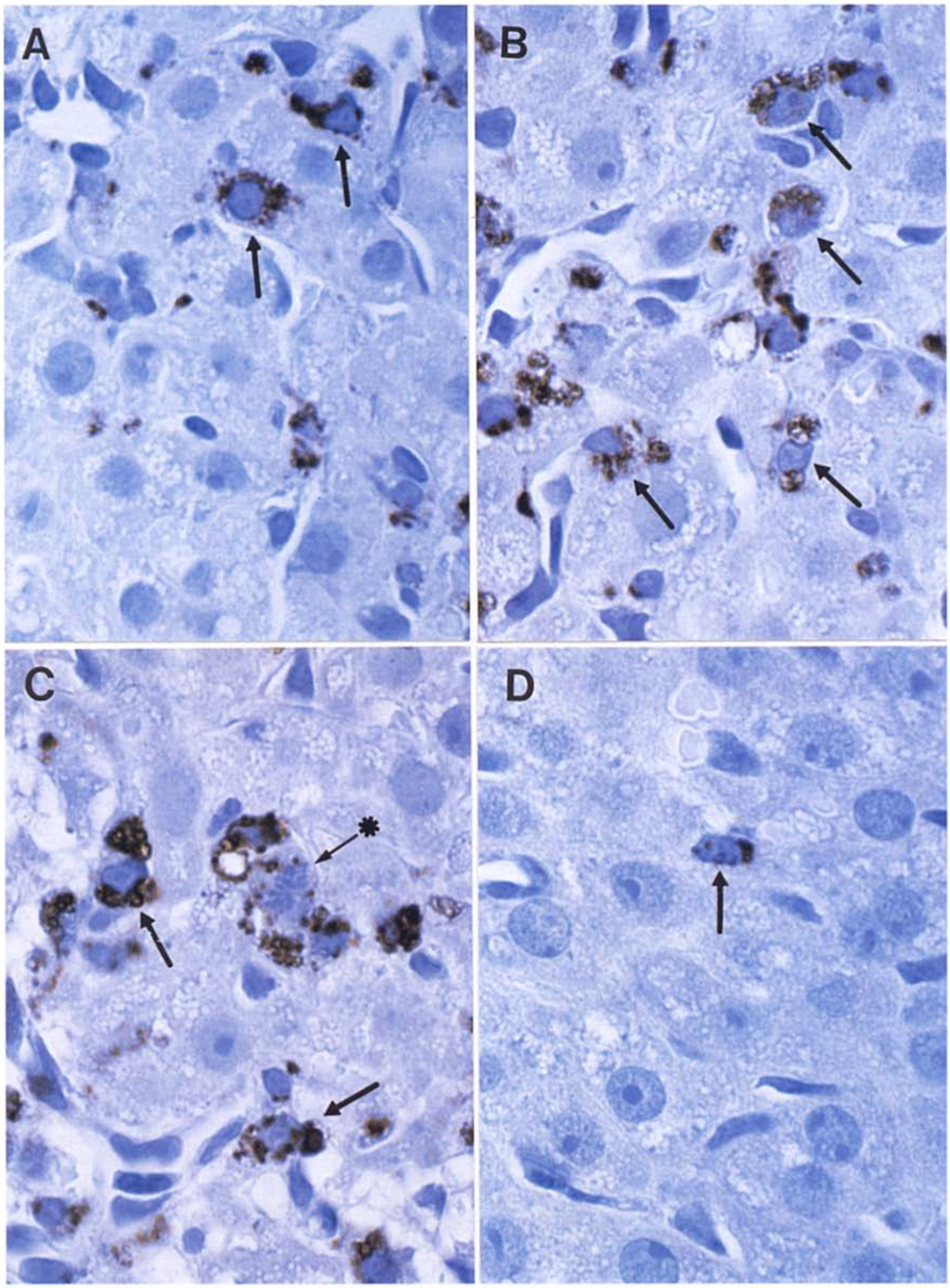

FIG. 6. Micrographs of regressing corpora lutea from cycling (**A**, **B**) and pregnant (**C**, **D**) rats treated with vehicle (**A**, **C**), oPRL (**B**), or CB154-lt (**D**). See the legend of Figure 2 for details on the various treatment groups. Sections were immunostained with ED1 monoclonal antibody and counterstained with hematoxylin. Macrophages are indicated by arrows. Mitotic macrophages (asterisk) can be observed. ×1000.

phages were scarce and showed morphological features similar to those present in newly formed corpora lutea, with an elongated nucleus and scanty cytoplasm. In PRL-treated cycling and vehicle-treated pregnant rats, high numbers of macrophages with large amounts of cytoplasm were present (Fig. 5, B and C). Mitotic figures were also observed (Fig. 6C). In contrast, in CB154-treated pregnant rats the number of macrophages was very low. These cells were extremely scarce in rats treated with CB154 from the morning of proestrus (Fig. 6D).

Quantitative data are shown in Figure 7. In cycling rats, the number of macrophages was decreased (a 70% fall)

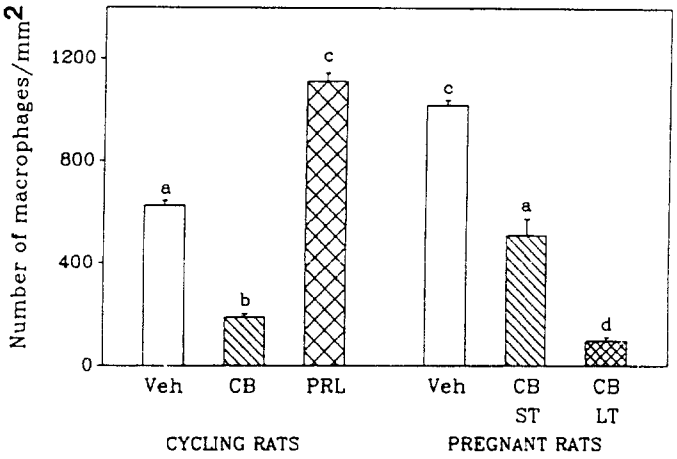

FIG. 7. Number of macrophages in regressing corpora lutea from cycling and pregnant rats. See the legend of Figure 2 for details on treatments. The data represent the mean ± SEM (n = 10 for vehicle-treated rats and n = 5 for the other groups). Different superscripts mean significant ($p < 0.01$) differences. ANOVA and Tukey's test.

after treatment with CB154 and was increased (by 75%) after treatment with PRL. In pregnant rats, the number of macrophages was higher than in cycling rats, and the numbers were decreased after treatment with CB154. The decrease was higher when treatment was started on the morning (a 90% fall) than on the evening (a 47% fall) of proestrus.

Data on basal serum PRL concentrations in cycling and pregnant rats at 1100 h on metestrus or the second day of pregnancy, after treatment with CB154, are shown in Table 1. Pregnant rats treated with several injections of CB154 (short and long treatments) showed significantly ($p < 0.05$) decreased basal PRL concentrations in relation to vehicle-injected rats.

TABLE 1. Serum PRL concentrations at 1100 h on metestrus (cyclic rats) or Day 2 of pregnancy (pregnant rats) after treatment with CB154 or vehicle (mean ± SEM for n = 5).

	Treatment	
Group	Vehicle	CB154
Cyclic	5.12 ± 2.45	2.68 ± 0.55
Pregnant (st)	12.36 ± 4.06	0.86 ± 0.06[a]
Pregnant (lt)	14.10 ± 3.70	0.90 ± 0.03[a]

[a] $p < 0.05$ vs. vehicle. ANOVA and Tukey's test. See *Materials and Methods* for details on CB154 treatment.

DISCUSSION

The effectiveness of PRL manipulation was evidenced by the well-known luteolytic effects of this hormone on regressing corpora lutea [1, 2, 5–7]. In this sense, luteolysis was enhanced in PRL-treated rats, whereas regressive changes were nearly absent in CB154-treated animals.

Proliferative Activity of Vascular Cells in Newly Formed Corpora Lutea

Pregnant rats showed a higher proliferative activity of the vascular luteal cells during the second day of pregnancy than the temporally equivalent cycling rats on metestrus. The bulk of proliferating cells corresponded to endothelial cells, as indicated by their location lining blood and lymphatic vessels, although a small number of proliferating cells corresponded to other cell types such as pericytes and macrophages. This agrees with previous studies indicating that the growth of the corpus luteum during the estrous cycle and early pregnancy is attributable mainly to the proliferative activity of endothelial cells related to neovascularization [29–31]. The data of the present study clearly indicated that the higher proliferative activity found in the corpus luteum of pregnant rats was dependent on the twice-daily PRL surges induced by mating, whereas it was not related to the preovulatory PRL surge. This was indicated by the presence of an equivalent proliferative activity of vascular luteal cells in pregnant and in cycling rats after PRL treatment that simulated the twice-daily PRL surges. Furthermore, whereas suppression of the preovulatory PRL surge did not decrease the proliferative activity in cycling rats, CB154 treatment decreased the proliferative activity in pregnant rats to the level in the cycling rats, irrespective of whether treatment was started on the evening of proestrus (suppressing only the twice-daily PRL surges) or on the morning of proestrus (suppressing both the preovulatory and the twice-daily PRL surges).

It is well established that the corpus luteum is dependent on PRL during the first week of pregnancy [3, 32]. Classical luteotrophic actions of PRL on the corpus luteum of early pregnancy include the maintenance of normal numbers of LH and estrogen receptors, inhibition of 20-α-hydroxysteroid-dehydrogenase, and an overall stimulation of protein synthesis [4, 32, 33]. This study provides evidence of an additional luteotrophic action of the twice-daily PRL surges through increases in the proliferative activity of endothelial cells, thus increasing the vascular supply to the growing corpus luteum. Whether this is a direct effect of PRL on endothelial cells or is mediated by other cell types remains to be determined.

The maintenance of the basal proliferative activity in both cycling and pregnant rats after CB154 treatment suggests that it is not dependent on PRL, and also that different mechanisms are involved in the control of basal and stimulated proliferative activity of the vascular cells.

Macrophages in Newly Formed Corpora Lutea

The number of macrophages in newly formed corpora lutea was not higher in pregnant rats, nor was it increased in cycling rats, after PRL treatment. This agrees with the lack of effect of PRL treatment on the number of macrophages in the corpus luteum of hypophysectomized immature rats [15]. However, suppression of PRL surges through treatment with CB154 significantly decreased the number of macrophages in both cycling and pregnant rats. The decrease in the number of macrophages was especially important in pregnant rats treated with CB154 from the morning of proestrus, which indicates that both the preovulatory and the twice-daily PRL surges regulate the macrophage population in newly formed corpora lutea. The reasons for the lack of an increase in the number of macrophages in PRL-treated or pregnant rats are not clear. The lack of response of macrophages to the twice-daily PRL surges in pregnant rats may constitute a safety mechanism to prevent luteolysis in the long-lived corpus luteum of pregnancy. Otherwise, differences in the number of macrophages between ethanol-treated cycling rats and pregnant rats injected with CB154 from the evening of proestrus (CB154-st), and between cycling rats injected with CB154 and pregnant rats injected with CB154 from the morning

of proestrus (CB154-lt), are probably due to suppressive effects of CB154 on basal PRL secretion.

The data of this study suggest that the basal proliferative activity of endothelial cells was not mediated by macrophages, since the number of proliferating cells was maintained in pregnant rats treated with CB154 from the morning of proestrus; these animals showed extremely low numbers of macrophages. However, these results do not exclude the possibility that macrophages could mediate the PRL-induced increase in the proliferative activity of endothelial cells found in pregnant rats or in cycling rats after PRL treatment. In this sense, the PRL-induced proliferative activity of endothelial cells was inhibited in CB154-treated pregnant rats, which showed decreased numbers of macrophages. Macrophages release several angiogenic growth factors [18], are activated by PRL in other systems [34], and are more abundant at early that at later stages of the development of the corpus luteum of pregnancy [21]. Additional studies should explore this possibility.

Macrophages in Regressing Corpora Lutea

Both the preovulatory and the twice-daily PRL surges appeared to be responsible for the increased numbers of macrophages in regressing corpora lutea. This was evidenced by the decrease in the number of macrophages in both cycling and pregnant rats after treatment with CB154. Furthermore, macrophages were extremely scarce in pregnant rats in which both the preovulatory and the twice-daily PRL surges were suppressed. Otherwise, PRL-treated cycling rats showed a number of macrophages that was similar to that in vehicle-treated pregnant rats. These results indicate that the preovulatory PRL surge induced the influx of macrophages into regressing corpora lutea and that this was amplified by the twice-daily PRL surges in pregnant rats. This agrees with previous results indicating the invasion of macrophages in regressing corpora lutea [13, 15] and with the role of PRL in these events [14, 15]. In agreement with the scarcity of macrophages in CB154-treated animals, the involution of the corpora lutea was inhibited, whereas it was accelerated in PRL-treated cycling rats and in pregnant rats that showed increased numbers of macrophages. Although it is difficult to discriminate between cause and effect, these results suggest a relevant role for macrophages in structural luteolysis. The presence of frequent mitotic figures in the macrophages indicates that the increase in the number of macrophages was due not only to the influx of monocytes/macrophages as previously proposed [15] but also to local expansion of the macrophage population through proliferation. It has been reported that PRL has a direct stimulatory effect on monocytes/macrophages [34, 35]. The differing morphological features of macrophages in vehicle-treated rats (large nucleus and cytoplasm) and CB154-treated rats (small nuclei and cytoplasm) suggest that PRL induced changes not only in the number of macrophages but also in their physiological status.

In summary, the twice-daily PRL surges induced by mating have a stimulatory effect on endothelial luteal cells by increasing their proliferative activity. Both the preovulatory and twice-daily PRL surges seem to be necessary for establishment of the macrophage population in the newly formed corpus luteum. Otherwise, the preovulatory PRL surge induced the influx of monocytes/macrophages into the regressing corpus luteum, and this effect was amplified by the twice-daily PRL surges induced by mating.

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