petitive inhibitor of concanavalin A such as 0.2*M* methyl α-D-glucoside must be added to block recombination of concanavalin A with serum glycoproteins in the column.

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Hormone-Dependent Differentiation of Immature Mouse Mammary Gland in vitro

Abstract. *Explants from the mammary glands of 3-week-old mice can be induced to synthesize casein in vitro in the absence of lobuloalveolar development. Maximum biochemical differentiation requires the presence of insulin, hydrocortisone, and prolactin in the culture medium. In contrast to explants from adult mice, the mammary epithelium of immature animals undergoes DNA synthesis and mitosis in vitro in the absence of exogenous insulin; however, such proliferation does not lead to the formation of differentiated daughter cells. Insulin acts in at least two ways during the proliferative phases of the cell cycle of differentiating mammary epithelial cells.*

Mammary gland epithelial cells in explants derived from pregnant or nonpregnant mature mice proliferate, assume a secretory appearance, and begin to synthesize certain milk proteins when cultured in the presence of insulin, hydrocortisone, and prolactin (*1–4*). This functional differentiation of the epithelial cells has been found to be necessarily coupled to their proliferation (*5–7*). Synthesis of DNA and subsequent cell division require only insulin; however, proliferation does not lead to differentiation unless hydrocortisone and prolactin are present in the culture medium. The sequence of action of the hormones in relation to the cell cycle in explants of mammary gland from pregnant mice has been described (*8*).

Although the relationships between hormone-dependent proliferation and differentiation have been characterized in the mature gland, the biochemical behavior of the immature gland in vitro has been less clear. We examined the capacity of the immature tissue to differentiate in vitro and compared the requirements for and responses to the hormones with those of the mature tissue.

Although the onset of "maturity" is variable, 21-day-old female weanling mice of the C3H/HeN strain are termed immature in our report. The glands of the 21-day-old mouse consist principally of branching ducts several cell layers thick with terminal buds. No alveoli are seen in serial sections, nor have any been seen in whole mounts in other laboratories (*9*).

Mammary epithelial cells in immature animals proliferate into the mammary fat; yet, as the animal matures, proliferation virtually ceases and does not resume until pregnancy. We confirmed this by injecting thymidine-H^3 (1 μc per gram of body weight) intraperitoneally and preparing autoradiographs of the mammary gland 24 hours later. Only about 1.5 percent of the epithelial cells from 3-month-old mice were labeled, whereas 18 percent of the epithelial cells from 3-week-old animals were labeled. It is unlikely that the results reflect a difference in pool size, because almost all the labeled nuclei had approximately 35 to 40 grains.

Synthesis of DNA in vitro by mammary epithelial cells from mature mice is completely dependent on the presence of insulin in the culture medium (*5–7*). In contrast, our studies indicate that DNA synthesis in vitro by mammary gland explants derived from immature mice [as reflected by the amount of thymidine-H^3 incorporated during 4-hour periods as previously described (*5*)] is quantitatively independent of the addition of insulin. Since these explants contain large numbers of fibroblasts whose DNA synthesis is insulin-independent, data on total incorporation of thymidine-H^3 must be interpreted with caution. To determine whether such incorporation of thymidine-H^3 into DNA reflected epithelial cell activity, we made autoradiographs of explants exposed to thymidine-H^3 (1 μc/ml) for 72 hours. Similar explants were cultured in the presence of colchicine (0.05 μg/ml) for 72 hours and were examined for mitotic activity.

The duct epithelium, especially the epithelium of smaller terminal branches, was heavily labeled, and mitotic figures were numerous in either the presence or absence of insulin in the cultures. In explants cultured without the addition of hormones, 12 percent of the cells were in metaphase (5000 epithelial cells counted); in those cultured in the presence of insulin, 9 percent were in metaphase (5000 epithelial cells counted); and in those cultured in the presence of hydrocortisone and prolactin, 11.5 percent were in metaphase (1000 epithelial cells counted). In contrast to tissue from mature mice (*5–7*) then, proliferation of mammary epithelial cells from immature animals occurs in vitro in the absence of exogenous insulin. However, proliferation in the absence of exogenous insulin does not lead to functional differentiation.

Figure 1 shows that explants of mammary glands of immature mice can be induced to synthesize the major casein components when cultured in the presence of insulin, hydrocortisone, and prolactin. During the first 24 hours, the synthesis of casein bands 2, 3, and 4 is virtually undetectable, but by the 4th or 5th day these proteins are synthesized at greatly accelerated rates in ratios similar to those observed in tissue from mature animals (*10*). Casein band 1, a more heavily phosphorylated component (*11*), is present from the start and is least affected by culture. This component persists longest in mature tissue cultured in incomplete hormone systems (*10*).

Figure 2 shows patterns of casein production during the 5th day of culture in several media. The full differentiative response is elicited only when the three hormones are present. Of the incomplete systems, only those containing insulin are even partially effective, and insulin alone is as effective as it is in combination with hydrocortisone or prolactin. In the absence of insulin, not even band 1 is sythesized.

The hydrocortisone-prolactin system (Fig. 2), in which proliferation occurs

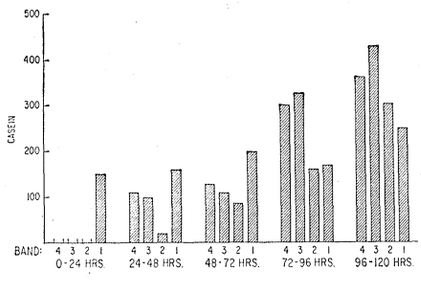

Fig. 1. Effect of culture on synthesis of the major casein components. Explants weighing 0.5 to 1 mg were prepared and cultured in sterile "Medium 199" (*2*). The medium contained 5 μg/ml each of crystalline beef insulin (Lilly), ovine prolactin (NIH Endocrinology Study Section), and hydrocortisone. Explants were exposed to P^{32}-labeled inorganic phosphate (75 μc/ml) during the times indicated and then were homogenized in the presence of mouse carrier casein. Total casein was isolated by precipitation with calcium and rennin and was further characterized with vertical starch-urea gel electrophoresis at *p*H 8.6 (*13*). Bars represent counts in gel sections corresponding to the four major casein bands with appropriate background subtracted (*10*). Ordinate refers to radioactivity in terms of counts per minute per centimeter per 10 mg of tissue.

in the absence of insulin, did not synthesize casein. Furthermore, when explants were cultured in such a hydrocortisone-prolactin system for 96 hours and insulin was then added during the final 24 hours, no effect on casein synthesis was observed. This suggests that actions of insulin other than postmitotic synergism with prolactin (*8*) and initiation of DNA synthesis (*7*) must occur before functional differentiation can be

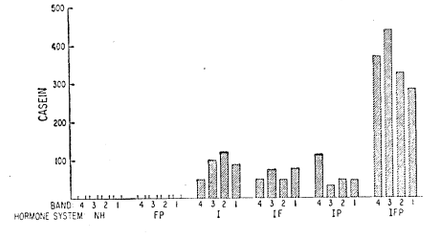

Fig. 2. Effect of culture on synthesis of casein components in incomplete hormone systems. Explants were cultured as in Fig. 1 but in the incomplete hormone system shown. Pulse labeling with P^{32}-labeled inorganic phosphate was from 96 to 120 hours in all cases. Because these experiments involve systems lacking insulin, the series was repeated with glucose replaced in the medium by D-fructose (100 mg/ml). No difference was noted. *NH*, no hormones; *I*, insulin; *F*, hydrocortisone; *P*, prolactin. Ordinate refers to radioactivity in terms of counts per minute per centimeter per 10 mg of tissue.

expressed. Insulin, then, exerts its influence on mammary tissue in at least three ways: (i) it is necessary for the initiation of DNA synthesis by epithelial cells of mature tissue as a prelude to functional differentiation (*5–7*); (ii) it must be present during the postmitotic action of prolactin (*8*) when phenotypic differentiation is expressed; (iii) it is required during the early proliferative phases of immature cells in some capacity other than initiation of DNA synthesis which, alone, cannot lead to differentiation.

Thus, even though immature tissue differs from the mature in displaying insulin-independent DNA synthesis and mitosis, such proliferation apparently does not lead to functional differentiation unless it occurs in the presence of insulin.

Although casein bands 2, 3, and 4 are not detectably synthesized by explants of immature mammary gland during the 1st day of culture in the presence of all three hormones, such explants synthesize α-lactalbumin and β-lactoglobulin. Tissue was exposed to C^{14}-labeled algal hydrolyzate (10 μc/ml). Explants were homogenized in mouse skim-milk carrier, and then α-lactalbumin and β-lactoglobulin were isolated by ammonium sulfate fractionation and electrophoresis on polyacetate strips as previously described (*2*). When the culture was exposed to the hydrolyzate from 0 to 24 hours, the rate of synthesis of α-lactalbumin was 310 count/min and of β-lactoglobulin 295 count/min per milligram of tissue. When the period of exposure was 72 to 96 hours, the rate of synthesis of α-lactalbumin was 880 count/min and of β-lactoglobulin 510 count/min per milligram of tissue. Thus, the emergence of the capacity to synthesize the various milk proteins is asynchronous in this immature tissue.

Ichinose and Nandi reported (*9*) that lobuloalveolar development in mammary explants from immature mice rarely occurs unless the mice have previously been primed with injections of estrogen, progesterone, prolactin, and growth hormone. Our work confirms this observation. Culture of unprimed explants in the presence of insulin, hydrocortisone, and prolactin elicits the biochemical differentiation described above, but there is little or no formation of alveolar structures. If, however, the animals are primed on each of 7 days with estradiol-17β (1 μg), progesterone (1 mg), and growth hormone (50 μg) before explantation of the mammary tissue into medium containing insulin, hydrocortisone, and prolactin, alveolar structures develop in vitro. Under these primed conditions, the rate of thymidine-H^3 incorporation into DNA was approximately doubled [perhaps due to a shortened S-phase (DNA synthesis) as suggested by Banerjee (*12*)], and the development of casein production was accelerated about 24 hours compared to tissue from unprimed mice. Thus, it is possible to dissociate structural development from biochemical development in vitro with mammary tissue from unprimed immature mice.

Our studies in vitro with mammary gland explants from immature mice revealed that: (i) the epithelium differs from that of the mature gland in that DNA synthesis and mitosis occur in the absence of exogenous insulin; (ii) this insulin-independent proliferation does not result in the appearance of differentiated daughter cells; (iii) addition of insulin to the cultures does not quantitatively affect DNA synthesis or mitosis but allows functional differentiation to occur when hydrocortisone and prolactin are present; (iv) emergence of the ability to synthesize casein bands 2, 3, and 4 is not synchronized with the appearance of whey-protein synthesis; and (v) it is possible in vitro to dissociate the capacity to synthesize secretory proteins from structural development of this tissue.

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20 September 1967