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Inhibition of Experimental Lactational Mammary Gland Growth in the Rat with Exogenous Estrogen and Progesterone (39822)

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Introduction. The onset of lactation involves numerous biochemical and ultrastructural changes in individual rat mammary gland cells, including an active period of mammary gland deoxyribonucleic acid (DNA) synthesis (1, 2). Since DNA content per mammary gland cell nucleus has been shown to remain constant (3) and no change in the connective tissue content has been noted during the development of the mammary gland (4), this increase in DNA most likely reflects an active phase of cellular proliferation. Some histological evidence for this has been provided (5).

Lactation in the rat normally does not occur until parturition. Numerous studies (6) have suggested that circulating levels of estrogen and/or progesterone during pregnancy serve to hold lactation in abeyance until parturition. The question then arises: Does the combination of estrogen and progesterone during pregnancy inhibit only the secretory capacity of the mammary gland, or does it also inhibit the mitotic activity that is associated with lactogenesis? The following investigation was designed to explore this question.

Materials and methods. Virgin female Sprague-Dawley rats (225-275 g) were housed in a temperature-controlled (23°), artifically lighted room (8 AM-10 PM) and were provided Wayne Lab Blox and water *ad libitum*. All 113 rats were ovariectomized and injected daily with 2 μg of 17β-estradiol-3-benzoate and 6 mg of progesterone (EP) in 0.1 ml of corn oil for 20 days to promote mammary development equivalent to that of late pregnancy. The rats were then divided into 11 treatment groups. In four of the groups we attempted to induce a lactational mammary growth phase by giving daily injections of either cortisol (C), prolactin (L), growth hormone (G), or a combination of all three hormones (C + L + G) for 3 days following the last injection of EP. In another series of four treatment groups we repeated the above, except that injections of EP were continued along with the injections of C, L, G, and C + L + G on Days 21-23. Three groups of rats were used as controls: (i) rats killed after 20-days EP treatment, (ii) rats killed after 23-days EP treatment, and (iii) rats injected with saline for 3 days after the 20-day EP treatment.

Cortisol (hydrocortisone acetate) was administered in saline suspension (250 μg/day) in 0.1-ml volumes. Prolactin (2 mg/day) and growth hormone (1.5 mg/day) were dissolved in saline and given in 0.15-ml volumes. All injections were made subcutaneously. Estradiol, progesterone, and cortisol were obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Prolactin and growth hormone were kindly provided by The National Institutes of Health (NIH-P-s8, NIH-GH-s9).

The structural and functional status of the mammary glands was estimated by measurement of DNA, ribonucleic acid (RNA), and nitrogen content. The six abdominal-inguinal glands were removed, minced, and defatted in 2:1 chloroform:methanol for 24 h, then in ether for 24 h. The lipid-free tissue was dried, weighed, and ground to a powder. DNA and RNA were extracted from 25-mg samples of mammary powder with hot trichloroacetic acid as described by Schneider (7). DNA content was estimated using Burton's diphenylamine method (8), and RNA by the orcinol procedure (9). All DNA values were expressed as milligrams of total DNA per 100 grams body weight, which takes into account the variability due to animal size. RNA values were expressed as a ratio of total mammary RNA to total DNA, which should be indicative of the

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RNA content per mammary gland cell. Nitrogen content was determined on 5-mg samples of mammary gland powder using the Kjeldahl microdetermination procedure of Lang (10) and was also expressed as a ratio of total nitrogen to total DNA. Differences between means were analyzed for statistical significance with Student's *t* test.

Results. When EP was administered for 20 days, then discontinued, and C, G, L, or C + G + L was given on the following 3 days, varying degrees of lactational activity resulted. We noticed that merely withdrawing EP and injecting saline for 3 days caused slight elevations in DNA, RNA, and nitrogen (Tables I–III). Consequently, we compared all treatment groups to the saline control rather than the group killed on Day 21. Nitrogen (Table I) was elevated in groups treated with C, L, and C + L + G. RNA (Table II) was elevated in groups given C alone or C + L + G. DNA (Table III) was elevated only in the group treated with C alone ($P < 0.005$). If EP was continued along with the treatments of C, L, G, or C + L + G, all of the hormonally induced increases in DNA, RNA, and nitrogen were inhibited (Tables I–III).

Discussion. Griffith and Turner (11) first demonstrated that a lactational-like increase in mammary gland DNA could be induced experimentally be priming virgin ovariectomized rats for 20 days, followed by a 3-day treatment with C, L, and G, alone and in combination. Using the same basic experimental design, our data revealed that such "experimental lactational growth" can be blocked by continued administration of EP. EP not only suppressed this experimental growth, but also prevented the induction of milk synthesis, as indicated by the low levels of RNA and nitrogen. Thus, results from

TABLE I. EFFECT OF ESTROGEN + PROGESTERONE (EP) ON MAMMARY GLAND NITROGEN/DNA RATIOS DURING EXPERIMENTAL LACTOGENESIS.

Treatment (Days 21–23)	No. of rats	EP Discontinued on Day 20	EP Continued to Day 23
Killed Day 21	18	2.77 ± 0.08[a]	
Saline	10	2.90 ± 0.11	
EP	10		2.82 ± 0.15
Cortisol	10, 10	3.92 ± 0.16*	2.75 ± 0.07**
Growth hormone	10, 10	3.24 ± 0.19	2.61 ± 0.19***
Prolactin	9, 8	3.41 ± 0.14****	2.63 ± 0.15*****
Cortisol + Growth hormone + prolactin	10, 8	3.54 ± 0.14****	2.61 ± 0.25***

[a] Mean ± SE.
* Significantly larger than saline control; $P < 0.001$.
** Significantly lower than corresponding treatment with EP discontinued; $P < 0.001$.
*** Significantly lower than corresponding treatment with EP discontinued; $P < 0.01$.
**** Significantly larger than saline control; $P < 0.01$.
***** Significantly lower than corresponding treatment with EP discontinued; $P < 0.005$.

TABLE II. EFFECT OF ESTROGEN + PROGESTERONE (EP) ON MAMMARY GLAND RNA/DNA RATIOS DURING EXPERIMENTAL LACTOGENESIS.

Treatment (Days 21–23)	No. of rats	EP Discontinued on Day 20	EP Continued to Day 23
Killed day 21	18	0.86 ± 0.04[a]	
Saline	10	1.04 ± 0.06	
EP	10		0.98 ± 0.04
Cortisol	10, 10	1.28 ± 0.04*	1.01 ± 0.03**
Growth hormone	10, 10	1.12 ± 0.07	0.80 ± 0.36***
Prolactin	9, 8	1.12 ± 0.06	0.89 ± 0.03***
Cortisol + growth hormone + prolactin	10, 8	1.30 ± 0.05*	1.17 ± 0.04****

[a] Mean ± SE.
* Significantly larger than saline control; $P < 0.01$.
** Significantly lower than corresponding treatment with discontinued EP; $P < 0.001$.
*** Significantly lower than corresponding treatment with discontinued EP; $P < 0.01$.
**** Significantly lower than corresponding treatment with discontinued EP; $P < 0.05$.

TABLE III. EFFECT OF ESTROGEN + PROGESTERONE (EP) ON MAMMARY GLAND DNA (mg/100 g body wt) DURING EXPERIMENTAL LACTOGENESIS.

Treatment (Days 21-23)	No. of rats	EP Discontinued on Day 20	EP Continued to Day 23
Killed Day 21	18	8.07 ± 0.16[a,*]	
Saline	10	8.80 ± 0.26	
EP	10		7.53 ± 0.24**
Cortisol	10, 10	10.71 ± 0.35***	8.63 ± 0.20****
Growth hormone	10, 10	9.04 ± 0.37	9.26 ± 0.35
Prolactin	9, 8	8.67 ± 0.13	8.45 ± 0.55
Cortisol + growth hormone + prolactin	10, 8	9.14 ± 0.36	8.62 ± 0.55

[a] Mean ± SE.
* Significantly lower than saline control; $P < 0.01$.
** Significantly lower than saline control; $P < 0.005$.
*** Significantly larger than saline control; $P < 0.005$.
**** Significantly lower than corresponding treatment with discontinued EP; $P < 0.001$.

this particular experimental model do seem to suggest that the inhibition of lactation during late pregnancy by circulating levels of estrogen and/or progesterone is associated with an inhibition of the lactational type of mammary gland growth.

The dose level of EP employed in our study was based on the work of Moon *et al.* (12), in which various dose levels and ratios of estradiol to progesterone were analyzed for the ability to stimulate development of mammary glands in ovariectomized female rats. A dose ratio of 1:3000, given daily for 19 days, yielded mammary gland DNA levels equivalent to those observed in normal rats during late pregnancy. Thus, with respect to their effects on mammary gland DNA content, the levels of EP used in our experiments did mimic normal pregnancy. Nevertheless, we are aware that most likely there are numerous differences in the total endocrine and metabolic condition of the experimental rats compared to normal pregnant and lactating rats. Our results do at least suggest that the increases in mammary gland DNA which occur during pregnancy in normal rats may be functionally different from normal lactational increases in mammary DNA, in that the former type of mammary growth is stimulated by EP whereas the latter may be inhibited by EP.

Some years ago it was first suggested that estrogen exerts a direct inhibition on lactation at the level of the mammary gland, rather than merely preventing the release of lactogenic hormones (13, 14). Recent studies (15, 16) involving direct estrogen implants have provided strong evidence for this suggestion. Our results might also be considered supportive of this concept. Since the inhibition by EP occurred while C, L, and G were given exogenously, the inhibition could not have resulted from simply blocking the endogenous release of these hormones.

One aspect of our study on experimental lactational growth needs emphasis for proper evaluation of the results. If we had used as our controls either the group treated with EP for 20 days or the group given EP for 23 days, we would have shown significant DNA increases in all groups: C, L, G, and C + L + G. It seems that either the withdrawal of Ep or the stress of the saline injections produced slight increases in DNA, as well as in RNA and nitrogen. Perhaps this was due to a release of endogenous hormones. Consequently, when compared to the saline controls, no significant increases in DNA were indicated in the groups treated with exogenous G, L, or C + G + L. This is interpreted as demonstrating that raising the levels of these hormones above endogenous levels results in no additional growth.

The effect of C alone on inducing increases in DNA, RNA, and nitrogen was quite evident, indicating lactational growth and secretory activity. Moreover, when the skins were removed from the rats, numerous pools of milk appeared on the surface of the mammary glands. The fact that the combination of C + L + G did not do at least as well as C alone was surprising, and little explanation for this can be offered at present.

Summary. Virgin ovariectomized rats were primed with a combination of estradiol and progesterone (EP) for 20 days to promote mammary gland development similar to that of normal pregnancy. EP injections were discontinued at Day 20 and we attempted to induce an experimental lactational mammary gland growth by giving cortisol (C), prolactin (L), and growth hormone (G), alone and in combination, for the next 3 days. The group treated with C alone exhibited the greatest degree of lactational activity with significant elevations in DNA, RNA, and nitrogen content of the mammary glands. If EP was administered simultaneously with C, G, and L, all hormonally induced increases in DNA, RNA, and nitrogen were inhibited. The results were interpreted as indicating that levels of EP during normal pregnancy not only hold milk secretion in abeyance until parturition, but also block the particular type of mitotic growth phase associated with early lactation in the rat.

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