MAINTENANCE AND DEVELOPMENT OF WHOLE MAMMARY GLANDS OF MICE IN ORGAN CULTURE*

EVELYN M. RIVERA

Department of Zoology and its Cancer Research Genetics Laboratory, University of California, Berkeley, California, U.S.A.

(Received 15 November 1963)

SUMMARY

Whole mammary glands from virgin female BALB/cCrgl mice were cultivated in a synthetic medium alone or with various hormone supplements, with or without addition of 5% rat serum. Except for one experiment lasting 12 days, all cultures were carried out for 5–6 days.

Regression of mammary glands occurred in synthetic medium alone and in synthetic medium supplemented with 5% serum only. When aldosterone, insulin, oestradiol and progesterone were added maintenance only was observed. When this combination was supplemented with prolactin and growth hormone, one or more of the following responses was found: more pronounced ductal dilatation than occurred in the absence of pituitary hormones, lateral bud formation, and irregular organization of duct endings. The addition of 5% serum to the latter medium resulted in alveolar development which was more apparent after 12 days than after 5–6 days. The alveolar cells were secretory but clear evidence for lobular organization was not obtained.

INTRODUCTION

Intact whole mammary glands from young female mice have been cultivated in partly defined (serum containing) media supplemented with hormones (Prop, 1961a; Koziorowska, 1962). Both lobulo–alveolar differentiation and epithelial proliferation have been described (Prop, 1961a; Koziorowska, 1962). Chemically defined media (without serum) have been used to study effects of hormones on fragments of immature mammary glands, isolated end buds, and primary ducts (Lasfargues, 1960; Elias, 1961, 1962; Rivera, 1963) and some growth responses were observed (Lasfargues, 1960; Rivera, 1963).

The present study was undertaken to determine the extent to which whole mammary glands from virgin female mice differentiate in a completely defined medium supplemented with hormones and also to determine the effect of serum in the medium. No study was made of the individual effects of the hormones used but the following hormones were tested in a variety of combinations: aldosterone, insulin, oestradiol, progesterone, prolactin, and growth hormone. The occurrence both of survival and of alveolar development is reported.

* Supported by U.S.P.H.S. grants S-TI-CA-5045 and CA-05388.
Cultures were made of the second thoracic pair of mammary glands from virgin female BALB/cCrjgl mice, taken between the ages of 4 and 5 weeks. This strain has not been used previously for a study of this kind. After the animal was killed by cervical dislocation, a midventral incision was made through the skin extending the entire length of the body. The skin in the thoracic region was swabbed with 70% ethanol and painted with a solution of gentian violet to permit visualization of the mammary parenchyma. The glands were exposed by loosening the skin on one side of the body and pinning it on a cork board. After removing the layer of muscle loosely attached to the surface of the second thoracic gland, the entire gland with its fat pad was carefully dissected out for explantation. The gland used for explantation was chosen randomly, and the contralateral gland was similarly removed but fixed and stained for determination of the initial extent of mammary development.

The criterion used to assess survival of the cultivated glands was maintenance of the ductal structure comparable to initial control glands; the criterion used to assess development was the increase in duct ramification and/or the development of alveoli. The whole mounted preparation of each cultivated gland was compared with that of its contralateral control (uncultivated). Histological comparisons were also made in some cases.

Culture medium

The basal culture medium was medium 199 (Hyland Laboratories, Los Angeles, California), a synthetic medium (Morgan, Morton & Parker, 1950). The preparation of the crystalline steroid hormones used (oestradiol-17β, Schering Corporation; progesterone, Schering Corporation; d-aldosterone, Ciba Pharmaceutical Products) has been described elsewhere (Rivera, Elias, Bern, Napalkov & Pitelka, 1963). The protein hormones used (insulin 20 i.u./mg., Dr O. K. Behrens, Eli Lilly and Co.; ovine prolactin 25–30 i.u./mg., Professor C. H. Li; bovine growth hormone 25–30 i.u./mg., Professor C. H. Li) were prepared as described in a previous paper (Rivera & Bern, 1961).

Although cortisol is the adrenal corticoid used in previous mammary gland studies both in vivo and in vitro, it is not a normal secretory product of the mouse adrenal (Hofmann, 1956). However, it has recently been observed that aldosterone has effects similar to cortisol in cultures of mouse mammary lobules (Rivera, 1964). In view of its normal occurrence in mice and its effectiveness in vitro, aldosterone was used in preference to cortisol in this study.

Insulin and aldosterone were included in all hormone-supplemented media to insure survival of the explanted glands (Elias, 1959; Prop, 1961a, Rivera, 1964). In two groups of experiments, medium containing aldosterone and insulin was further supplemented with oestradiol and with oestradiol plus progesterone, respectively, to determine the effects, if any, of ovarian steroids in the absence of pituitary hormones. In a third group the medium was supplemented with aldosterone, insulin, oestradiol and progesterone, prolactin and growth hormone in an attempt to approximate the optimal hormonal milieu for lobulo–alveolar development in vitro, the combination of the latter four hormones having been shown to be effective in vivo.
Organ cultures of whole mammary glands

(Nandi, 1958). Serum was investigated for its effects in the absence of added hormones and in the presence of the preceding hormone combination. The serum was obtained from hypophysectomized male Long–Evans rats and was added in a final concentration of 5%.

The hormone concentrations were in the range found minimally effective in cultures of mammary lobules (Rivera, 1964): aldosterone (1 μg./ml.), insulin (5 μg./ml.), oestradiol (0-01 μg./ml.), progesterone (1 μg./ml.), prolactin (1 μg./ml.), and growth hormone (1 μg./ml.). In one series of experiments the concentrations of prolactin and growth hormone were increased to 50 μg./ml.

Culture method

The watch-glass method of Fell and Robison, as adapted for liquid media by Chen (1954), was used. A mixture of 5% CO₂: 95% O₂ was bubbled through distilled water in the incubator, and the pH of the medium was maintained at about 7-4. The medium was changed on the 3rd day and the experiments were terminated on the 5th or 6th day; in one experiment the medium was changed every 2 days after the first medium change and the cultures were terminated at the end of 12 days. At the end of incubation the cultures were preserved in 15% formalin.

Whole gland preparations

The mammary glands were prepared by a technique developed by Craig & Wilson (1937), as modified in the Cancer Research Genetics Laboratory. After allowing an adequate time for fixation, the glands were defatted in acetone, hydrated, stained with iron-haematoxylin, dehydrated, cleared in toluene, and stored in methyl salicylate.

RESULTS

Mammary development in 4 to 5-week-old virgin female BALB/cCrjgl mice

(uncultivated glands)

The second thoracic pair of mammary glands consisted of irregularly branched ducts terminating in numerous end buds, which were usually swollen and had densely-stained ‘caps’ (Pl. 1, figs. 1, 3, 7 and 9) but were sometimes small (Pl. 1, fig. 5; Pl. 2, figs. 15 and 17). The caps of the end buds represent several layers of undifferentiated epithelial cells (Elias, 1962). Littermates within each age group showed some variation in the extent of mammary duct branching and the area of mammary fat pad occupied. However, the overall differences in the extent of mammary development between 4–5 weeks was not as pronounced as reported for mice between 4–6 weeks of age (Flux, 1954).

Responses of whole mammary glands in vitro

(1) Cultivation in unsupplemented synthetic medium

Six glands from 4-week-old mice were each cultivated for 6 days. Mammary gland regression occurred following culture in medium without added hormones (Pl. 1, cf. figs. 1 and 2). End buds and terminal ducts were no longer evident, and the larger ducts showed only as indistinct outlines.
(2) Effect of medium containing aldosterone + insulin + oestradiol

Ten glands from 5-week-old mice were each cultivated for 6 days. The structural integrity of the glands was maintained but some of the ducts were slightly dilated, and the duct walls appeared thinner than in the controls (Pl. 1, cf. figs. 3 and 4). The responses of the end buds were not uniform: in some instances dilatation occurred and decrease in size was noted in others. As a rule, the end bud caps were no longer seen.

(3) Effect of medium containing aldosterone + insulin + oestradiol + progesterone

Ten glands from 5-week-old mice were each cultivated for 6 days. Cultures were maintained and the general morphology was similar to that seen in the absence of progesterone, as described above (Pl. 1, cf. figs. 5 and 6).

(4) Effect of medium containing aldosterone + insulin + oestradiol + progesterone + prolactin + growth hormone

Twenty-eight glands from mice 4 to 5 weeks old, were each cultivated for 5 or 6 days. Mammary cultures were maintained and showed one or more of the following responses: ductal dilatation, formation of small lateral buds, and irregular indentation of the duct endings (Pl. 1, cf. figs. 7 and 8, 9 and 10). As in glands cultured without pituitary hormones, most of the end bud caps evident before culture were not seen. The lumina of many end buds contained densely staining material (Pl. 1, figs. 8 and 10).

An increase in pituitary hormone concentration to 50 µg./ml. did not improve the responses of the mammary glands.

(5) Effect of serum-supplemented medium

Eight glands from 4-week-old mice were each cultivated for 6 days. In medium 199 supplemented only with 5% serum, the ducts were usually preserved but were considerably thinner than before culture (Pl. 2, cf. figs. 11 and 12). The end buds appeared to be undergoing degeneration, and the general picture was that of early regression.

(6) Effect of serum-supplemented medium containing aldosterone + insulin + oestradiol + progesterone + prolactin + growth hormone

Glands from twenty-four mice, 4 to 5 weeks old, were cultivated; twelve glands for 6 days and twelve glands for 12 days. After 6 days, irregular bud-like structures were seen at the periphery of duct walls and duct ends (Pl. 2, cf. figs. 13 and 14). After 12 days, these structures appeared alveolar (Pl. 2, cf. figs. 15 and 16). The histological appearance of these alveoli resembled that of alveoli seen in mammary lobules in mice before lactation. The cells were vacuolated and the lumina of the alveolar structures contained stainable secretion (Pl. 2, cf. figs. 17 and 18).

DISCUSSION

The regression of whole mammary glands in unsupplemented synthetic medium and in synthetic medium supplemented only with 5% serum was in accord with previous observations (Prop, 1961a; Koziorowska, 1962). The integrity of portions of the large ducts and the complete disappearance of the terminal ducts and end buds
in synthetic medium indicate that the growing extremities of the mammary tree were more sensitive to the hormonal deficiency than were the large ducts. Observations on cultures of isolated portions of immature glands lend support to this view of differences in hormone-dependence in different parts of the mammary gland: end buds degenerated after cultivation with medium 199 alone (Elias, 1962), whereas isolated primary ducts retained their integrity for a comparable period in vitro (Rivera, 1963).

Whole glands could be maintained in a medium supplemented with aldosterone, insulin, and oestradiol with or without progesterone, but no further development occurred. In accord with the negative results of Prop (1961b) oestradiol and progesterone produced little or no effect in the concentrations used, in as much as the ductal maintenance observed in these experiments could possibly be attributed to the aldosterone and insulin present (Prop, 1961a). It is, however, possible that insulin may have suppressed an action of oestradiol as suggested by Koziorowska (1962). Oestrogen alone has been reported to stimulate growth of the mammary epithelium and, in combination with progesterone, to cause slight lobular development (Lasfargues, 1960). Comparison with previous work is, however, limited by the use of different basal culture media and different strains of mice.

The main distinction between glands cultured with prolactin and growth hormone (in the presence of aldosterone, insulin and ovarian steroids) and those cultured without the pituitary hormones lies in the appearance of lateral duct buds and in the pronounced ductal dilation in some of the glands cultivated with pituitary hormones. Although the development of ductal buds, as indicated in Pl. 1, fig. 10, shows that some degree of differentiation may be obtained in the above medium, it is uncertain whether this finding represents truly normal differentiation since it is not usually seen in vivo. Conceivably, lateral budding can be stimulated at more sites on the mammary tree in vitro than in the intact animal. The greater degree of ductal dilatation in mammary glands cultivated in the presence of pituitary hormones may be a reflexion of the response to prolactin and growth hormone, although insulin plus cortisol (Prop, 1961a) or oestradiol alone (Koziorowska, 1962) may also elicit this response.

The use of medium containing serum in addition to pituitary and other hormones resulted in differentiation of a more advanced type than occurred in the absence of serum. The development of alveoli was more apparent after 12 days than after 6 days in vitro in the presence of serum. Extending the culture period of glands cultivated with various combinations of hormones but without serum might also have resulted in alveolar development correspondingly greater than that seen after the shorter period but, unfortunately, the present investigation could not cover the point. However, as judged from the results after 5–6 days in culture, serum unquestionably increases the response of the glands to the hormonal supplement. Although the alveolar structures which developed in vitro were not organized into discrete lobules as seen in mammary glands from pregnant mice, their secretory appearance suggests that the component cells were responding as true alveolar epithelium. The ineffectiveness of serum alone indicates that serum may provide essential growth factors whose activity in mammary differentiation requires the presence of hormones in the culture medium.
I wish to express my appreciation to Professor Howard A. Bern for his valuable suggestions regarding this study, to Dr Satybrata Nandi and Dr Joel J. Elias for their advice, to Miss Donna Brown for skilled assistance, and to Mr John Soubier for the whole gland photography.

REFERENCES


DESCRIPTION OF PLATES

PLATE 1

Mounts of mouse mammary gland stained with iron-haematoxylin. × 11.

Fig. 1. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing numerous end buds.

Fig. 2. Whole mount of mammary gland contralateral to that shown in fig. 1, cultivated for 6 days with unsupplemented medium '199'. Note regression of the gland.

Fig. 3. Whole mount of mammary gland, uncultivated, from a 5-week-old female, showing numerous end buds.

Fig. 4. Whole mount of mammary gland contralateral to that shown in fig. 3, cultivated for 6 days with medium containing aldosterone, insulin, and oestriadiol. Note maintenance with slight duct and end bud dilatation and disappearance of end bud caps.

Fig. 5. Whole mount of mammary gland, uncultivated, from a 5-week-old female, showing small, inconspicuous end buds.

Fig. 6. Whole mount of mammary gland contralateral to that shown in fig. 5, cultivated for 6 days with medium containing aldosterone, insulin, oestriadiol, and progesterone. Note similarity to fig. 4.
Fig. 7. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing well-developed end buds.

Fig. 8. Whole mount of mammary gland contralateral to that shown in fig. 7, cultivated for 6 days with aldosterone, insulin, oestradiol, progesterone, prolactin, and growth hormone. Note dilatation, lateral buds, and densely-stained contents of end buds.

Fig. 9. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing well-developed end buds.

Fig. 10. Whole mount of mammary gland contralateral to that shown in fig. 9, cultivated with medium as described for fig. 8. Note lateral buds and irregular shapes of some duct endings.

Plate 2

Fig. 11. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing small and large end buds. × 11.

Fig. 12. Whole mount of mammary gland contralateral to that shown in fig. 11, cultivated for 6 days with medium supplemented with 5% serum, showing regressed appearance. × 11.

Fig. 13. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing well-developed end buds. × 11.

Fig. 14. Whole mount of mammary gland contralateral to that shown in fig. 13, cultivated for 6 days with aldosterone, insulin, oestradiol, progesterone, prolactin, and growth hormone and 5% serum. Note irregular budding at duct endings. × 11.

Fig. 15. Whole mount of mammary gland, uncultivated, from a 4-week-old female, showing small and large end buds. × 11.

Fig. 16. Whole mount of mammary gland contralateral to that shown in fig. 15, cultivated for 12 days with medium as described for fig. 14. Note alveolar development in lower half of gland. × 11.

Fig. 17. Section through small duct ending from mammary gland shown in fig. 15 to show appearance before culture. × 400.

Fig. 18. Section through duct ending with alveoli from mammary gland shown in fig. 16 to show secretory appearance. × 400.