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Immunohistochemical localization of prolactin in functioning and regressing *corpus luteum* of pituitary autotransplanted rats

J. Martin de las Mulas[1], E. Aguilar[2] and J.E. Sánchez-Criado[2]

'Department of Pathology, Faculty of Medicine, University of Cordoba, Spain;
'Department of Physiology, Faculty of Medicine, University of Cordoba, Spain

Summary. In an attempt to shed light on the intimate mechanism by which prolactin (PRL) switches from supporting *corpus luteum* (CL) progesterone secretion (P) to promote structural regression of the CL, day 2 (metestrous) autopituitary transplanted (APTr) rats were used. In APTr rats the CL is under the only control of PRL since an almost complete absence of LH and FSH exist. The experimental group was given bromocriptine (CB-154: 0.4 mg/day) on days 12, 13 and 14 of the cycle and 0.25 ml of ethanol from day 15 to day 21. The control group was given CB-154 from day 12 to day 21. Rats were hemiovariectomized on day 12 to assess the morphological characteristics of the active CL. PRL and P were determined by RIA on days 12, 15 and 22. On day 12, both PRL and P levels were higher than 80 ng/ml (luteotrophic action of PRL). On day 15, due to treatment with CB-154, the levels of both hormones had fallen below 7 ng/ml (functional luteolysis). On day 22, PRL levels were again high (> 50 ng/ml) in the shortly CB-154-treated rats and low (< 5 ng/ml) in the controls; the P levels were lower than 5 ng/ml in both groups.

PRL-induced structural luteolysis in the experimental group (hyperprolactinemic) was assessed by the structural characteristics and by the CL weight loss on day 22 in comparison with that exhibited by control rats. The immunohistochemical staining of both endogenous and total PRL in the lutein cells showed that the internalization of PRL is not modified by the functional state of the CL, nevertheless the intracellular redistribution of the internalized hormone varied in relation with the PRL action on the CL (luteotrophic, day 12 vs luteolytic, day 22). These results seem to indicate that intracellular mechanisms rather than receptor content determine CL response to PRL.

Key words: *Corpus luteum* - Prolactin - Progesterone - Luteolysis

Offprintrequests to: Dr. Josb E. Sánchez-Criado, Department of Physiology, Faculty of Medicine, Avda. Menbndez Pidal s/n, Universidad de Cordoba, 14004 Cbrdoba, Spain

Introduction

Prolactin (PRL) is the major luteotrophic hormone in the rat. It acts in such a way during pseudopregnancy (Smith et al., 1975), early pregnancy (Morishige and Rothchild, 1974) and lactation (Ford and Yoshinaga, 1975). Similarly, PRL also acts supporting progesterone secretion by the *corpus luteum* (CL) in hypophysectomized rats treated with exogenous PRL shortly after ovulation (Malven, 1969), and in day 2 autopituitary transplanted rats (Everett, 1956). On the contrary, PRL acts luteolytically on the CL once it has ceased to secrete progesterone (structural or morphological luteolysis). PRL induces morphological luteolysis in the *corpora lutea* of cyclic rats (Billeter and Fluckiger, 1971), pregnant rats after parturition (Uilenbroeck et al., 1982) or hypophysectomized rats treated with exogenous or endogenous PRL by means of pituitary graft (Sloan and Malven, 1969), whenever functional luteolysis has occured.

The mechanism by which PRL action switches from supporting CL progesterone secretion to promoting CL regression is poorly understood. Well-controlled immunohistochemical (IHC) methods used to detect PRL in its target tissues have proved to be good tools for morphofunctional studies, as demonstrated in milk secretory cells from lactating rats (Nolin and Witorsch, 1976; Nolin and Bogdanove, 1980).

Thus, IHC localization and distribution of immunoreactive products to PRL in actively secreting and regressing lutein cells, using the autopituitary-grafted rat model, seems to be worth investigating.

Materials and methods

General

Adult virgin female Wistar rats raised in our laboratory were used. They were maintained four to a cage under controlled light (12L/12D, light on at 0700) and temperature (20° C), with free access to Sanders rat chow and tap water. Oestrous cycles were monitored by vaginal smears taken six days a week by saline lavage between 0900 and 1100 and only rats that had at least two consecutive four day cycles were selected for this experiment. Day of vaginal oestrous was designated as day 1 of the cycle.

Experimental procedures

All surgery was done under clean but not aseptic conditions using ether as anesthesia. Hypophysectomy was carried out through the parapharyngeal approach on day 2 of the cycle (metestrus). The recovered pituitary was grafted beneath the left kidney capsule as a source of endogenous PRL. On day 12 autopituitary-transplanted (APTr) rats were hemiovariectomized. Right ovary weight and number of CL were noted.

To induce functional luteolysis 0.4 mg of bromocriptine mesilate (Sandoz, Basle, Switzerland) was injected sc. on days 12, 13 and 14. From day 15, half of the rats received daily bromocriptine injections throughout the experiment (Control group). In the remaining rats, 0.25 ml of ethanol (70%) (the solvent of bromocriptine) was daily given (Experimental group).

On day 22 of the cycle, rats were sacrificed with an overdose of ether. At autopsy, left adrenal and left ovary weight and the number of CL were recorded. The pituitary *fossa* were checked for remnants of hypophysis and overall appearance of the pituitary graft. Ovaries and a piece of the kidney with the pituitary graft were kept for histological processing.

Blood samples

On days 12, 15 and 22, less than 1 ml of blood was taken by direct jugular venopuncture under light ether anesthesia. Blood was collected in centrifuge tubes and allowed to clot at 4° C.

Thereafter, the sera were separated and stored at -20° C until assayed for PRL and progesterone.

Radioimmunoassays

Serum PRL levels were measured as recommended in the directions supplied with the NIAMDD kits (Bethesda, Maryland, USA) and expressed in terms of the corresponding RP-3 reference preparation.

Serum progesterone levels were measured using specific antiserum (GDN-337) supplied by Dr. Gordon Niswender (Colorado State University, Fort Collins, USA), as described previously (Gibori et al., 1977).

To avoid interassay variations, all samples were measured in the same assay. The intra-assay coefficients of variations were 9 and 10% for PRL and progesterone, respectively. Results are expressed in ng/ml.

Excluded animals

Nine rats which did not show an appropriate pituitary transplant (size, colour, vascularization and normal histological appearance), completeness of hypophysectomy or a decrease in the adrenal weight higher than three times the standard deviation of that exhibited by the same age intact rats (14.9 + 2.5 vs 34.3 + 2.5 mg) were not included in the results. Four additional rats were also excluded because they showed either day 12 serum levels of PRL and progesterone below 70 ng/ml or day 15 or 22 serum levels of progesterone over 14 ng/ml, suspicious of absence of CL rescue by the transplant or absence of functional luteolysis by PRL deprivation, respectively.

Histological procedures. Immunohistochemistry

Ovaries and grafted pituitaries collected at autopsy were fixed in Bouin's solution for 15-18 h, washed in 70% ethanol, dehydrated and embedded in paraffin. Sections were cut serially at 5 μm, mounted on albumin coated slides and cleared of paraffin and rehydrated prior to use.

In order to control the correct functioning of the model, pituitary and ovarian sections from every rat were stained with Hematoxilin and Eosin; the viability of the graft (Fig. 1A) and signs of morphological regression between day 12 and day 22 ovaries were assessed.

The immunohistochemical method used to localize PRL is the peroxidase anti-peroxidase (PAP) method (Taylor, 1978). Grafted pituitary sections were used to control the technique before it was applied to day 12 and day 22 (experimental) ovarian sections. The working dilution of the anti-rat PRL serum (APRL), after testing a battery ranging from 1:100 to 1:1000, was selected on the basis of the greatest amount of immunoreactive PRL cells that could be detected with the lowest background staining, and established at 1:400 (Fig. 1B, C). After this, the specificity of the IHC detection of PRL was demonstrated by the total absence of staining when APRL was absorbed with highly purified rat PRL (ABS PRL; 8, 16 and 24 μg/ml of rat PRL incubated overnight at 4° C with 500 μl of APRL 1:400 each) (Fig. 1D). The possibility of unspecific binding of swine or rabbit sera, used in other steps of the technique, to rat tissues, was ruled out by the absence of staining after replacement of APRL with 0.05 M Tris.

Table 1. Serum levels of Prolactin and Progesterone on days 12, 15 and 22 in day 2 autopituitary-transplanted rats[1].

Groups	Prolactin (ng/ml)			Progesterone (ng/ml)		
	day 12	day 15	day 22	day 12	day 15	day 22
Control	80.5 ± 7.1 (11)	7.0 ± 1.1 (11)	5.2 ± 0.5 (6)	105.5 ± 6.9 (11)	5.2 ± 0.8 (11)	4.6 ± 0.8 (6)
Experimental			54.1 ± 4.0* (5)			7.0 ± 1.8 (5)

[1] Values are means ± S.E.M.; number of determinations are shown in parenthesis.

* $p < 0.001$ compared with control group (Sudent's t-test). Control rats were injected with bromocriptine from day 12 to day 21. Experimental rats were injected with bromocriptine from day 12 to day 14.

For the localization of endogenous PRL in lutein cells, three sections were placed in the same slide: one from the left and one from the right ovary of each rat, to allow for direct comparison of IHC procedures, and a third one from its grafted pituitary, run as a control; three slides, numbered I, II and III, and containing consecutive sections from one another, were used per rat. Slide I tissues were firstly layered with APRL, slide II tissues with ABS APRL and slide III tissues with 0.05 M Tris. Dilutions and incubation times of reagents used in the technique are as follows: normal swine serum (Sigma Chemical Company) 1:30, 30 min; rabbit APRL (NIAMDD, anti rat PRL S-8), 1:400, 48 h; swine anti-rabbit immunoglobulins (Dakopatts Corporation), 1:50, 30 min; and rabbit PAP complex (Dakopatts Corporation), 1:100, 30 min. All incubations were carried at room temperature in a moisturized environment. To form visible reaction products, tissues were reacted with 3,3'-diaminobenzidine (Sigma Chemical Company), 0.75 mg/ml in 0.05 M Tris containing 0.003% hydrogen peroxide, for about 5 min. 0.05 Tris was used as washing agent, as well as the diluting agent of all sera used.

In some cases, a fourth additonal slide (number IV) was used per rat, in which luteal tissues were incubated for 48 h at room temperature with rat PRL (NIAMDD, PRL I-5, 1 µg/ml) before applying APRL. This allowed detection of both, endogenous and total, endogenous plus exogenous PRL in direct adjacent serial sections.

Verification of structural luteolysis

Evaluation of structural luteolysis was calculated by average weight loss of the left ovary (day 22) in relation to the weight of the right ovary (day 12), and by the histological characteristics of the CL. In pituitary-grafted rats, a strong correlation between ovary and CL weight exists, and there are no differences between right and left ovaries (Sloan and Malven, 1969; Sanchez-Criado et al., 1984).

Statistical analysis

Statistical comparison of PRL and progesterone levels and average weight loss was made by Students' t-test.

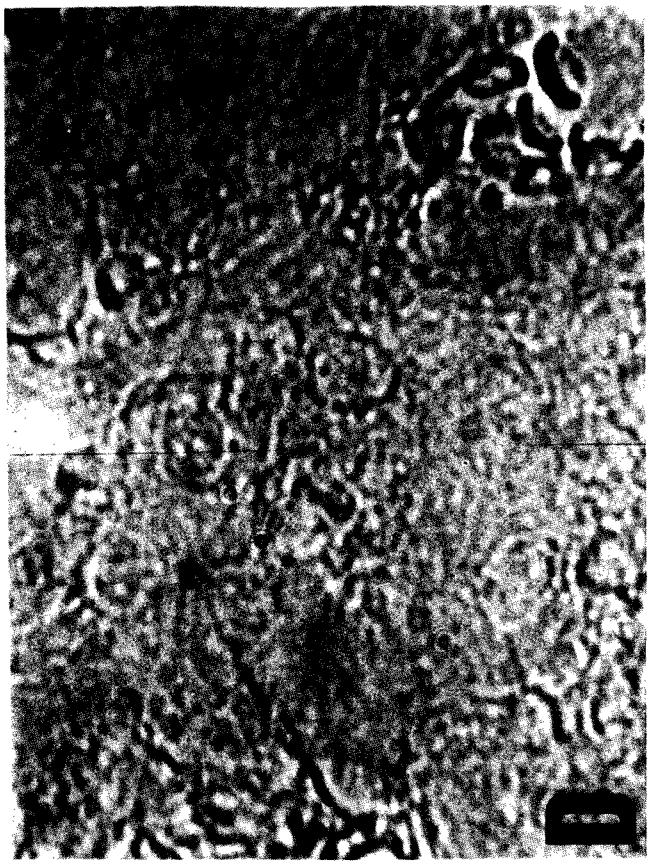
Results

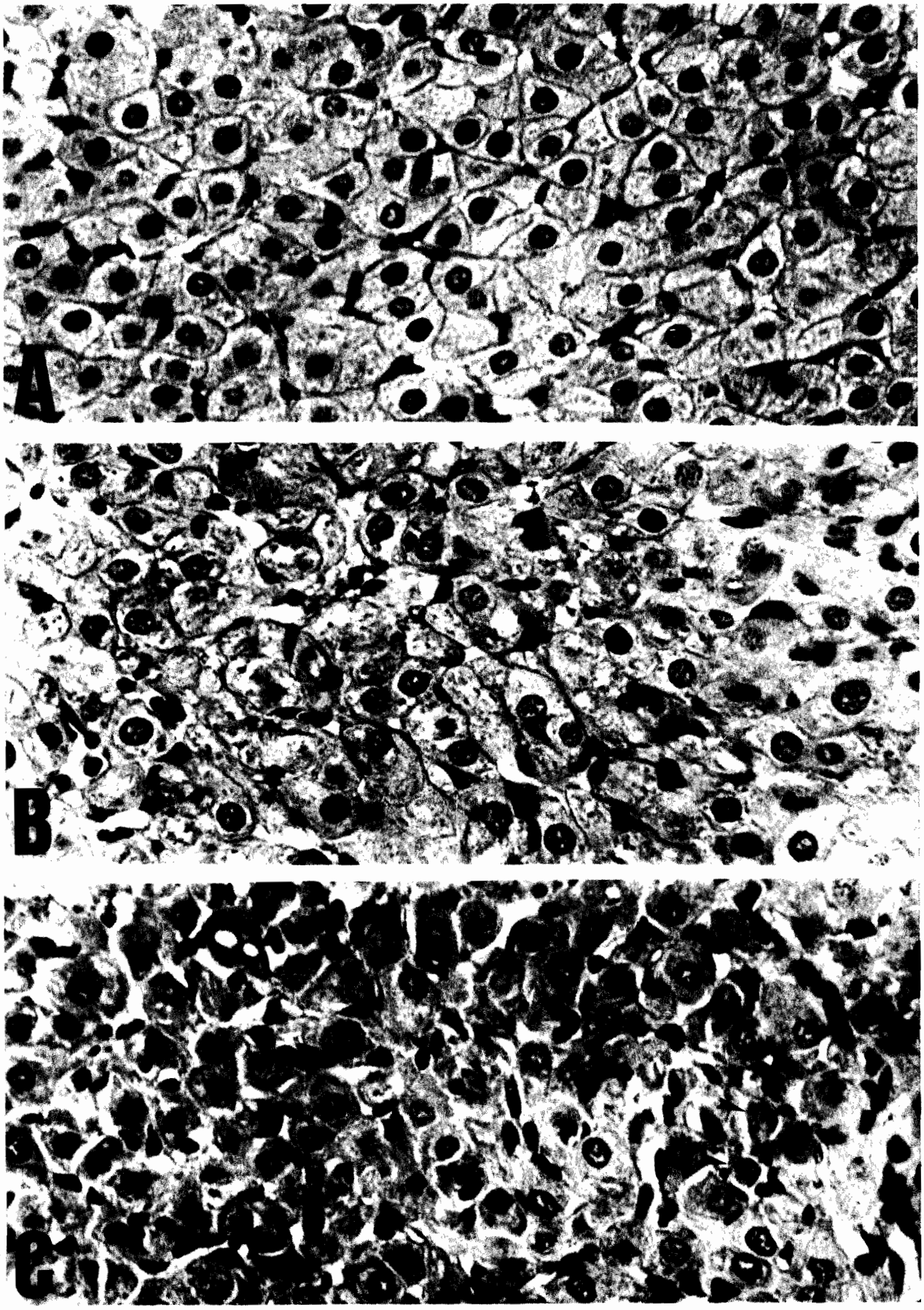
Serum levels of PRL and progesterone in autopituitary-transplanted rats

Serum levels of PRL and progesterone on days 12, 15 and 22 in day 2 APTr rats are shown in Table 1. On day 12, both hormone levels were high due to the pituitary graft-*corpora lutea* unit working well. On day 15, as a consequence of the treatment with bromocriptine, PRL as well as progesterone decreased

Fig. 1. Histological and immunohistochemical features of the auto-grafted pituitary (day 22 hyperprolactinemic rats). Pituitary tissue without inflammatory changes or signs of necrosis can be identified overlaying kidney structures and beneath the kidney capsule (c) (H.E. ×200) (A). When tested for PRL with the PAP method, great numbers of immunoreactive cells distributed throughout the graft are detected (APRL 1:400; ×400) (B); some of them are hypertrophic (C, arrows) and PRL is located in the cytoplasm (C, arrowheads are nuclei) (×1,000). There is no staining when the ABS APRL is applied (×1,000) (D).

Fig. 2. Verification of PRL-induced structural luteolysis on histological grounds. Only last generation CL are shown. Pictures A, B, and C belong to day 12, day 22 control and day 22 experimental ovaries, respectively. Only the CL of B and C show signs of morphological regression, but these are comparatively enhanced in day 22 experimental CL, where smaller lutein cells, many of them with deeply eosinophilic cytoplasm, are intermingled with more connective tissue elements. Arrowheads (C): eosinophilic bodies with basophilic inclusions (H.E. ×400).

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B
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Table 2. Macroscopic aspects of PRL-induced structural luteolysis[1].

Groups	Number of *corpus luteum*		Average of ovarian weight lost (mg)*
	day 12	day 22	
Control	6.9 + 0.5 (11)	5.7 + 0.8 (6)	3.6 + 0.4 (6)
Experimental		6.6 + 0.7 (5)	13.2 + 1.2** (5)

[1] Values are means + S.E.M.; Number of rats are shown in parenthesis

* It was calculated by the average of differences between right ovary weight on day 12 and left ovary weight on day 22

** $p < 0.001$ compared with control group (Student's t-test). Control rats were injected with bromocriptine from day 12 to day 21. Experimental rats were injected with bromocriptine from day 12 to day 14

to basal levels (functional lyteolysis). On day 22, rats treated with the vehicle from day 15 to 21 (experimental group) exhibited hyperprolactinemia in relation to rats treated with bromocriptine (control group).

Effect of the experimental hyperprolactinemia from day 15 to day 22 upon the morphological characteristics of the CL

On macroscopic examination, four to nine CL could be identified per ovary, regardless of the group studied (Table 2), but striking differences existed between day 12 and day 22 (experimental) CL weight ($P < 0.001$) (Table 2). Day 22 (control) CL weight loss, though evident, has no statistical significance.

Histological examination of the ovaries revealed a crop of CL (last generation CL) the morphological appearance of which varied between, but not within, the groups; there were smaller structures, sometimes composed exclusively of degenerated luteal cells trapped in a connective tissue mass, indistinguishable from ovarian stroma (previous generation CL); some secondary and tertiary (but not Graafian) follicles, most showing signs of atresia, could also be observed.

Day 12 CL (last generation) had large, tightly arranged luteal cells with clearly defined poligonal borders, fine granular cytoplasms and round dark nuclei (Fig. 2A). Both control and experimental day 22 CL showed signs of regression; smaller luteal cells with less clear (Fig. 2B) to blurred (Fig. 2C) borders and microvacuolated cytoplasms were intermingled with slight (Fig. 2B) to moderate (Fig. 2C) amounts of connective tissue elements. Luteal cells with picnotic nuclei and homogeneization and shrinkage of the cytoplasms, as well as deeply eosinophilic, round bodies with occasional basophilic inclusions (Fig. 2C, arrowheads) were exclusively found in the CL of day 22 hyperprolactinemic rats. Thus, experimental hyperprolactinemia hastens the morphological regression of CL.

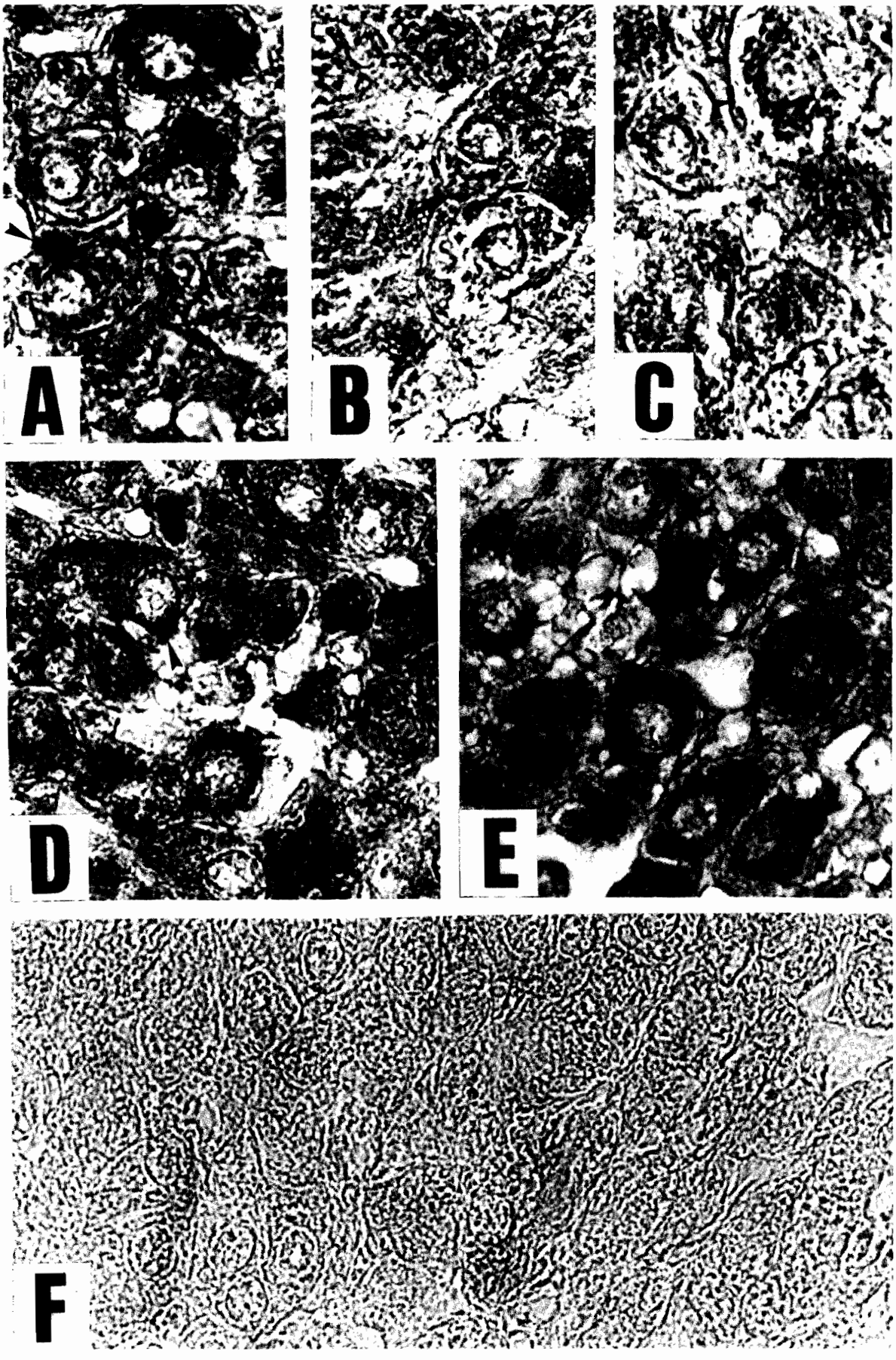
Immunohistochemical detection of PRL

Great numbers of immunoreactive PRL cells, homogeneously distributed throughout the graft, were detected in the pituitary gland of day 22 hyperprolactinemic rats (Fig. 1B). These cells stained both moderately and intensely with the APRL incubation sequence, some of them being clearly hypertrophic (Fig. 1C). No pituitary staining occured in ABS PRL controls (Fig. 1D).

In day 12 CL, products immunoreactive to PRL were found in the cytoplasm of all lutein cells. PRL staining was moderate, varied in intensity from cell to cell and revealed different patterns of hormone distribution; thus, PRL was found as unipolar or bipolar cytoplasmic aggregations, or less frequently, not found at all near the cell borders and nuclei; occasionally, it was homogeneously distributed throughout the cytoplasm (Fig. 3A, B, C).

In day 22 CL from hyperprolactinemic rats, all cells were immunoreactive to PRL products, and differences in intensity of staining from cell to cell were slight (Fig. 3D, E). Distribution of the hormone was quite uniform throughout the cytoplasm, regardless of the intensity of staining, though some occasional cells still showed cytoplasmic aggregations of PRL (Fig. 3D, arrowhead). In general, PRL staining was more intense in day 22 than in day 12 lutein cells. PRL was absent from the nuclei of both day 12 and day 22 lutein cells and cytoplasmic PRL staining was abolished when the ABS APRL control sequence was applied to tissue sections (Fig. 3F).

Fig. 3. Endogenous PRL in day 12 (A, B, C) and day 22 experimental (D, E) CL. All lutein cells are immunoreactive to PRL in both groups. The specificity of the staining is assessed by its absence when ABS PRL is used (F). In day 12 CL there are differences in staining intensity from cell to cell, and the hormone can be found as unipolar (A, arrowhead) or bipolar (A and B, arrowheads) cytoplasmic aggregations, or as linear absences from near cell borders (C, arrowheads), and nuclei (B); less frequently, it is homogeneously distributed throughout the cytoplasm (A). In day 22 experimental lutein cells, the differences in staining intensity from cell to cell are slight, and the hormone is homogeneously distributed in the cytoplasms of the majority of the cells, with some of them still showing cytoplasmic aggregations of PRL (D, arrowheads). The overall staining intensity is weaker in day 12 than in day 22 experimental CL (A, B, C, compared with D, E) (PAP, APRL 1:400) (× 1,000).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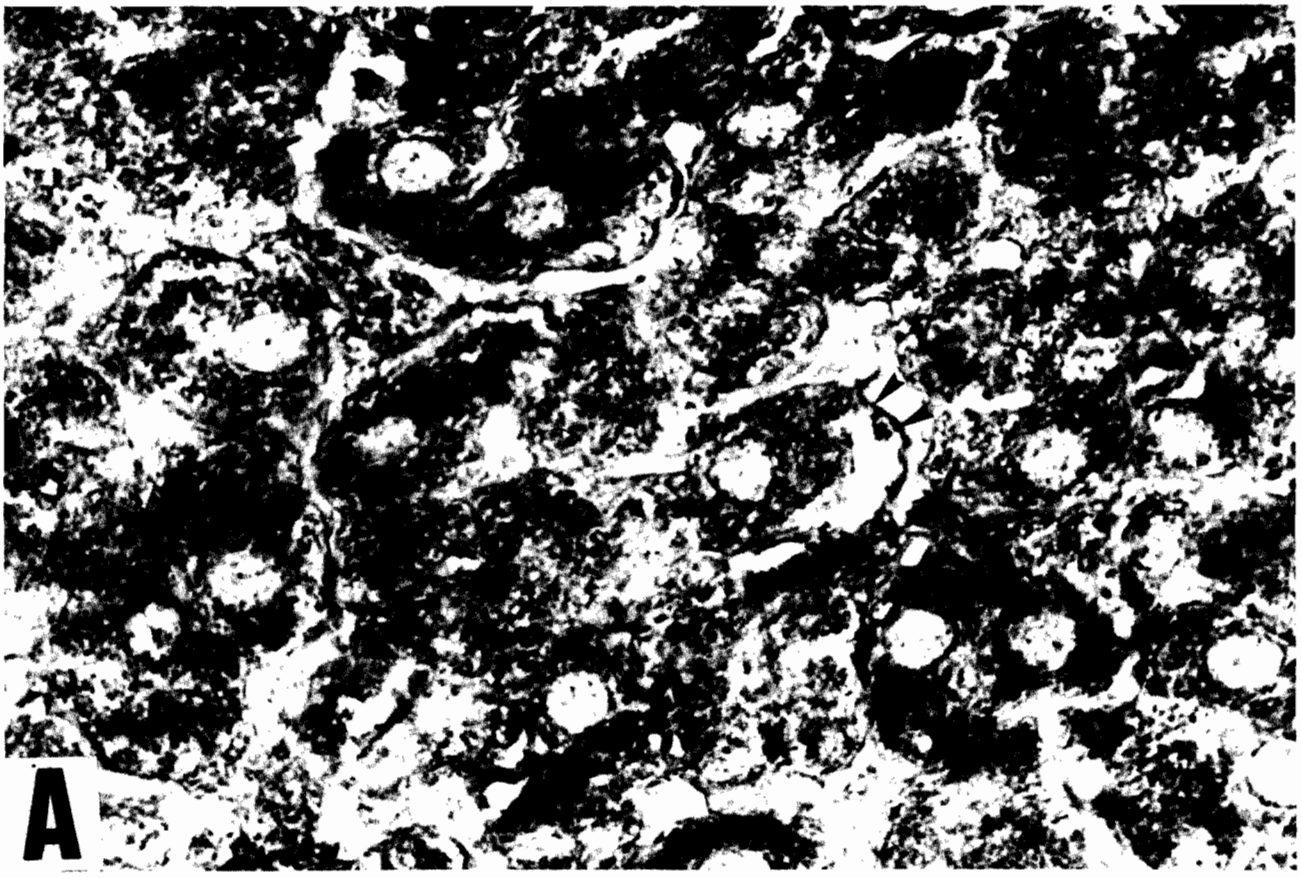
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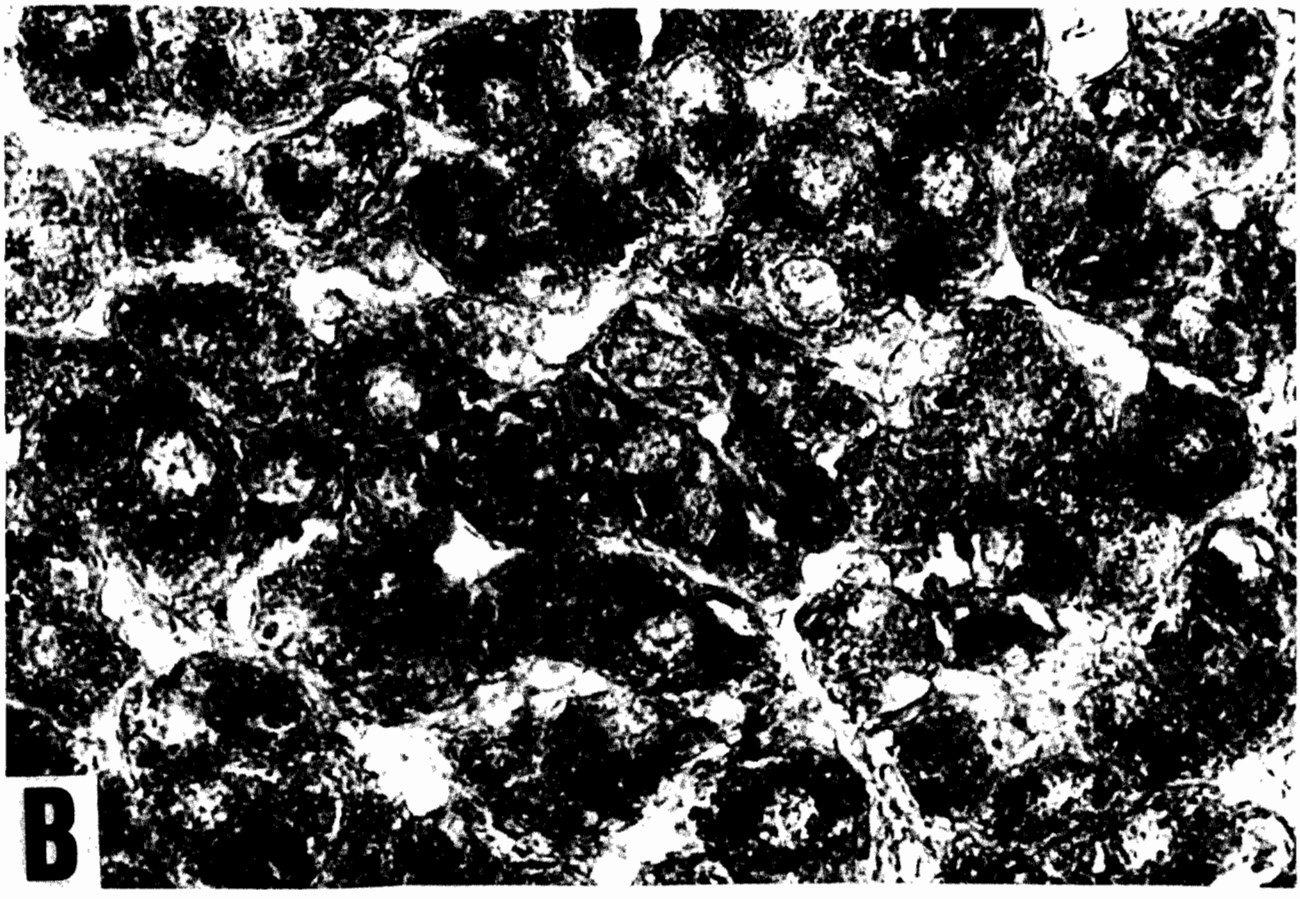
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When tests for total PRL (endogenous plus exogenous) were carried out, APRL-dependent staining was intensified in day 12 lutein cells (Fig. 4A), though not in day 22 (experimental) ones (Fig. 4B). Both day 12 and day 22 CL had similar amounts of total PRL on IHC grounds. By contrast, when PRL and APRL were applied to sections simultaneously, as a mixture (ABS APRL), staining was abolished, as in previous experiments (Fig. 3F).

Discussion

The autotransplantation of the pituitary on the second day of the cycle results in a prolongation of the life span of the CL (Everett, 1954). The transplant is associated with high and constant levels of PRL and the virtual absence of FSH and LH (Everett, 1956). This model allows us to investigate the effects of PRL on the CL without interference from other pituitary hormones.

On day 12 of day 2 autopituitary transplanted rats, CL progesterone secretion supported by PRL is associated with different patterns of cytoplasmic distribution ("heterogeneous pattern") of both endogenous and total (endogenous plus exogenous) PRL within the luteal cells (Figs. 3. 4). Similar findings have been reported in intact lactating rats, either 72 hours post-partum (Dunaif et al., 1982) or on day 15 of lactation (Nolin and Bogdanove, 1980) with subtle modifications attributable to differences in the specific binding of APRL when different fixatives are used (Salih et al., 1979) and to differences in the model (autopituitary transplanted rats vs. intact lactating rats).

Luteolysis is a complex process which involves different hormones. Although the intimate mechanism through which the CL regresses is not known, luteolysis takes place in at least two stages. In the first, the secretion of progesterone ceases, either by deprivation of its hormonal support (Morishige and Rothchild, 1974) (Table 1) or by an active form through uterine prostaglandins (Labhsetwar, 1974) and/or pituitary hormones (Rothchild, 1965; Gordon and Sherwood, 1982). The second stage in the luteolytic process is a form of morphological regression of the CL (structural luteolysis). Once the CL has lost the ability to make progesterone, the *corpora lutea* persists morphologically for 13 to 17 days (Long and Evans, 1922). The rate at which the CL undergoes regression depends on PRL. PRL induces morphological regression of the CL of cyclic rats (Wuttke and Meites, 1971; Van der Schoot and Uilenbroek, 1983), in hypophysectomized PRL-treated rats (Malven, 1969; Sloan and Malven, 1969) and of the CL of pregnancy in lactating rats (Long and Evans, 1922; Malven et al., 1967).

On day 22 of day 2 autopituitary transplanted rats treated with bromocriptine from day 12 to day 14, PRL secreted by the pituitary graft acted upon the *corpora lutea* already without capacity to secrete progesterone (Table 1) accelerating the rate of structural regression (PRL-induced luteolysis) (Table 2, Fig. 2) (Sánchez-Criado et al., 1984). This *corpora lutea* undergoing the luteolytic effect of PRL from day 15 showed a different pattern of distribution of immunohistochemically reactive products to PRL when compared to that exhibited by day 12 CL. It is basically a "homogeneous pattern" of distribution of endogenous as well as total PRL (Figs. 3D, E, 4B). This homogeneous pattern has also been demonstrated during the rat estrous cycle (Dunaif et al., 1982), where PRL is responsible for luteolysis (Billeter and Fluckiger, 1971).

The incorporation of PRL by milk secretory cells (MSC) of intact lactating rats depends on their functional state; thus, while resting or inactive MSC (without capacity of milk secretion) do not internalize PRL, actively secreting ones of the same animal do. These findings show that those MSC lacking the capacity to secrete milk products do not utilize the available circulating PRL (Nolin and Witorsch, 1976). In our study, endogenous PRL was found in all lutein cells of both day 12 CL (active lutein cells, Fig. 3A, B, C) and day 22 CL (inactive lutein cells, Fig. 3D, E) which are exposed to similar high-circulating levels of PRL (80.5 vs 54.1 ng/ml; no statistical differences between these two values exist, Table 1). Our results indicate that the internalization of PRL is not modified by the functional state of the CL (luteotrophic vs luteolytic).

On the contrary, the second stage of the incorporation of PRL by the lutein cells, the intracellular redistribution of the internalized hormone (Kelly et al., 1984), varies significantly in relation to the action exercised by PRL on the CL, a fact that has been previously addressed on IHC grounds in intact lactating rats (Dunaif et al., 1982).

In summary, the results of the present experiment indicate: a) that the intracellular distribution of PRL changes in connection with its different actions in the virtual absence of LH and FSH and b) that the products immunoreactive to total PRL (endogenous plus exogenous), as a reflection of the receptor content (Kurzon and Sternberger, 1978) and in agreement with the results of other authors in postovulatory CL (Richards and Williams, 1976), do not differ regardless of the effect of PRL (Fig. 4), which seems to indicate that intracellular mechanisms, e.g. intraluteal prostaglandin synthesis (Sánchez-Criado et al., 1984; Ueda et al., 1985), intraluteal concentration of progesterone (Rothchild, 1981), rather than receptor content, appear to be affecting the response of lutein cells to PRL.

Fig. 4. Total PRL (endogenous plus exogenous) in day 12 (A) and day 22 experimental (B) CL. While APRL-dependent staining has been intensified in day 12 lutein cells (A), it has not been in day 22 experimental (B). The overall staining intensity of both day 12 and day 22 experimental CL is similar. The patterns of distribution of the hormone in the cytoplasms of all lutein cells are parallel to those of endogenous PRL. Note differences in staining intensity from cell to cell, as well as linear absences near the cell borders, in day 12 CL (A, arrowheads). (PAP, PRL 1 μg/ml plus APRL 1:400) (× 1,000).

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