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Prolactin Receptor Expression in Monolayer Cultures of Rabbit Mammary Epithelial Cells: Pre- and Postpartum [^{125}I]-Prolactin Binding Activity

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Expression of specific [^{125}I]-prolactin-binding sites under culture conditions has been investigated for isolated mammary epithelial cells from virgin, pregnant, and lactating rabbits. Primary monolayer cultures were obtained by sequential enzymatic dispersion of mammary tissue followed by 48 hr incubation in a medium selective for epithelial cells. Scatchard analyses of binding data obtained from these cultures indicated a single class of receptor sites, the affinity constant of which ($2.5 \times 10^9\ M^{-1}$) did not vary significantly during mammary development. The number of prolactin receptors, however, expressed by virgin and early pregnant epithelial cells was significantly increased over those from late pregnancy or lactation. Less differentiated cells also respond to growth in pregnant rabbit serum with an increase in specific [^{125}I]-prolactin binding. The diminished receptor expression by cells obtained after 17 days of pregnancy coincides with the attainment of secretory capacity in the animal, and may reflect the influence of the low serum prolactin or high progesterone levels circulating during the last trimester in the rabbit, or be the cultural expression of secretory differentiation.

Key words: prolactin, receptors, cell culture, mammary gland, rabbit

With the onset of pregnancy, intense cellular proliferation and differentiation commences in the rabbit mammary gland and is regulated by concomitant actions of progesterone, glucocorticoids, and prolactin. Progesterone greatly influences early lobuloalveolar development [1], but antagonizes eventual prolactin-induced lactogenesis by both inhibition of lactogen secretion and by direct effects on the mammary epithelial cell [2]. Prolactin alone, injected into pseudopregnant rabbits or added to the culture medium of mammary gland explants, is capable of initiating lactogenesis [3,4]. Glucocorticoids, themselves inactive, potentiate the activities of prolactin [4]. Prolactin is mitogenic for mouse and rat mammary epithelial cells in vitro [5,6], and

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stimulates early pregnant rabbit mammary gland DNA accumulation in vivo [7], suggesting an early mammotropic role for prolactin which presumes interaction with mammary stem cell receptors [6]. Later accumulation of casein mRNA and α-lactalbumin [8] appears to follow binding of lactogen to a specific receptor on the epithelial cell membrane. Both early and late activities are thus dependent upon the developmental status of the prolactin receptor.

We have recently characterized expression of specific prolactin receptors by isolated rabbit mammary epithelial cells in primary monolayer culture [9,10]. Prolactin interaction with the mammary epithelial cell receptor was saturable and reached steady state at 37°C or 24°C and pH 7.4 within 2 to 3 hr, with a high-affinity binding constant similar in magnitude to physiologic concentrations of serum prolactin. Binding was reversible to a great extent; at 24°C 50% of bound hormone dissociated within 2 hr. The present study was undertaken to characterize changes in in vitro prolactin receptor expression by mammary epithelial cells as a result of hormonal and developmental conditions during pregnancy and lactation in the rabbit. Cellular heterogeneity has been minimized by our dissociation and culture procedure.

MATERIALS AND METHODS

Materials

Modified Eagle's medium containing 0.8 mM D-valine in place of the L-isomer (D-valine MEM), penicillin-streptomycin-fungizone mixture, and trypsin-EDTA solution were obtained from Grand Island Biological Co. Bovine prolactin (bPRL; NIH-B-3, 24.1 IU/mg) was obtained from the Hormone Distribution Program, NPA-NIAMDD. Bovine insulin (24.0 IU/mg), hydrocortisone, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), BSA (fraction V powder), and hyaluronidase (Type I; lot #127C0429, 510 NFU/mg) were from Sigma Chemical Co. Collagenase (CLS III; lots #47S257 and #47S269, 100–250 U/mg) was obtained from Worthington Biochemical Co, and Pronase (B grade; lots #600367 and #701677; each 45 PUK/mg) from Calbiochem. Carrier-free $Na^{125}I$ (13–17 mCi/μg) was from Amersham-Searle and Enzymobead reagent from Bio-Rad. Other chemicals were reagent grade.

Sera

A single lot of dialyzed calf serum (lot #R975209, Gibco) was used for supplementation throughout this study. Rabbit serum was obtained by cardiac puncture of an anesthetized 26-day pregnant animal, dialyzed against two changes of 100 vol each 0.85% NaCl for a total of 8 hr, and filter sterilized before use. Bovine serum PRL was measured by radioreceptor assay according to Shiu et al [11]. Rabbit serum PRL was assayed by rPRL radioimmunoassay kit (Dr. A.F. Parlow, Hormone Distribution Program, NIAMDD; rPRL, 25.0 IU/mg) according to prescribed procedure. Serum progesterone levels were assessed using rabbit antiprogesterone-11α-BSA and goat antirabbit IgG (Miles) by the RIA method of Youssefnejadian et al [12]. Levels of PRL and progesterone in dialyzed calf serum were 2.6 ± 0.6 ng/ml and 0.9 ± 0.1 ng/ml, respectively. Dialyzed rabbit serum contained 32.5 ± 9.8 ng prolactin and 3.2 ± 0.7 ng progesterone per ml.

Preparation of Mammary Epithelial Cell Cultures

Virgin, or timed pseudopregnant, primigravid, or lactating New Zealand white rabbits were euthanized, and inguinal, abdominal, and thoracic mammary glands were removed. A suspension of predominantly ductal and alveolar epithelial cells was obtained by sequential digestion with 0.125% collagenase-0.1% hyaluronidase in 4% BSA, followed by 0.1% pronase, according to established procedure [9,10]. Dissociated cells suspended in D-valine MEM with antibiotics and 5% dialyzed calf or rabbit serum were distributed in 2 ml volumes to 35 × 10-mm Costar culture dishes (Bellco) at a seeding density of 1.0 ($\pm$0.2) × 10^5 cells/cm^2. Incubation was for at least 48 hr at 37°C with 5% CO_2 in air.

Radioiodination of Hormone

Bovine PRL was labeled by enzymatic iodination in the presence of immobilized lactoperoxidase and glucose oxidase. Hormone (10 μg) was incubated for 60 min at 24°C in 120 μl of a reaction mixture consisting of 80 mM phosphate buffer (pH 7.2), 0.5 mg Enzymobead reagent, 1.0 mCi $Na^{125}I$, and 12 mM β-D-glucose. Specific activities of batches of tracer used in this study were determined by acid precipitation [13] and varied between 48.4 and 97.1 μCi/μg. Separation of bound from free ^{125}I was accomplished on a column, 0.9 × 60 cm, of Sephadex G100 eluted with 25 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 0.1% BSA. The peak fraction of iodinated monomer was stored in 100 μl aliquots at −70°C for no longer than 2 wk before use. Integrity of the iodinated hormone was verified utilizing a standard rabbit mammary gland membrane preparation by the method of Shiu et al [11]. Typically, 18–20% of the added labeled hormone (0.25 nM; ~100,000 cpm; >96% TCA precipitable) was bound by an excess of membrane (1.5 mg protein) in 500 μl Tris-HCl buffer, pH 7.2, per tube in the absence of homologous hormone, and 72–80% of hormone bound was specific.

Prolactin Binding Assay for Rabbit Mammary Epithelial Cell Monolayers

Spent medium from confluent monolayers was replaced with 3 ml of 25 mM HEPES-buffered Earle's balanced salt solution (HEPES-EBSS), pH 7.4, and monolayers were incubated for 2 hr at 24°C. For assay, buffer was replaced with 0.5 ml HEPES-EBSS, pH 7.4, containing 1.5% BSA and concentrations of [^{125}I]-bPRL from 0.01 to 6.0 nM (10 μCi/ml) in the presence or absence of excess homologous hormone (435 nM). The binding reaction was carried out for 2 hr at 24°C, dishes were washed five times with 2 ml ice-cold HEPES-EBSS, pH 7.4, with 1.5% BSA, and cells were dissolved in 0.5 ml 1N NaOH with 0.02% SDS. Contents of each dish were transferred to respective tubes with a further 1 ml H_2O wash, and radioactivity counted on a Beckman Gamma 300 spectrometer with a counting efficiency for ^{125}I of 58%.

Specific prolactin binding is defined as the difference between [^{125}I]-bPRL bound to cells incubated with and without excess unlabeled hormone, and is expressed as a function of cell number. Monolayers carried through the assay procedure without tracer were dissociated with 1 ml 0.05% trypsin-0.02% EDTA and suspended in Isoton diluent (Curtin-Matheson) for counting and sizing by Coulter counter. Cell

volume measurements using the counter were made manually, and calibrated with standard latex beads.

Rabbit mammary epithelial cell monolayers utilized in the present studies typically contained between 2.5×10^5 and 3.5×10^5 cells/dish, varying with the preparation. Specific [^{125}I]-bPRL binding was a linear function of cell number between 1.0×10^5 and 5.5×10^5 cells (data not shown).

Specific binding to cells in monolayer represented 65% of the total hormone bound. Of the nonspecific binding, a small component representing 13–16% of bound (0.0025% of added) tracer was due to nondisplaceable, nondissociable adsorption of hormone to the plastic dish. The data from triplicate determinations after correction for nonspecific binding was analyzed according to the method of Scatchard [14] with least-squares linear regression analysis. Student's paired t-test was utilized to measure reliability of the correlation coefficient.

RESULTS

Dissociation involving the sequential enzyme digestion procedure utilized for the present study has been reported to yield suspensions which are enriched >90% for epithelial cells [5,15]. Monolayers reach confluency after 48 hr of culture in the epithelial cell-selective medium [16].

When prolactin binding capacity was measured as a function of increasing [^{125}I]-bPRL concentration in 48-hr cultures of epithelial cells obtained at two stages of gestational mammary development, different saturation curves were obtained (Fig. 1). Saturation was reached at 3–4 nM [^{125}I]-bPRL using cells from both days 14 and 21 of pregnancy, but at this concentration prolactin binding to cells from early pregnant gland (14 day) was three-fold that of cells of late pregnancy. Scatchard [14] analysis (Fig. 2) of the binding data obtained from Figure 1 indicated a single class of prolactin-binding sites in both cases. The increased binding at day 14 of pregnancy was due to an increased number of binding sites rather than to a significant change in affinity for the hormone. The affinity constant (K_a) for binding to cells from 21-day pregnant gland was $1.75 \times 10^9\ M^{-1}$, with $r = 0.91$, and $P < 0.001$. The intercept of the regression line with the abscissa yielded an estimate of 4,700 sites/cell. The K_a for binding to cells from day 14 of pregnancy was $2.74 \times 10^9\ M^{-1}$, with $r = 0.93$, and $P < 0.001$, and about 10,100 sites/cell calculated from the abscissa intercept.

Scatchard [14] analysis of prolactin binding to rabbit mammary epithelial cells obtained from virgin, pseudopregnant, pregnant, and lactating glands yielded the affinity constants shown in Table I. Regardless of mammary gland status, affinity constants obtained from cultured epithelial cells ($n = 14$) were distributed between 1.33 and $3.92 \times 10^9\ M^{-1}$, with a mean ± SE of $2.50\ (\pm 0.59) \times 10^9\ M^{-1}$. For individual analyses, $r > 0.85$ and $P < 0.01$. Total [^{125}I]-bPRL-binding capacities calculated from the intercepts of individual regression lines with the abscissa are summarized in Figure 3. The number of receptor sites expressed by cells in culture was greater for cells of virgin, 14- or 17-day pregnant gland than for cells obtained later in pregnancy or during lactation. The average number of receptor sites per virgin, 14-day, or 17-day pregnancy cell is significantly different from that of the pregnancy cell at or beyond 18 days (9,817 ± 1,366 versus 5,701 ± 710 sites, respectively, $P < 0.005$ by two-tailed Student's t-test, unpaired), with no change in the affinity constants ($2.80 \pm 0.30 \times 10^9\ M^{-1}$ for early pregnancy versus 2.62 ±

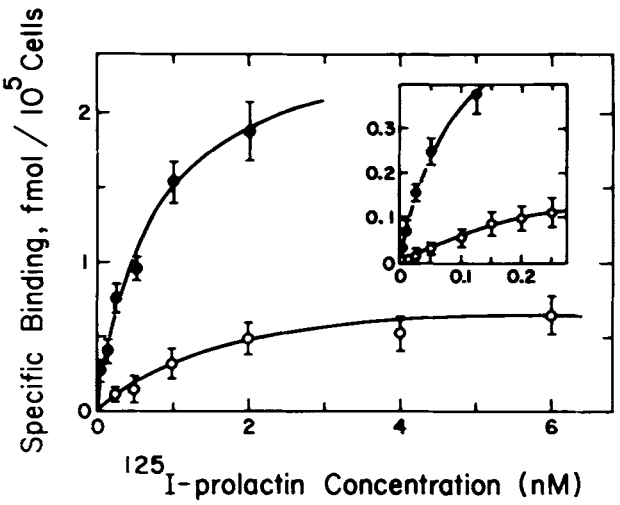

Fig. 1. Specific binding of [^{125}I]-bPRL to rabbit mammary epithelial cells of early pregnancy (day 14 = ●) and of later pregnancy (day 21 = ○) as a function of [^{125}I]-bPRL concentration. Monolayers cultured for 48 hr and containing about 3.0 × 10^5 cells were incubated with increasing concentrations of [^{125}I]-bPRL (abscissa) in the presence or absence of 5 μg (435 nM) unlabeled bPRL for 2 hr at 24°C, as detailed in Materials and Methods. Each point represents the mean ± SE of triplicate determinations. (Inset) Enlarged lower portion of saturation curves. Ordinate and abscissa are the same as for main figure.

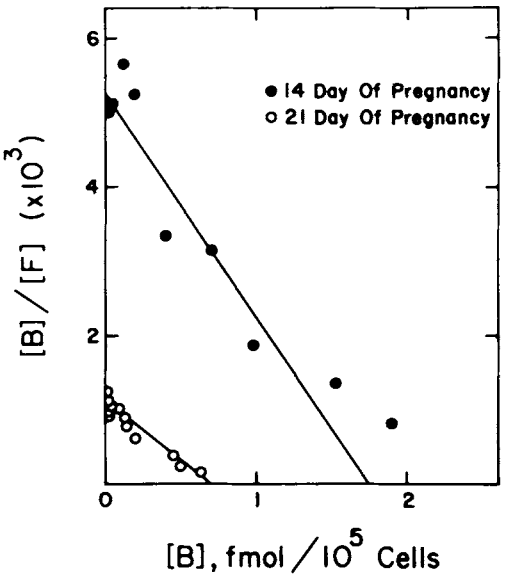

Fig. 2. Scatchard [14] plots of data presented in Figure 1. Affinity constant (K_a) for pregnancy day 21 receptor = 1.75 × $10^9 M^{-1}$, with r = 0.91 and P < 0.001. Intercept of line with abscissa indicates about 4,700 receptor sites/cell. For pregnancy day 14 receptor, K_a = 2.74 × 10^9 M^{-1}, r = 0.93 and P < 0.001, with about 10,100 sites/cell.

TABLE I. Influence of Source of Mammary Epithelial Cells Obtained for Culture on the Affinity Constant (K_a) of ^{125}I-Prolactin Binding to RMEC*

Source of RMEC[a]	K_a($\times 10^9 M^{-1}$)
Nuliparous virgin	
experiment 1	1.85
experiment 2	2.88
Primiparous	
14 days, experiment 1	2.74
14 days, experiment 2	1.47
17 days, experiment 1	1.33
17 days, experiment 2	2.21
18 days	2.78
19 days	3.39
20 days, experiment 1	1.85
20 days, experiment 2	3.92
21 days	1.75
23 days	2.33
26 days	1.35
27 days	2.70
29 days	3.17
Pseudopregnant	
20 days	2.59
Postpartum	
2 days	3.08
4 days	2.76
Mean ± SEM	2.50 ± 0.59

*Values were obtained as the slope of the line generated by Scatchard [14] analysis of the saturation data. Average K_a of duplicated experiments was utilized in statistical computations.
[a]Rabbit mammary epithelial cells.

$0.64 \times 10^9 M^{-1}$ for late pregnancy, $P > 0.2$). The number of sites observed at 20 days of pseudopregnancy was the same as that of the equivalent stage of pregnancy (5,400 sites/cell, $r = 0.98$). Analysis by Coulter counter of cell volume from early ($3,230 \pm 300 \mu m^3$) and late ($3,530 \pm 150 \mu m^3$) pregnancy cultures offered no evidence that increased cell volume accompanied increased receptor number ($P > 0.2$; two-tailed t-statistic, unpaired).

We assessed the effect of pregnant rabbit serum on regeneration of surface prolactin receptors of early and late pregnancy cells, and on the fate of receptors regenerated in the presence of calf serum. Supplementation of 14-day pregancy cells with serum from a 26-day pregnant rabbit resulted in 68% greater prolactin-binding activity when compared to that with calf serum (Fig. 4A,B). Additionally, pregnant rabbit serum not only maintained existing levels of prolactin binding, but enhanced by 20% that of cells subsequently exposed to it (Fig. 4B). These observations are consistent with that of a 12.5-fold greater prolactin level in the rabbit serum and were obtained in the absence of additional proliferative response. By contrast, calf serum

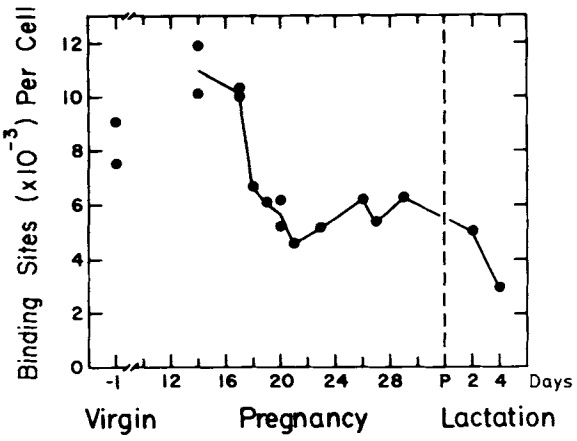

Fig. 3. Expression of prolactin receptors (as binding sites per cell) in cultured rabbit mammary epithelial cells obtained from glands throughout pregnancy and early lactation, and maintained in the presence of calf serum. The values were obtained by analysis of Scatchard [14] plots of saturation data of which Figures 1 and 2 are representative.

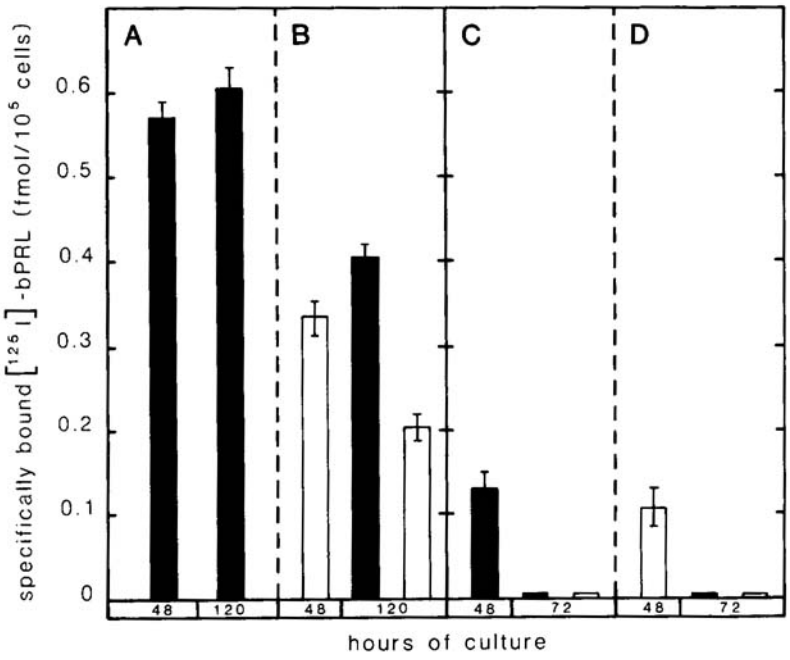

Fig. 4. Effect of serum supplementation on specific [^{125}I]-bPRL binding to cultured mammary epithelial cells from 14-day (A,B) and 26-day (C,D) pregnant rabbits. Cells were seeded in medium supplemented to 5% with either calf serum (□) or serum from a rabbit at day 26 of pregnancy (■). At 48 hr of culture, cells ($\sim 2.5 \times 10^5$) were used for assay or medium was replaced with fresh medium containing either supplementation. At the times indicated, binding to monolayers (mean ± SE, n = 3) was assessed as described in Materials and Methods using 0.2 nM [^{125}I]-bPRL alone and in the presence of unlabeled hormone (435 nM).

was less successful in maintaining prolactin binding to early- or late-pregnancy cells. Homologous serum did not appreciably increase prolactin binding to cells from late pregnancy over that achieved with calf serum (Fig. 4C,D), suggesting diminished capacity to respond to lactogen. Results obtained using early postpartum mammary epithelial cells were similar to those of late pregnancy (data not shown). Addition of insulin plus hydrocortisone (each 500 ng/ml) to serum-supplemented medium used to establish monolayers did not augment prolactin binding after 48 hr. Binding of [^{125}I]-bPRL to 14-day pregnancy cells under these conditions was 0.52 $\pm$ 0.03 fmol/10^5 cells with pregnant rabbit serum, and 0.33 $\pm$ 0.05 fmol/10^5 cells with calf serum; that to 26-day pregnancy cells was 0.11 $\pm$ 0.02 fmol/10^5 cells with homologous serum, and 0.10 $\pm$ 0.03 fmol/10^5 cells with calf serum.

DISCUSSION

While Sakai et al [17] have demonstrated similar specific prolactin-binding and affinity constants for epithelial cells dissociated solely by collagenase and for mammary tissue slices, work of others has suggested that extensive membrane alterations occur with sequential enzyme digestion, requiring time for membrane regeneration [15,18]. Our study was performed using cells that had been in culture for at least 48 hr, with the intent of allowing sufficient time for regeneration of membrane macromolecules [15]. These cells express receptor sites that meet the physicochemical requirements for identification as specific prolactin receptors [9,10].

Previous studies indicating mitogenic stimulation of rabbit mammary ductal development by prolactin in vivo could not completely rule out indirect effects [19–21]. Demonstration of prolactin receptor number to a greater extent on the surface of mammary gland epithelial cells cultured from virgin or early pregnant animals than at later times strongly supports a role for the lactogen in ductal and lobuloalveolar development. "Lactogenic" receptors of multipotential stem cells may function as somatotropic sites, although this remains to be conclusively established. We have been unable to demonstrate competition by rabbit growth hormone for prolactin binding to midpregnant rabbit mammary epithelial cells, though human growth hormone competes to a limited extent [10].

Transformation of epithelial cells from basal to secretory status is characterized by a differentiation process required for formation of the new phenotype. In the rabbit, differentiated function, evidenced by an increase in the concentration of casein mRNA, appears between days 18 and 25 of pregnancy [22]. At the same time, the first increase in the rate of lactose biosynthesis is observed [23], and the rate and pattern of lipid synthesis changes to that characteristic of rabbit milk [24]. The decrease in prolactin receptor expression we observed after in vitro maintenance of cells obtained later than day 17 of pregnancy coincides, then, with this final stage of mammogenesis. Our findings are analogous to that of Suard et al [18] that dissociated rabbit mammary epithelial cells could be separated into "differentiated" cells with a low number of sites, and "undifferentiated" cells with elevated receptor level consistent with virgin status.

The etiology of the reduced receptor number during late pregnancy is unclear. It is doubtful that it could be the result of receptor occupancy since enzyme treatment should have cleared hormone from the cell surface, and in view of the relatively low level of prolactin in the calf serum supplement. At midpregnancy, progesterone levels

peak and remain elevated until just prior to parturition [25]. Progesterone suppresses the replenishment of mammary cell prolactin receptor induced by glucocorticoid [26] and inhibits the increase of lactogen receptor seen after prolactin injection in pseudo-pregnant rabbit mammary gland [27]. Hence, activity of the steroid in vivo may contribute to biochemical events leading to reduced prolactin binding ability in culture. Receptor down-regulation in response to the physiological decline in serum prolactin during the last trimester to the lowest levels demonstrated during gestation [28] could itself contribute to reduced prolactin binding to mammary epithelial cells [29–31]. Receptor levels in late pregnancy cells remain low, even under conditions which stimulate binding to cells of early pregnancy gland. Such conditions also fail to maintain existing late pregnancy receptors. These results indicate that receptor upregulation, in contrast to down-regulation, is a long-term process, and suggest that de novo biosynthesis may play the major role in replenishment of surface receptors at this later time.

We have further observed that receptor number does not rise in epithelial cells cultured subsequent to parturition, at a time when, with serum prolactin elevated and progesterone decreased, an increase in prolactin binding to membrane fractions and freshly isolated cells has been reported by others [18,32]. These cells also fail to respond positively to pregnant rabbit serum. One explanation could involve the differentiated state of postpartum cells. If, as seems likely, altered receptor expression in cultured mammary epithelial cells reflects an increasing population of differentiated epithelial cells in the gland [33], more rapid dedifferentiation and receptor loss may occur during adaptation of these highly differentiated cells to culture. A corollary would be that reduced activity of peroxisomal D-amino acid:oxygen oxidoreductase (deaminating; EC 1.4.3.3.) accompanies terminal epithelial secretory differentiation. Since our culture system makes use of the selective effect of D-valine for specialized cells of epithelial origin, low prolactin binding to postpartum cells could indicate that the rate-limiting reaction for synthesis of new receptor protein has become that catalyzed by D-amino acid: oxygen oxidoreductase. Receptor regeneration, however, does take place in these cells. Alternatively, there may be a requirement for sustained elevation of prolactin for activation of lactogen receptor induction, as implicated in experiments reported by Posner [34]. A very rapid increase in serum prolactin to high levels ($\sim$200 ng/ml) occurs at parturition in the rabbit [18,28], and cells removed from this milieu for 48 hr of culture may lose receptors in response to a paucity of lactogen [29,30,34]. This loss may also occur on early pregnancy cells, but elevated [^{125}I]-bPRL binding at this stage would reflect initially higher receptor levels.

Study of cultured rabbit mammary epithelial cells can be expected to contribute valuable insights into the onset of differentiation, and into the regulation of lactogenic receptors by individual or combined physiological agents. Responses of the system to sera from several stages of pregnancy, as well as to prolactin and other hormones, are subjects of current study. In this regard, use of metabolic inhibitors to explore the role of de novo protein synthesis in the induction process should prove to be of value.

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