Hormonal Requirements for Survival and Growth of Mouse Primary Mammary Ducts in Organ Culture.* (28783)

EVELYN M. RIVERA (Introduced by Howard A. Bern)
Department of Zoology and Cancer Research Genetics Laboratory, University of California, Berkeley

Organ cultures of mammary glands from immature mice require hormones for maintenance and growth both in chemically-defined protein-free medium and in medium containing serum(2,3). Embryonic mouse mammary rudiments underwent some development in a plasma clot-embryo extract medium(4) or in chemically-defined medium(5). Explants of adult mouse mammary tissue consisting primarily of ducts (from nonpregnant, postweanling mice) could be maintained *in vitro* in a medium supplemented only with insulin (6). However, the cultivation of isolated primary ducts from immature mice has not been attempted previously. Accordingly, this report concerns the hormonal requirements for survival in organ culture of ducts taken from immature female mice of the BALB/cCrgl strain.

Materials and methods. Tissues for explantation were removed from female BALB/cCrgl mice between 3 and 5 weeks of age by cutting through the primary mammary duct first as close to the nipple as possible and then at a point just before its first major bifurcation. The explants were approximately 1 × 1 × 1.5-2 mm. Several samples were preserved to determine the initial histologic appearance of the ducts.

Culture media. The basal culture medium used was synthetic medium "199." Amorphous beef insulin (I), with an activity of 20 units/mg, provided by Dr. O. K. Behrens, was dissolved in a small volume of 0.005 N HCl. To this solution an appropriate volume of medium "199" was added to yield the desired stock concentration. The protein hormone solution was sterilized by passage through a millipore filter with an average porosity of either 0.45 or 0.65 μ. Aliquots of the filtrates were then added to the final culture medium in amounts to yield the desired final concentration of hormones. The nonesterified steroid hormone, cortisol (F), was prepared as previously described(7). Control medium consisted of nonsupplemented medium "199."

Culture method. The culture method has been described(7). The medium was changed on the third day of cultivation, and the cultures were either terminated on the 5th day, or in the case of 12-day cultures, the medium was changed every 2 days following the first medium change. At time of termination, the tissues were fixed in Bouin's solution, dehydrated and cleared, paraffin-embedded, sectioned serially at 7 μ, and stained by a modified Masson's method(8).

Results. Histology of primary ducts at time of explantation. The primary ducts possess a lining of epithelium 2 cell layers in thickness, supported by collagen fibers and adipose tissue (Fig. 1-2). The epithelial cells bordering the lumen were cuboidal in shape, whereas the subjacent basal cells were irregularly distributed and oval in shape. Although Feyrter(9) considered these basal cells to be undifferentiated myoepithelial cells, other workers(9,10) indicate that the large mammary ducts possess a double-layered epithelium. Small lateral buds were evident on all primary ducts (Fig. 1).

Cultures of primary ducts (Table I). I. *Five-day cultures.* After cultivation in hormone-free medium "199," the cells of the ductal epithelium did not appear so well preserved as in the initial control tissues (Fig. 3). Pyknosis was evident in some cells; in others, reduction in size was noted. Many cells appeared to be detached from the basement membrane. Thus, in nonsupplemented medium "199," regressive changes occurred in primary ducts by 5 days *in vitro*. In contrast to the above picture, explants were well maintained after 5 days in medium "199" supplemented with insulin (10 or 100 μg/ml)

* Supported by U.S.P.H.S. grants.

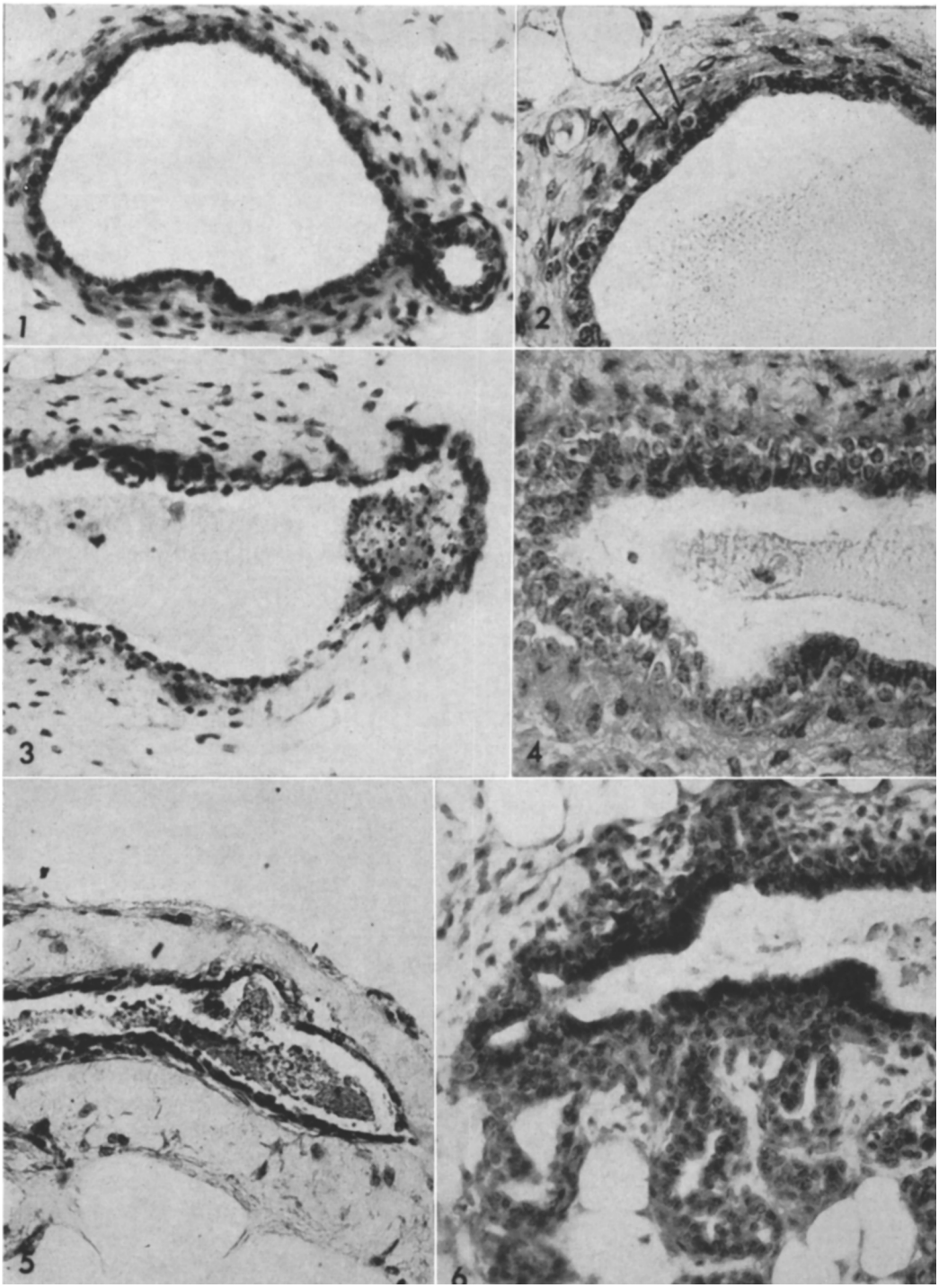

FIG. 1. Section through primary mammary duct showing appearance before culture. × 270.

FIG. 2. Higher magnification view of tissue shown in Fig. 1; basal cells indicated by arrows. × 400.

FIG. 3. Section through duct cultivated for 5 days in hormone-free medium "199." Note reduction in size of cells bordering ductal lumen and sloughing. × 270.

TABLE I. Response of Primary Mammary Ducts to Hormones in Organ Culture.

	Medium "199" + hormones (μg/ml)		No. of mice providing explants	Total No. of explants	Histologic appearance
	Cortisol	Insulin			
5-day cultures	—	—	3	6	Early regression
	—	10	9	10	Survival
	—	100	6	14	"
	8	100	7	18	"
12-day cultures	No hormones		2	6	Regression
	Insulin, 10 μg/ml		3	6	Survival with hyperplasia

or with insulin plus cortisol (100 + 8 μg/ml, respectively). The lining epithelial cells appeared taller and more numerous than before culture. In addition, the basal cells, occurring in control tissue as a poorly-defined layer of cells, appeared more distinct after culture (Fig. 4). The collagenous stroma was also increased in amount (Fig. 2 and 4). Insulin, in the lower concentration used, 10 μg/ml, was an adequate hormonal supplement for explant survival. Addition of cortisol (8 μg/ml), did not improve the response to insulin.

II. *Twelve-day cultures.* The ductal epithelium of tissues cultivated with hormone-free medium "199" was reduced to 1-2 layers of flattened cells, many of which possessed pyknotic nuclei (Fig. 5). Degenerated cells were frequently present in the duct lumen. In contrast to the picture of regression seen in hormone-free medium, explants cultivated in insulin-containing (10 μg/ml) media not only appeared viable, but the basal cells were even more numerous after 12 days than after 5 days in culture (Fig. 4 and 6). In 5-day cultures, the stromal portion was usually easily distinguishable from the epithelial cell layers, whereas after 12 days, it was often not possible to define exactly the boundary between the basal epithelium and the underlying stroma, owing to an increase in the number of fibroblasts. In addition, the epithelial cell layer lining the duct lumen appeared somewhat more prominent after 12 days than after 5 days in culture. It therefore appeared that an increase both in the epithelial cells and in the stromal components occurred *in vitro*. Although it could not be finally ascertained that true secondary ducts had developed in culture, some development of this type was strongly indicated (Fig. 6).

Discussion. This study shows that addition of insulin to synthetic medium "199" allowed survival of BALB/cCrgl primary mammary ducts in organ culture. Ducts cultured in insulin-free medium "199" for 5 days showed regressive changes, and by 12 days, the regression was even more pronounced. Insulin proved adequate at a concentration of 10 μg/ml, and addition of adrenal steroid to the culture medium did not improve ductal survival. The minimal effective concentration of insulin was not determined, but data obtained from cultures of prelactating tissue (Rivera, in preparation) indicate that it is probably lower than 10 μg/ml.

These findings provide further evidence for the differences in hormone-dependence of different parts of the mammary gland, indicated in previous studies(6,11,12). Prelactating alveoli require corticoid in addition to insulin for survival in organ culture, whereas ducts from adult nonpregnant glands survive with insulin alone. Whole mammary glands from young virgin mice cultivated in the absence of insulin underwent regression even in media containing serum(2,3).

Interpretations of the results obtained from cultures of embryonic mammary rudiments

FIG. 4. Section through duct cultivated for 5 days with insulin, 10 μg/ml, showing well-maintained ductal epithelium and prominent basal cells. × 400.

FIG. 5. Section through duct cultivated for 12 days with hormone-free medium "199." Note regressed appearance of ductal epithelium. × 270.

FIG. 6. Section through duct cultivated for 12 days with insulin, 10 μg/ml, showing hyperplasia of basal cells as well as "crowding" of cells bordering ductal lumen. × 270.

have been conflicting. The occurrence of ductal differentiation in mammary rudiments in a medium composed of plasma and embryo extract has led to the conclusion that the early stages of mammary development were not dependent on hormones(4). However, growth of mammary rudiments in synthetic medium has been reported to be promoted primarily by somatotropin(5). The influence of insulin on embryonic mammary tissue has yet to be investigated; however, the medium employed by Hardy(4) could certainly have contained minimal amounts of insulin and/or other hormones and cannot, therefore, be considered established as hormone-free.

The hyperplastic (proliferative) response observed in mammary ducts after 12-day cultivation with insulin is apparently due to the growth-promoting influence of this hormone, which has been established for various tissues *in vitro*(13,14,15,16). Insulin also causes intra-acinar epithelial hyperplasia in organ cultures of mouse prostate(17). The cells which responded maximally to insulin in primary mammary duct cultures were the basal epithelial cells. In these cultures, the basal cells are presumably subject to higher concentrations of insulin than occur *in vivo*, resulting in more rapid proliferation than would occur normally in the intact animal. The difference in the availability of insulin may explain the hyperplasia of the basal cells *in vitro* and their lack of prominence in mammary ducts *in vivo*.

The identity of the basal cells is not finally resolved. Mayer and Klein(9) describe the lining of mammary ducts as possessing two types of cells: an outer layer of myoepithelial cells resting on the basement membrane and a layer of epithelial cells that defines the lumen. The excretory ducts, on the other hand, possess a double-layered epithelium, consisting of a basal layer of cuboidal cells which are not myoepithelial and a superficial layer of columnar cells. There are no reports in the literature on the prenatal and prepubertal development of myoepithelium. Linzell (10) found with silver-staining that the myoepithelium lay outside the basal cells as a third layer in the duct wall in lactating glands. The general appearance of the basal cells described in this study and their reactions in culture strongly suggest a true epithelial nature.

Summary. Primary mammary ducts from immature BALB/cCrgl mice were cultured in synthetic medium "199" with and without hormone supplementation for 5 and 12 days. Tissues cultivated in hormone-free medium showed epithelial regression. The addition of insulin, 10 μg/ml, prevented the degenerative changes occurring in explants cultivated without hormones. In the presence of insulin, proliferation both of epithelial cells and of subjacent stromal elements was prominent. Neither a 10-fold increase in insulin concentration nor the presence of cortisol modified the histologic response to 10 μg/ml of insulin.

I am indebted to Professor Howard A. Bern for valuable advice and criticism, and to Dr. J. J. Elias and Dr. S. Nandi for their suggestions. The microtechnical assistance of Miss Lillian Pissott and the photomicrographic work of Mr. Victor Duran are gratefully acknowledged.

1. Lasfargues, E. Y., *Compt. rend. Soc. Biol.*, 1960, v154, 1720.

2. Prop, F. J. A., *Pathol.-Biol.*, 1961, v9, 640.

3. Koziorowska, J., *Acta Med. Polona*, 1962, v3, 237.

4. Hardy, M., *J. Anat.*, 1950, v84, 388.

5. Lasfargues, E. Y., Murray, M. R., *Dev. Biol.*, 1959, v1, 413.

6. Rivera, E. M., Bern, H. A., *Endocrinology*, 1961, v69, 340.

7. Rivera, E. M., Elias, J. J., Bern, H. A., Napalkov, N. P., Pitelka, D. R., *J. Nat. Cancer Inst.*, 1963, v31, 671.

8. Lilly, R. D., *Histopathologic Technic and Practical Histochemistry*, 1954, Blakiston Co., Inc., p348.

9. Mayer, G., Klein, M., in *Milk: The Mammary Gland and its Secretion*, Academic Press, 1961, v1, p47.

10. Linzell, J. L., *Physiol. Rev.*, 1959, v39, 534.

11. Elias, J. J., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 500.

12. Elias, J. J., Rivera, E. M., *Cancer Res.*, 1959, v19, 505.

13. Leslie, I., *Biochem. J.*, 1952, v52, 21.

14. Chen, J., *J. Physiol.*, 1954, v125, 148.

15. Paul, J., Pearson, E., *J. Endocrinol.*, 1960, v21, 287.

16. Hay, M. F., *J. Physiol.*, 1958, v144, 490.

17. Franks, L. M., *Exp. Cell Res.*, 1961, v22, 56.

Received September 16, 1963. P.S.E.B.M., 1963, v114.