**DISSOCIATION OF CYTOLOGICAL AND FUNCTIONAL DIFFERENTIAL IN VIRGIN MOUSE MAMMARY GLAND DURING INHIBITION OF DNA SYNTHESIS**

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**SUMMARY**

Epithelial cells in mammary gland explants from mice assume a secretory appearance and synthesize the milk proteins, casein and α-lactalbumin, when cultured in the presence of insulin, hydrocortisone and prolactin. In cells from the glands of mature virgin animals such syntheses are known to require DNA synthesis. Addition of cytosine-β-D-arabinofuranoside to the explant cultures suppresses both hormonally induced DNA synthesis and enhanced production of milk protein. To determine the level at which this block in terminal differentiation occurs, epithelial cell pellets were prepared from virgin mouse mammary gland explants cultured with various combinations of insulin, hydrocortisone and prolactin, and subsequently examined by light and electron microscopy. We observed that the epithelial cells cultured in the presence of all three hormones developed fully, cytologically and ultrastructurally, even in the absence of DNA synthesis in vitro. Likewise, these cells were able to incorporate [3H]uridine into RNA efficiently and to incorporate amino acids into acid-precipitable polypeptides at levels equivalent to the untreated controls. However, immunoprecipitation of newly synthesized casein peptides showed that no new synthesis of casein occurred in cells prevented from synthesizing DNA. These data show uncoupling of cytological development and synthesis of milk protein in mammary explants from mature virgin mice inhibited from synthesizing DNA.

**INTRODUCTION**

The proliferatively dormant epithelial cells from mammary glands of mature virgin mice (Owens, Vonderhaar & Topper, 1973; Vonderhaar & Topper, 1974) must synthesize DNA in vitro before they can become fully differentiated and produce enhanced amounts of phosphorylated caseins and lactose synthetase activity in response to insulin, hydrocortisone and prolactin (I, HC and PRL). Actively proliferating cells from glands of pregnant mice (Owens et al. 1973) as well as the non-proliferating mammary cells from post-lactational animals (Vonderhaar & Topper, 1974) are able to produce enhanced levels of casein and α-lactalbumin in vitro even in the absence of DNA synthesis. In this respect, mammary tissue from mature virgin mice is different from that in mice in other developmental states.

The synthesis and secretion of milk proteins are the result of multiple discrete steps, which are controlled by the interaction of several peptide and steroid hormones (Topper & Freeman, 1980). It is necessary for the mammary epithelial cells to:
(1) synthesize and process the specific milk-protein mRNAs; (2) fabricate rough endoplasmic reticulum; (3) translate the messenger RNA on bound polysomes; and then (4) process (i.e. cleave the signal peptide sequences, phosphorylate and glycosylate casein and glycosylate α-lactalbumin); and (5) ultimately to package the proteins for secretion.

By using the methods of electron microscopy, sensitive enzymic detection of active α-lactalbumin and specific immunoprecipitation of the newly synthesized casein peptides, it is now possible to examine many of these steps of milk-protein production. Specifically, we have extended our previous studies to show that mammary epithelial cells from mature virgin mice develop distinct ultrastructural characteristics in response to various combinations of insulin, hydrocortisone and prolactin in vitro. In particular, we found that in the presence of all three hormones, histological and ultrastructural development occurs, resulting in nearly all cells having a secretory appearance even in the absence of both DNA synthesis and induction of synthesis of the major milk proteins. Thus it is clear that cytological development can be dissociated from functional differentiation in this system.

MATERIALS AND METHODS

Chemicals

Porcine zinc insulin was a gift from the Eli Lilly Company. Ovine prolactin (NIH-P-S-12) was a gift from the National Institutes of Health Hormone Distribution Center. Hydrocortisone was obtained from ICN Pharmaceuticals, Inc. Cytosine-β-D-arabinofuranoside (araC), UDP-galactose, ATP and bovine serum albumin were purchased from Calbiochem. Crude collagenase I was purchased from Worthington Biochemical Corp. Phosphate-buffered saline (PBS) and Medium 199 were obtained from Grand Island Biological Company. [14C]-UDP-galactose (298 mCi/mmol), [35S]methionine (977 Ci/mmol), [3H]-labelled L-amino acid mix, [5,6-H]uridine (38-3 Ci/mmol) and [methyl-3H]thymidine (38-7 Ci/mmol) were purchased from New England Nuclear. Goat anti-rabbit immunoglobulin G (IgG) immunobeads were obtained from BioRad Laboratories. Bovine α-lactalbumin and galactosyl transferase were purchased from Sigma.

Organ culture

Virgin C3H/HeN female mice, 3–5 months old, were used. The abdominal mammary glands were removed aseptically and cultured as described previously (Elias, 1957; Juergens, Stockdale, Topper & Elias, 1965). The pooled explants from the tissues of 6 to 13 animals were cultured in Medium 199 containing combinations of insulin (I; 5 μg/ml), hydrocortisone (HC; 1 μg/ml), and prolactin (PRL; 1 μg/ml). Where indicated, araC was added at 15 μg per ml. This level of araC had previously been determined to inhibit DNA synthesis by over 95% without toxic side effects (Owens et al. 1973). The culture medium was changed routinely after 24 and 72 h. In each experiment, part of the pooled explants from a group of animals was used for various biochemical analyses while the remainder was prepared for electron microscopy.

Preparation of epithelial cell pellet

A fraction enriched in mammary epithelial cells was obtained from freshly excised tissue or from explants by modification of methods described previously (Topper, Oka & Vonderhaar, 1975). Fat cells were removed by incubating up to 600 mg tissue in 5 ml of Medium 199 (pH 7-4) containing 15 mg of crude collagenase per ml and 4% bovine serum albumin (BSA).
Incubation was in a shaking 37 °C water bath for 30 to 45 min. At 5-min intervals the contents of the incubation vessels were aspirated into Pasteur pipettes; on the final 2 times using fine-bore pipettes. The dispersed cells were collected by centrifugation for 5 min at 800 g at room temperature and washed 3 times with PBS. This gentle disruption procedure allows many ductal and alveolar structures to remain intact (Wicha, Liotta & Kidwell, 1979).

Preparation of mammary cells for light and electron microscopy

Epithelial cell pellets or intact mammary explants were fixed overnight at 4 °C in 3 % formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M-sodium cacodylate buffer (pH 7.4). The explants or cell pellets were trimmed, then rinsed thoroughly in 0.1 M-sodium cacodylate prior to postfixation for 1 h in Dalton's (1955) chrome osmium fixative. The specimens were then stained en bloc with 0.05 % uranyl acetate at pH 4.9, dehydrated through a graded series of ethanol and propylene oxide, infiltrated and subsequently embedded in Epon-Araldite (Mollenhauer, 1964). For each experimental or control group at least 8 blocks were sectioned and studied by light and electron microscopy. Sections, 1-2 micron thick, were cut from the block with glass knives, picked up on glass slides, dried, and stained with toluidine blue–azure II and examined under a light microscope. Comparable areas in all groups were selected for electron microscopic examination. Profiles of at least 300 epithelial cells were evaluated ultrastructurally in each experimental group. Sections with a silver–silvergold interference colour were cut for electron microscopy with a diamond knife on a LKB Ultrotome III. The sections were picked up on Formvar–carbon coated grids and stained with uranyl acetate and lead citrate. The stained sections were viewed in a Siemens Elmiskop 102 at 60 or 80 kV accelerating voltage. Electron image photographs were taken at initial magnification of 3000–25000 diameters.

DNA synthesis

Epithelial DNA synthesis was measured by a modification of a method described previously (Friedberg, Oka & Topper, 1970). Cultures were pulsed with 0.1 μCi[^H]thymidine per ml for 72 h followed by a 20-h chase with 10 μg unlabelled thymidine per ml. Explants were then collected, weighed and either treated with collagenase to make a cell pellet before autoradiography or washed with acetone and cold trichloroacetic acid to determine incorporation of radioactivity into acid-precipitable material.

RNA synthesis

Total RNA synthesis was measured as described previously (Green & Topper, 1970). Explants were labelled for 72 h with 10 μCi[^H]uridine per ml after which the tissue was collected, weighed and defatted with acetone. Following 3 washes with cold 5 % trichloroacetic acid the tissue was solubilized with Protosol (New England Nuclear) and radioactivity in acid-precipitable material was determined in a liquid scintillation spectrometer.

Autoradiographic analysis of DNA synthesis

Cells labelled with [^H]thymidine were fixed and embedded as described above for light microscopy. Sections, 1–2 micron thick, were cut, dried on gelatin-coated glass slides and dipped in NTB-2 Kodak liquid emulsion. The slides were stored in the dark for an appropriate period of time and subsequently developed in Kodak D-19 solution. Distribution of the autoradiographic grains was determined on unstained sections under a Zeiss photomicroscope equipped with phase optics.

Lactose synthetase activity (α-lactalbumin)

Lactose synthetase activity was measured in the presence of excess exogenous UDP-galactosyl transferase by a modification of a previously described procedure (Ono & Oka, 1980). The homogenization buffer was supplemented with 0.1% Triton X100. Higher concentration of this detergent interfered with this enzyme activity (M. Bhattacharjee & B. K. Vonderhaar, 1980).
unpublished data). Homogenates were centrifuged at 20 000 g for 20 min and the lipid-free supernatant used for assay. A standard curve using bovine α-lactalbumin was run in all cases and results are expressed as bovine α-lactalbumin equivalents.

**Determination of casein-peptide and total protein synthesis**

Casein synthesis was measured by the incorporation of labelled amino acids using an indirect immunoprecipitation assay. Full characterization of the casein antibody will appear elsewhere (Smith & Vonderhaar, 1981). In brief, the antibody was prepared from caseins purified from mouse milk by DEAE-Sephalac chromatography. An IgG fraction from immunized rabbits was prepared, which precipitates all 4 known mouse caseins (both phosphorylated and non-phosphorylated forms). The casein antibody does not cross-react with mouse serum proteins or with purified mouse α-lactalbumin in immunodiffusion tests.

To determine the level of specific milk-protein synthesis, mammary gland explants were labelled with radioactive amino acids for the indicated period of time. Each group of explants was homogenized in 10 vol. (v/w) of ice-cold PBS (0-01 M-natrium phosphate, pH 7-4, 0-14 M-NaCl) containing 2 % Triton X100 and 25 μg/ml of phenylmethylsulfonylfluoride (PMSF) using a Polytron PCV-Z-110 fitted with a micro-tip. The homogenate was centrifuged at 20 000 g for 20 min and the lipid-free supernatant removed carefully. In some experiments, this supernatant was further spun at 105 000 g for 1 h prior to assay. The indirect immunoprecipitation was performed with triplicate samples. To ensure that saturating levels of antibody were achieved, increasing amounts of the primary rabbit total anti-mouse casein IgG were used. Parallel trapping controls were included for each point by the addition of an excess of non-radioactive mouse casein. Fifty to 100 μl of supernatant was used in each tube with a final reaction volume of 250 μl. After incubation at 4 °C for 20-24 h, the secondary antibody (goat anti-rabbit IgG immunobeads) was added and the reaction continued for an additional 3 h at 37 °C. The beads and associated immune complex were then centrifuged for 20 min at 1000 g and the recovered pellet washed once with PBS containing 0-1 % BSA. The samples were then transferred to scintillation vials and counted in the presence of 10 ml Aquasol (New England Nuclear) using a liquid scintillation spectrometer.

The incorporation of labelled amino acids into total protein was determined on triplicate 10-μl aliquots of the supernatants precipitated by trichloroacetic acid at 4 °C following hydrolysis of aminoacyl-tRNA at 90 °C for 10 min. A Lowry protein analysis (Lowry, Rosebrough, Farr & Randall, 1951) was also performed on pellets precipitated in the same way to decrease the concentration of interfering detergent. The difference of radioactivity between the immunoprecipitated sample and the trapping control was divided by the amount of incorporation of label into total protein and multiplied by 100 to yield the percentage of casein synthesis.

**RESULTS**

When explants from mammary glands of mature virgin mice are cultured in the presence of insulin, hydrocortisone and prolactin, various biochemical events occur, including induction of DNA and casein synthesis and the activities of lactose synthase and glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase (Owens et al. 1973). Previously, inhibition of DNA synthesis by the addition of araC or fluorodeoxyuridine (FdUrd) to the cultures was shown to prevent hormonal stimulation of the production of the specific milk-proteins but not the ubiquitous enzymes Glc-6-P and GlcA-6-P dehydrogenases (Owens et al. 1973). The complete lack of inhibition of these latter enzyme activities supports the conclusion that the suppression of milk-protein production is not due to toxic side effects of araC or FdUrd, but rather that terminal differentiation of the virgin mouse mammary gland in vitro is coupled to DNA synthesis (Owens et al. 1973; Vonderhaar & Topper, 1974). These previous studies, however, were limited in that they used relatively
insensitive assays for casein (rennin-Ca\textsuperscript{2+} precipitation of \textsuperscript{32}P-labelled proteins) and a-lactalbumin (lactose synthetase activity in the presence of endogenous galactosyl transferase (Owens \textit{et al.} 1973; Vonderhaar & Topper, 1974). Therefore, in order to confirm our earlier observations and define more precisely the level at which the inhibition of functional differentiation does occur, more sensitive biochemical assays were employed.

**Table 1. Synthesis of DNA, total protein and casein peptides in mammary gland explants from virgin mice**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>DNA synthesis (c.p.m./mg tissue)</th>
<th>Protein synthesis (c.p.m./mg protein)</th>
<th>Casein synthesis (% of total protein synthesis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured (0-5 h)</td>
<td>—</td>
<td>3122 ± 182</td>
<td>1.25 ± 0.04</td>
</tr>
<tr>
<td>I/HC</td>
<td>—</td>
<td>2874 ± 110</td>
<td>1.34 ± 0.05</td>
</tr>
<tr>
<td>I/HC/PRL</td>
<td>—</td>
<td>3367 ± 212</td>
<td>1.21 ± 0.04</td>
</tr>
<tr>
<td>I/HC/PRL + araC</td>
<td>—</td>
<td>920 ± 6</td>
<td>—</td>
</tr>
<tr>
<td>Cultured</td>
<td></td>
<td>6369 ± 92</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>NH</td>
<td>920 ± 6</td>
<td>3122 ± 182</td>
<td>1.25 ± 0.04</td>
</tr>
<tr>
<td>I/HC</td>
<td>2338 ± 18</td>
<td>8181 ± 204</td>
<td>5.06 ± 0.07</td>
</tr>
<tr>
<td>I/HC/PRL + araC</td>
<td>112 ± 4</td>
<td>7210 ± 148</td>
<td>1.30 ± 0.04</td>
</tr>
</tbody>
</table>

Pooled explants from 8 mature virgin mice were cultured in the absence of hormones (NH) or in the presence of various combinations of insulin (I), hydrocortisone (HC), prolactin (PRL) and cytosine-\textbeta-D-arabinofuranoside (araC) as described in Materials and Methods. \[^{35}S\]methionine (25 \(\mu\)Ci/ml) was added either from 0 to 5 h (uncultured) or 68-72 h (cultured) of culture. \[^{3}H\]thymidine (0-1 \(\mu\)Ci/ml) was added at the time of explantation and DNA synthesis was determined after 72 h of culture as described in Materials and Methods. Casein immunoprecipitation assays were performed on triplicate aliquots of the 105,000 \(g\) supernatant prepared from the tissue, using saturating levels of primary antibody. Total protein synthesis and the % of total protein synthesis that is casein were determined as described in Materials and Methods.

The synthesis of specific immunoprecipitable casein peptides was examined in explants of mature virgin mammary glands both in the presence and absence of araC (Table 1). After 72 h, explants cultured in the presence of I/HC/PRL showed a nearly 4-fold increase in the level of casein synthesis over the baseline level seen in tissue cultured for only an initial 5-h period. It is also clear that casein synthesis declined after 72 h in I/HC. In several experiments, the decrease in basal casein synthesis in the I/HC system ranged from 15 to 40%. The reduction of casein synthesis in the absence of PRL is consistent with results which have shown a stabilizing effect of PRL on casein messenger RNA (Houdebine, Devinoy & DeLouis, 1978; Guyette, Matusik & Rosen, 1979). In the presence of I/HC/PRL and araC for 72 h, the level of casein synthesis remained unchanged from the baseline level. Thus, under conditions where DNA synthesis was inhibited by over 95% and total protein synthesis unaffected, induction of casein synthesis over the level in the resting gland was not observed. Similar results were obtained when either \[^{3}H\]proline or \(^{3}H\)-labelled L-amino acid mix was used to label the peptides, when the 20,000 \(g\) supernatant was used, or when FdUrd was added to inhibit DNA synthesis (data not shown).
When explant cultures of mature virgin mammary glands were examined for the induction of $\alpha$-lactalbumin similar results were obtained. Using a sensitive assay designed to detect low levels of enzymically active $\alpha$-lactalbumin in the presence of excess purified bovine milk galactosyl transferase, significant induction of this milk-protein activity was seen after 96 h in culture in the presence of I/HC/PRL (Table 2). However, enzymically active $\alpha$-lactalbumin was not detected in uncultured tissue (not shown), in tissue cultured in I/HC, nor in tissue cultured in I/HC/PRL and araC, even though total RNA synthesis was unaffected. Therefore, induction of enzymically active $\alpha$-lactalbumin, as well as casein synthesis, is dependent on DNA synthesis.

As with biochemical differentiation, cytological and ultrastructural development of the mammary gland in culture occurs in discrete steps, which are the result of the

Table 2. Synthesis of DNA, RNA and $\alpha$-lactalbumin in mammary gland explants from virgin mice

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>DNA synthesis (c.p.m./mg tissue)</th>
<th>RNA synthesis (c.p.m./mg tissue)</th>
<th>Casein synthesis (% of total protein synthesis)</th>
<th>$\alpha$-Lactalbumin synthesis (ng/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH</td>
<td>396 ± 6</td>
<td>61296 ± 129</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>I/HC</td>
<td>—</td>
<td>86682 ± 69</td>
<td>0.74</td>
<td>N.D.*</td>
</tr>
<tr>
<td>I/HC/PRL</td>
<td>1269 ± 15</td>
<td>104316 ± 149</td>
<td>5.8</td>
<td>9.5</td>
</tr>
<tr>
<td>I/HC/PRL + araC</td>
<td>94 ± 2</td>
<td>104679 ± 292</td>
<td>0.88</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Pooled explants from 12 mature virgin mice were cultured in the absence of hormone (NH) or in the presence of various combinations of insulin (I), hydrocortisone (HC), prolactin (PRL) and cytosine-$\beta$-D-arabinofuranoside (araC) as described in Materials and Methods. Total RNA and DNA synthesis were determined after 72 h exposure of cultures to [H]uridine (10 $\mu$Ci/ml) or [H]thymidine (0.1 $\mu$Ci/ml), respectively, as described in Materials and Methods. The level of enzymically active $\alpha$-lactalbumin was determined after 96 h of culture as described in Materials and Methods. All other details are as described in the legend to Table 1 except the casein assay was performed on the 20,000 g tissue supernatant.

* N.D., not detectable; below the level of sensitivity of assay (i.e. 0.5 ng/mg tissue).

Figs. 1-8. Photomicrographs of 1–2 $\mu$m sections of mammary epithelial cell pellets fixed in paraformaldehyde, postfixed in osmium tetroxide, embedded in Epon-Araldite, and stained with toluidine blue-azure II. All tissue is from mammary glands of 3 to 5-month-old virgin C3H/HeN mice.

Fig. 1. Portion of a mammary duct within a cell pellet prepared from uncultured explants of mammary glands. $\times$ 750.

Fig. 2. Section through a mammary duct within a cell pellet prepared from explants cultured for 72 h in the presence of insulin alone. $\times$ 890.

Fig. 3. Cross-section through a developing alveolus within a cell pellet prepared from explants cultured for 72 h in the presence of insulin and hydrocortisone. There is a noticeable increase in cytoplasmic volume over uncultured and insulin-treated cells. $\times$ 890.

Fig. 4. Cross-section of a small alveolus within a cell pellet prepared from explants cultured for 72 h in the presence of insulin, hydrocortisone and prolactin. The cells contain large secretory droplets and in some areas (arrow) vesiculation of the cytoplasm is noticeable. $\times$ 890.
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1

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actions of several hormones acting alone or in concert with others (Mills & Topper, 1970; Topper & Freeman, 1980). In an attempt to elucidate the level at which milk-protein synthesis is inhibited in the systems with DNA synthesis blocked, ultrastructural development of the virgin mouse mammary epithelial cells was examined in detail under various culture conditions. In all cases, lactose synthetase activity was monitored and frequently casein production was examined in parallel to affirm the validity of the experimental procedures. DNA synthesis was always monitored to establish that, in the presence of the inhibitor (araC), [3H]thymidine incorporation in the epithelial cells was reduced to less than 5% of the unblocked system. Because the virgin mammary gland consists of relatively few epithelial cells embedded in a large fat pad, the tissue was treated with collagenase to produce an epithelium-enriched cell pellet for examination by electron microscopy. This procedure allowed for the examination of several hundred cells per experimental group and did not alter the results of either the ultrastructural or biochemical analyses.

Initially, 1–2 micron thick sections from Epoxy-embedded mammary gland explants or epithelial-enriched cell pellets were examined under phase optics following staining with azure II–toluidine blue. The cell pellets were found to be equivalent to intact explants with respect to the integrity of the epithelial components. Even after treatment with collagenase, it was possible in many instances to identify the ductal epithelial cells and to differentiate them from the alveolar epithelium. In section, the ducts were often cut longitudinally or obliquely while the alveoli were nearly always cut transversely because of their more nearly spherical shape.

Light microscopic examination of the mammary epithelial cell pellet derived from uncultured (Fig. 1) or insulin-treated explants (Fig. 2) showed closely packed low columnar and cuboidal cells with no evidence of intracellular lipid or secretory droplets. The nuclei were irregular in outline, relatively dense and centrally located. The surrounding cytoplasmic volume was small. In cell pellets from I/HC-treated

Figs. 5–8. One to 2 μm thick sections of mammary epithelial pellets prepared from explants incubated in the presence of [3H]thymidine from the onset of culture in the presence of various combinations of insulin, hydrocortisone, prolactin and cytosine-β-D-arabinofuranoside (araC; 15 μg/ml). The label was present for the initial 72 h of culture followed by a 20 h chase with cold thymidine. The sections were processed for light microscopic autoradiography as described in Materials and Methods.

Fig. 5. Epithelial cells from mammary explants cultured in the presence of I and HC, showing multiple autoradiographic grains distributed primarily over epithelial cell nuclei. × 890.

Fig. 6. Epithelial cells from mammary explants cultured in the presence of I/HC and araC showing little evidence of [3H]thymidine incorporation. × 890.

Fig. 7. Mammary epithelial cells from explants cultured in the presence of I/H and PRL showing [3H]thymidine incorporation and the accumulation of intracellular lipid droplets (arrowhead). × 890.

Fig. 8. Mammary epithelial cells from explants cultured in the presence of I/HC/PRL and araC showing little incorporation of [3H]thymidine into DNA. Nevertheless, intracellular lipid droplets appear along with evidence of vesiculation of the cytoplasm. × 890.
explants (Fig. 3) the ductal and alveolar epithelium showed a distinctly greater cytoplasmic volume. Some of the nuclei were more regular in outline and possessed prominent nucleoli. Intracellular lipid droplets were occasionally observed. Epithelial cells in explants cultured in the presence of 1/HC and PRL (Fig. 4) also had a significant increase in cytoplasmic volume over that observed in the 1/HC-treated cells. This was largely due to the accumulation of large intracellular secretory droplets in nearly all of the alveolar epithelium. Practically all epithelial cells including the ductal epithelium possessed a vacuolated cytoplasm; however, the ductal epithelium did not appear to collect the secretory material in large intracellular droplets as did the alveolar cells. Extra-cellular material could be seen in the ductal and alveolar lumina, presumably an accumulation of secretory products. Interestingly, in contrast to the situation in mid-pregnant mammary gland explants, only a few of the large lipid droplets were found in the lumen. Overall, secretory activity did not appear as complete in 1/HC/PRL-treated virgin mammary gland as in similarly treated mid-pregnant glands (Mills & Topper, 1970).

In cells from explants that had received hormonal stimulation in the combinations described above, but where araC was added at zero time, no differences in the major

Fig. 9. A portion of a duct within an epithelial cell pellet prepared from explants of uncultured mammary tissue from mature virgin mice. The cells were small, with scant cytoplasm, which contained a few mitochondria, occasional profiles of rough endoplasmic reticulum and scattered microfilaments. × 15,000; bar, 1.0 μm.
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histological features were observed although [3H]thymidine incorporation into DNA (Figs. 5–8) was reduced to 5% or less of the untreated controls and no induction of lactose synthetase activity was observed.

Fig. 10. After 72 h of culture in the presence of insulin alone, the mammary epithelium retains an ultrastructural appearance similar to that of untreated mammary gland (compare with Fig. 9). × 25,000; bar, 1.0 μm.

These observations were confirmed upon examination of the ultrastructure of these cells by electron microscopy. Previous studies have defined the major features of mammary epithelial ultrastructural development under various hormonal stimuli using mid-pregnant mammary tissue as the model (Mills & Topper, 1970). Our results indicate that the response of the virgin mammary epithelium is qualitatively similar to that seen in mid-pregnant glands and, furthermore, that blockage of DNA synthesis does not retard in any discernible way, the morphological differentiation of the cytoplasm of the mammary epithelium.

The virgin mouse mammary gland epithelial population is largely ductal. However, previous studies have shown that these cells can produce milk proteins nearly as efficiently as mixed populations of ductal and alveolar cells when explants of the glands are cultured with the appropriate hormones (Voytovich & Topper, 1967; Vonderhaar, 1977; Vonderhaar & Greco, 1979). Uncultured virgin mammary epithelium and cells from explants cultured for 72 h in the presence of insulin were examined
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by electron microscopy and found to be essentially identical. In the cell pellets from insulin-treated explants, the cytoplasmic/nuclear volume was relatively low. In the cytoplasm, there were few mitochondria and little rough endoplasmic reticulum, abundant ribosomes, numerous but randomly oriented microfilaments and a prominent but rudimentary perinuclear Golgi apparatus (Figs. 9, 10). In the cells cultured in the presence of insulin and hydrocortisone there was a noticeable increase in the cytoplasmic/nuclear ratio, the nucleoli were more prominent and mitochondria appeared more numerous (Figs. 11, 12). However, the most striking feature was an increase throughout the cytoplasm of profiles of rough endoplasmic reticulum and the appearance of electron-dense bodies resembling lysosomal structures. Little intracytoplasmic lipid was found in any of these epithelial cells. In cells from explants cultured for 72 h with I/HC and PRL, the cisternae of the rough endoplasmic reticulum in essentially all of the cells became dilated with moderately electron-dense material (Figs. 13, 14). Apical accumulation of lipid, usually in one very large droplet, occurred within the cytoplasm of the alveolar cells. Ductal epithelium contained only occasional small lipid droplets, although its rough endoplasmic reticulum consisted largely of dilated cisternae containing flocculent electron-dense material. The alteration of the Golgi region in the alveolar cells was difficult to assess, largely because of the lipid droplet in the perinuclear region. In the epithelial cells with small amounts of lipid, the Golgi apparatus was seen in the apical portion of the cells and secretory droplets could be seen developing in Golgi vesicles (Fig. 13). These phenomena of concentration and storage in the virgin explants, however, appear to be less well-defined than those described in mid-pregnant explants under similar conditions (Mills & Topper, 1970).

To ensure that these ultrastructural features were not the result of our manipulation of the tissue during preparation of the epithelial cell pellets, we examined identically treated intact explants. All of these cytoplasmic changes were evident in the intact virgin mammary gland explants whether or not they were treated simultaneously with araC to block DNA synthesis (Fig. 15). The cells with DNA synthesis blocked exhibited a qualitatively and quantitatively identical response to that of the hormonal induction of cytoplasmic differentiation (Figs. 13, 14). Of 300 I/HC/PRL-treated epithelial cells examined in cultures with DNA synthesis blocked and unblocked in each of 4 experiments, 240–270 (80–90%) possessed the ultrastructural appearance of cells actively synthesizing secretory products, emphasizing that the secretory appearance of the blocked cells was not an isolated event. Thus, morphological and ultrastructural development of virgin mouse mammary tissue can occur equally in cells cultured in the presence of I/HC/PRL with and without the addition of araC. This

Figs. 11, 12. Epithelial cells prepared from explants cultured in the presence of I and HC, whether or not DNA synthesis occurred, show a significant increase in cytoplasmic volume. Attendant with this alteration was the more prominent appearance of rough endoplasmic reticulum, lysosomal bodies and a more prominent Golgi apparatus. These changes were equally characteristic of the tissue whether araC was present (Fig. 12) or absent (Fig. 11). ×15,000; bar, 1 μm.
response is observed even though they are unable to respond to the hormones with induced synthesis of the milk proteins as determined in parallel cultures.

DISCUSSION

These results indicate that mammary tissue from mature virgin mice cultured in the presence of insulin, hydrocortisone and prolactin is capable of developing fully cytologically and ultrastructurally in the absence of DNA synthesis in vitro. Those cells that are prevented from synthesizing DNA by the addition of araC to the cultures have a secretory appearance that is indistinguishable from that of their unblocked counterparts. Yet the blocked cells are not able to differentiate fully biochemically as defined by hormonally enhanced production of milk proteins.

The absence of biochemical differentiation is not the result of non-specific toxicity of the DNA inhibitors. Full terminal differentiation is achieved in cells from primiparous non-pregnant animals even in the presence of inhibitors of DNA synthesis (Vonderhaar & Topper, 1974; Smith & Vonderhaar, 1981). In addition, the araC-blocked explants are able to maintain the baseline level of casein synthesis, and total RNA and protein synthesis apparently is not affected even after 72 h in the presence of the drug.

The anomalous cytological development of a secretory appearance in mammary epithelium in the absence of biochemical differentiation (i.e. milk-protein synthesis) is less perplexing when viewed together with the biochemical findings summarized in Tables 1 and 2. During culture, there is a corresponding increase in the rate of synthesis of protein, DNA and RNA in response to the presence of hormones, which is indicative of a general increase in anabolic metabolism. Very small differences in this overall response (except for DNA) are observed between blocked and unblocked explants. Only milk-protein(s) synthesis is adversely affected, suggesting a specific role for DNA synthesis in the regulation of these differentiated cell products. Therefore the morphological changes observed properly reflect the increased metabolic activities of the explants that appear directed towards the preparation of new secretory products.

The ultrastructural data reported herein indicate that 80–90% of the epithelial cells from the I/HC/PRL + araC-treated explants are secretory in appearance and cannot be distinguished from their unblocked counterparts. This observation cannot be due to the presence of the major classes of caseins as we were unable to detect induced levels of synthesis of the 4 major casein(s) as we were unable to detect induced levels of synthesis of the 4 major casein peptides using our casein-specific antibody.

Figs. 13, 14. Epithelial cells from I/HC/PRL-treated mammary gland explants invariably possessed a hyperactive Golgi area and an abundant amount of rough endoplasmic reticulum whose cisternae were distended with moderately electron-dense material. Secretory materials were occasionally detected in small collecting vesicles (arrow) in the apical cytoplasm. Increased surface activity as indicated by the large number of microvilli was also prevalent. Cells prevented from undergoing DNA synthesis (Fig. 14) developed an identical cellular morphology compared to those cultured in the absence of araC (Fig. 13). x 15000; bar, 1 μm.
Regulation of mammary differentiation

The control of synthesis of the other major milk protein, α-lactalbumin, may not be coordinately regulated with that of casein (Vonderhaar, Owens & Topper, 1973; Nardacci & McGuire, 1977; Ono & Oka, 1980). However, if it is assumed that casein and α-lactalbumin are made by the same cells, it is possible that the secretory appearance of the blocked cells may be partially attributable to the presence of α-lactalbumin, but in an enzymically inactive form. In this, as well as previous reports (Owens et al. 1973; Vonderhaar & Topper, 1974; Vonderhaar et al. 1978), the detection of α-lactalbumin has relied on its ability to function in the lactose synthetase assay. In addition, the induced synthesis of other minor milk components such as secretory piece (Kühn & Kraehenbuhl, 1979), EGF (Hirata & Orth, 1979) or lactoferrin (Segars & Kinkade, 1977) may not require DNA synthesis and thus may account for the secretory appearance of the blocked cells.

A reasonable conclusion from these and previous observations is that DNA synthesis is required for effective milk-protein synthesis by adult virgin mouse mammary epithelium in culture. But why DNA synthesis should be required for functional differentiation in these cells is not known. It is not clear whether the DNA synthesis required is related to cellular DNA replication during S-phase leading to cytokinesis or to a specialized function or modification of DNA, or to DNA synthesis related to gene amplification, or the rearrangement of intervening sequences. These mechanisms are currently under investigation.

We wish to thank Ms Antoinette E. Greco for assistance in preparing the explant cultures and performing the lactose synthetase assays, and Ms Joan Nixon for assistance in the autoradiographic and electron microscopic studies. We gratefully acknowledge the participation of and thoughtful discussions and suggestions by Drs J. M. Rosen and R. J. Pauley throughout this project and particularly during the preparation of this manuscript.

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Fig. 15. As a check for artefacts resulting from the preparation of the epithelial cell pellet, I/HC/PRL-treated explants were fixed directly and the glandular epithelium located via thick-sectioning following embedding. Secretory droplets were found in the ductal lumen and, as noted, in the cell pellets; the cytoplasm was vesicular due to the distended cisternae of the rough endoplasmic reticulum. ×18000; bar, 1·0 μm.
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(Received 27 April 1981)