

# Effects of Perinatal Testosterone on Mouse Mammary Duct Morphology and Lobule Induction *in Vitro*<sup>1</sup>

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## ABSTRACT

The effects of several hormone supplements to the chemically defined medium were assessed in mammary explants from C57BL/Crgl mice that had been given injections of 25  $\mu$ g testosterone on each of the first 5 days of life and in mammary tissues of uninjected controls. Tissues were organ cultured the fourth week of life after 4, 6, and 9 days of pretreatment with 1  $\mu$ g 17 $\beta$ -estradiol plus 1 mg progesterone. Alveolar and lobular development were similar in tissues of perinatally testosterone-injected (T) mice and their controls after *in vivo* pretreatment and after 4 or 9 days of pretreatment followed by culture in all media tested. Both alveolar and lobular development were inhibited in tissues from T mice after 6 days of pretreatment followed by culture with optimal medium for lobular differentiation (17 $\beta$ -estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin, and thyroxine). After 6 days of pretreatment, explants from T mice responded similarly to controls after culture with suboptimal media (insulin and thyroxine, or estrogen, progesterone, aldosterone, insulin, and thyroxine). Perinatal injection of testosterone, a treatment which has been reported to promote mammary tumorigenesis, did not promote *in vitro* differentiation of mammary lobules; indeed, significant inhibition of lobule formation was noted at 6 days of pretreatment. A 6-day minimal threshold effect for expression of pretreatment may be involved.

Duct diameter increased in explants from T mice after culture in several medium and pretreatment groups. Differences in duct responses were markedly uniform throughout the explant in cultured tissues of T mice. In view of the fact that mammary ducts have been implicated as sites of origin of preneoplastic and neoplastic change, duct responses are of particular interest in relation to subsequent promotion by various endogenous and exogenous factors of the neoplastic potential of mammary tissues of perinatally testosterone-injected mice.

## INTRODUCTION

Tumor incidence and hormone responsiveness may be altered in a variety of target tissues as the result of exposure of the conceptus or newborn to steroid hormones (2, 3, 10, 22). Diethylstilbestrol, 17 $\beta$ -estradiol, various progestins, and testosterone have been implicated both in humans and

in animal model systems (1, 4, 8, 21).

Female mice that have been given injections of estradiol as newborns develop more mammary dysplasias (27) and tumors (8, 14). Prior to developing neoplasms, mammary glands of E<sup>3</sup> mice grow faster *in vivo* (24) and develop more lobules *in vitro* in response to exogenous hormones (25, 28). Testosterone has been used to suppress lactation in women who do not wish to breast-feed or who wish to alleviate postpartum engorgement in order to nurse (23). In contrast, systemic injection of testosterone into newborn rats stimulates the mammary glands and induces cystic, secretory, milky alveoli (20). T rats develop fewer mammary tumors later in life after systemic feeding of chemical carcinogen (18). Female mice given injections of testosterone as newborns have an increased incidence of spontaneous mammary tumors late in life (4).

The following experiments were performed to show whether mammary explants of T mice (like those of E mice) were more sensitive to hormones in organ culture early in life and whether similar mammary pathological mechanisms might therefore have been invoked by the respective perinatal treatments.

## MATERIALS AND METHODS

**Animals.** Newborn and immature female C57BL/Crgl mice were studied. This strain lacks and is resistant to infection with murine mammary tumor virus (16). Mice were housed, 5/cage, with their mothers. With the exception of uninjected controls, all mice were given daily s.c. injections of 25  $\mu$ g of testosterone in 0.02 ml of aqueous microsuspension in the interscapular region for 5 consecutive days, starting within 24 hr after birth. This regimen results in 100% ovary-independent, vaginal cornification (9).

**Pretreatment.** Beginning at 4 weeks of age, control and experimental mice were given daily s.c. injections of 1  $\mu$ g of 17 $\beta$ -estradiol and 1 mg of progesterone in 0.2 ml of aqueous microsuspension (7) for 4, 6, or 9 days.

**Experimental Groups.** In 26 groups, 727 explants from 530 mice were examined. Pretreated mice were killed by cervical dislocation 24 hr after the last hormone injection. Several mammary glands from each mouse were immediately fixed in 15% formalin to determine the effect of pretreatment on morphology. The remaining glands were grown in organ culture with various hormone supplements. Each experiment was repeated at least once. A list of various control and experimental groups follows: (a) mammary morphology in nonpretreated control and neonatally testosterone-injected mice at 4 weeks of age, the age at

<sup>3</sup> The abbreviations used are: E, host or donor injected with 25  $\mu$ g of 17 $\beta$ -estradiol per day for each of the first 5 days of life; T, host or donor injected with 25  $\mu$ g of testosterone per day for each of the first 5 days of life.

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which pretreatment was begun for organ culture studies, or at 5.5 weeks of age, the age at which pretreatment was completed and glands were cultured; (b) effect of 4, 6, or 9 days of pretreatment with estrogen plus progesterone on *in vivo* mammary morphology of control and neonatally testosterone-injected mice; (c) effect of 4, 6, or 9 days of pretreatment followed by culture on *in vivo* mammary morphology of normal and neonatally testosterone-injected mice.

The basic culture medium was supplemented with: (a) insulin and thyroxine; (b) prolactin, growth hormone, insulin, and thyroxine; (c) estrogen, progesterone, aldosterone, insulin, and thyroxine; (d) estrogen, progesterone, aldosterone, prolactin, growth hormone, insulin, and thyroxine.

Hormones used for injection and in culture media were as described by Warner (26), and whole mammary glands were similarly explanted and cultured. At Day 2.5 to 3 of incubation, medium was removed and replaced with fresh medium. Tissues were fixed in Tellyesniczky's fluid on Day 5.

**Culture Medium.** Chemically defined Waymouth's Medium MB 752/1 (Hyland Labs, Los Angeles, Calif.) plus 0.002% phenol red, to which penicillin G (50 IU/ml) and L-glutamine (350 µg/ml) had been added, was the basic medium for all cultures. Hormones [17β-estradiol (0.001 µg/ml); progesterone or aldosterone (1 µg/ml); prolactin, growth hormone, insulin, or thyroxine (5 µg/ml)] were added to the basic medium (6). Thyroxine was dissolved in 0.001 N NaOH and sterilized by passing it through a 0.45-µm Millipore filter.

**Evaluation of Results.** Mammary glands were fixed, stained as whole mounts with iron hematoxylin, examined, and photographed in methyl salicylate (12). Results of

experiments were evaluated on the basis of gross morphological differences between the tissues of the various groups examined (28). Lobular or alveolar development was graded from 0 to +++, subjectively, on whole-mount preparations: 0, no lobules or no alveoli; +, few small lobules or few alveoli in less than one-half the area of the explant; ++, lobules or alveoli in about one-half the explant; +++, one-half or more of the explant had lobules or alveoli typical of an early lactating mammary gland. Ducts were classified as normal, thin, or dilated, according to whether they were greater, less, or equivalent in diameter in comparison with ducts of normal, unpretreated, and noncultured controls. Fig. 6 illustrates a normal-diameter duct as found in experimental tissue.

The Mann-Whitney *U* test was used to determine statistical significance (19).

**RESULTS**

There were no significant differences in alveolar or lobular development between tissues from neonatally testosterone-injected mice and uninjected controls at 4 weeks of age (initial controls), at 5.5 weeks of age (final controls), or after pretreatment with estrogen plus progesterone for 4, 6, or 9 days prior to culture. Morphology of normal controls has been published previously (25, 28). Cultured explants also had similar numbers of lobules and alveoli in T and uninjected control groups (Charts 1 and 2; Table 1) with the following exceptions. After 6 days of pretreatment followed by culture with optimal medium (17β-estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin and thyroxine), T group explants developed fewer alveoli and fewer lobules than did explants from the uninjected control

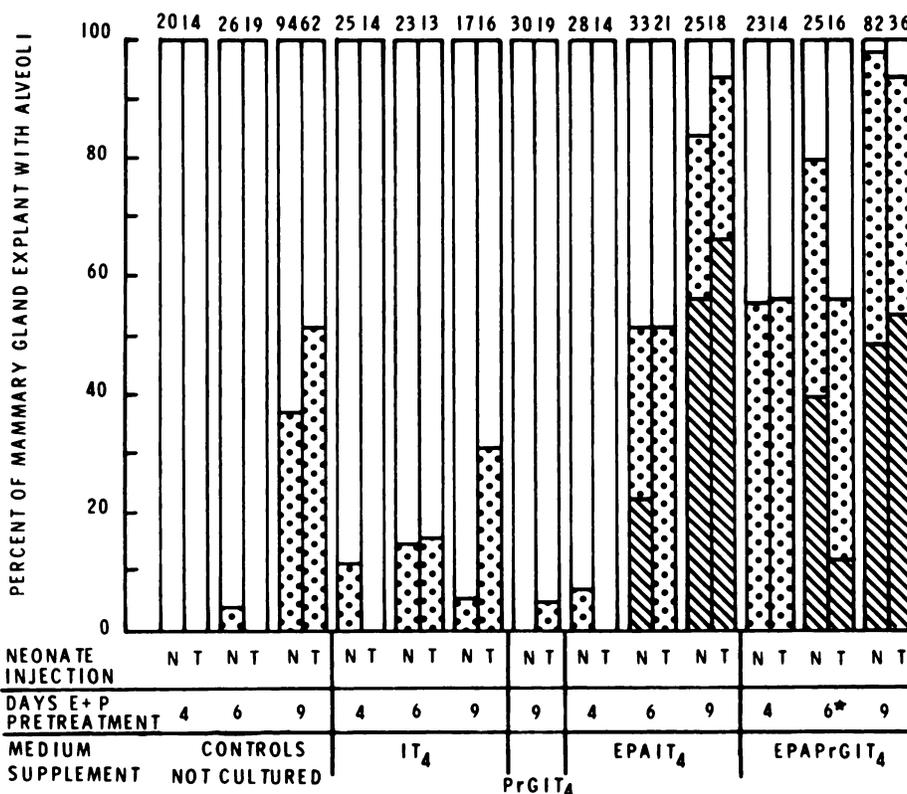


Chart 1. Effects of injection of newborn female C57BL/Crgl mice with testosterone on mammary alveolar development after 4, 6, or 9 days of hormone pretreatment of donors followed by subsequent organ culture with various hormone-supplemented media. Mice were given daily injections of 25 µg of testosterone for each of the first 5 days of life (T). N, uninjected controls. Pre-treatment was begun at 4 weeks of age. One µg of 17β-estradiol and 1 mg of progesterone in aqueous microcrystal suspension were injected s.c. for 4, 6, or 9 consecutive days. The following hormones were added to the culture medium: 17β-estradiol (E) (0.001 µg/ml); progesterone (P) (1 µg/ml); aldosterone (A) (1 µg/ml); prolactin (Pr) (5 µg/ml); growth hormone (G) (5 µg/ml); insulin (I) (5 µg/ml); thyroxine (T<sub>4</sub>) (5 µg/ml). Numbers above the bar, number of explants per group, open bars, no alveoli, dotted bars, percentage of explants less than one-half of which had alveoli; hatched bars, one-half of the area of these explants had alveoli. The Mann-Whitney *U* test was used to determine significance among groups. \*, *p* < 0.05.

Chart 2. Effects of injection of newborn female C57BL/Crgl mice with testosterone on mammary lobuloalveolar development after 4, 6, or 9 days of hormone pretreatment of donors followed by subsequent organ culture with various hormone-supplemented media. *Open bars*, no lobules; *dot- ted bars*, percentage of explants less than one-half of which were lobular; *hatched bars*, one-half of the area of these explants had lobules; *solid bars*, percentage of explants which had one-half or more lobular development. The Mann-Whitney *U* test was used to determine significance among groups. ★,  $p < 0.05$ . For experimental procedures, dosages, and abbreviations, see legend to Chart 1.

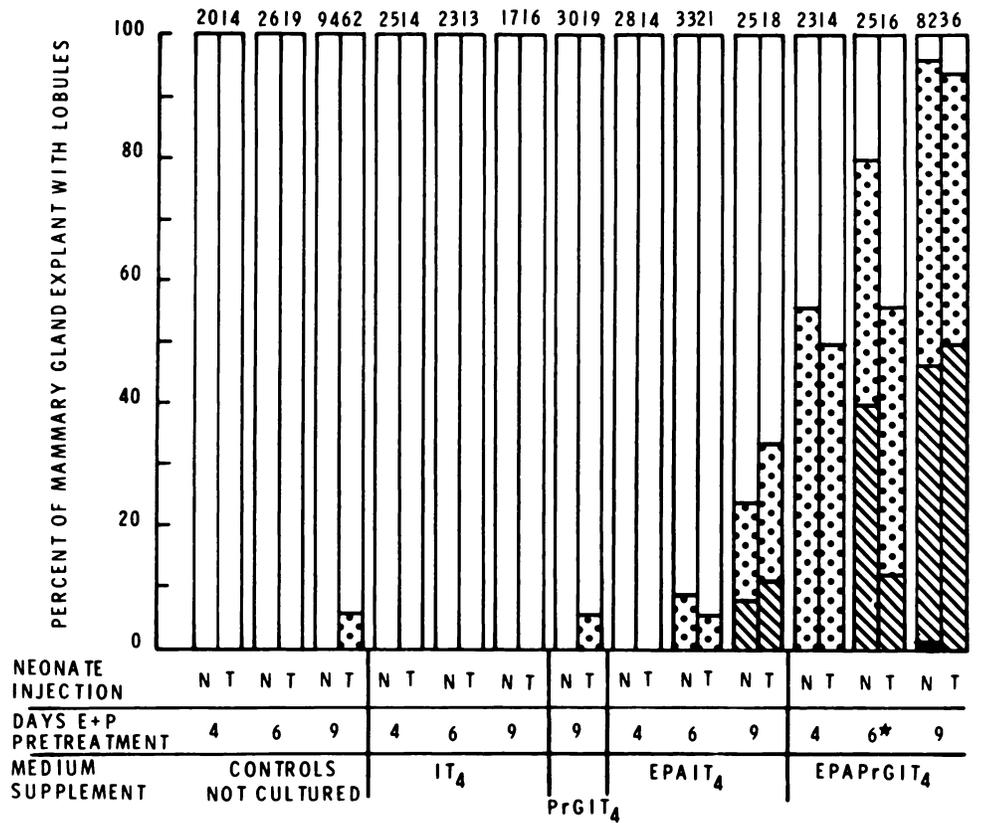
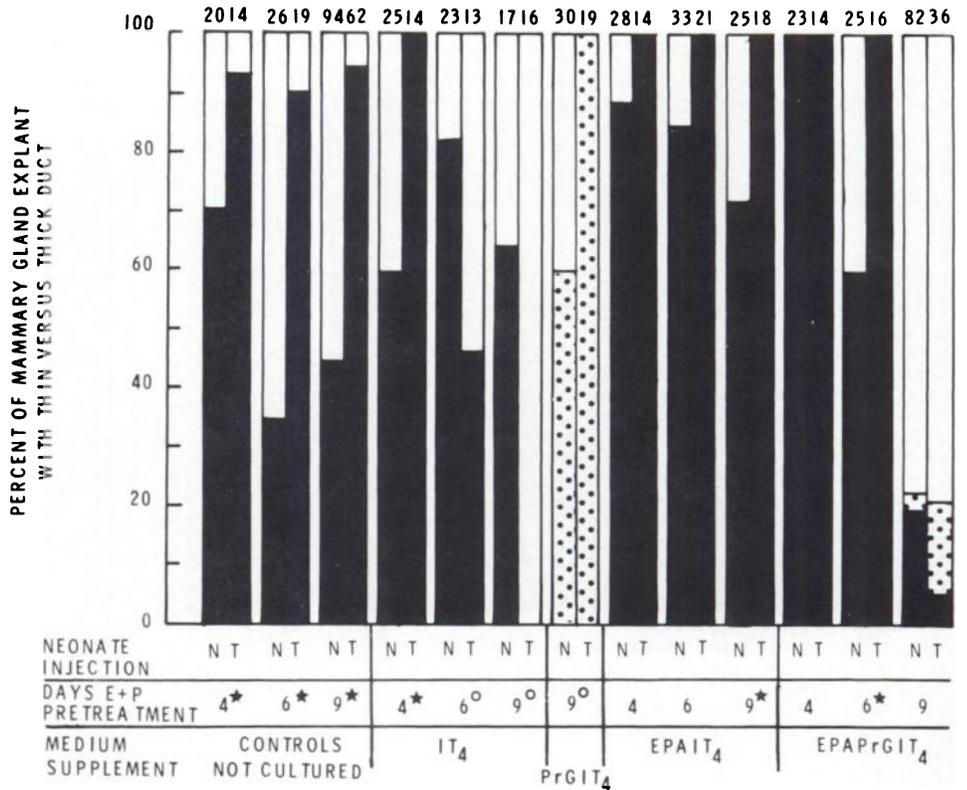


Chart 3. Effects of injection of newborn female C57BL/Crgl mice with testosterone on mammary ductal dimensions after 4, 6, or 9 days of hormone pretreatment of donors followed by subsequent organ culture with various hormone-supplemented media. *Dotted bars*, percentage of explants with thin ducts; *solid bars*, percentage of explants with dilated ducts; *open bars*, percentage of explants with normal ducts. The Mann-Whitney *U* test was used to determine significance among groups. ★,  $p < 0.05$  for normal < testosterone-treated mice; °,  $p < 0.05$  for normal > testosterone-treated mice. For experimental procedures, dosages, and abbreviations, see legend to Chart 1.



group ( $p < 0.05$ ) (Charts 1 and 2; Table 1).

Duct dimensions of T explants and explants from uninjected controls varied significantly before and after culture after 4, 6, or 9 days of pretreatment (Chart 3; Table 1). After

4 days of pretreatment followed by culture, dilated ducts were more frequent in T explants in all media tested (Figs. 1 to 4). Increased diameter of dilated ducts was due primarily to fluid accumulation in the lumina (Fig. 3). Response

Table 1

Comparison of effect of duration of pretreatment on alveolar, lobular, and ductal morphology in mammary explants from neonatally testosterone-injected versus control C57BL mice after culture with various hormone supplements

C57BL/Crg1 (T) mice were given for the first 5 days of life daily injections of 25 µg of testosterone. Control (N) mice were not given injections. Pretreatment was begun at 4 weeks of age, and 1 µg of 17β-estradiol and 1 mg of progesterone were injected for 4, 6, or 9 consecutive days. See "Materials and Methods" for doses of hormones added to the culture medium.

Neonate injection	Duration of <i>in vivo</i> pre-treatment (days)	Medium supplement	No. of ex-plant	Alveoli present		Lobules present		Duct	
				Response	% of ex-plant	Response	% of ex-plant	Response	% of ex-plant
N	4	<sup>b</sup>	20	0 <sup>c</sup>	100	0	100	N <sup>d</sup>	30 <sup>e</sup>
T	4	<sup>b</sup>	14	0	100	0	100	D	70
N	6	<sup>b</sup>	26	0	96	0	100	N	7 <sup>d</sup>
T	6	<sup>b</sup>	19	0	100	0	100	D	93
N	9	<sup>b</sup>	94	0	63	0	100	N	65 <sup>d</sup>
T	9	<sup>b</sup>	62	0	48	0	95	D	35
N	4	IT <sub>4</sub>	25	0	84	0	100	N	10 <sup>d</sup>
T	4	IT <sub>4</sub>	14	0	100	0	100	N	89
N	6	IT <sub>4</sub>	23	0	87	0	100	D	55 <sup>d</sup>
T	6	IT <sub>4</sub>	13	0	85	0	100	N	45
N	9	IT <sub>4</sub>	17	0	94	0	100	N	6 <sup>d</sup>
T	9	IT <sub>4</sub>	16	0	69	0	100	D	94
N	9	PrGIT <sub>4</sub>	30	0	100	0	100	N	40 <sup>d</sup>
T	9	PrGIT <sub>4</sub>	19	0	95	0	95	D	60
N	4	EPAIT <sub>4</sub>	28	0	93	0	100	N	0 <sup>e</sup>
T	4	EPAIT <sub>4</sub>	14	0	100	0	100	Th	100
N	6	EPAIT <sub>4</sub>	33	0	43	0	91	N	11
T	6	EPAIT <sub>4</sub>	21	0	49	0	95	D	89
N	9	EPAIT <sub>4</sub>	25	0	16	0	76	N	0
T	9	EPAIT <sub>4</sub>	18	0	6	0	77	D	100
N	4	EPAPrGIT <sub>4</sub>	23	0	44	0	44	N	28 <sup>d</sup>
T	4	EPAPrGIT <sub>4</sub>	14	0	43	0	50	D	72
N	6	EPAPrGIT <sub>4</sub>	25	0	20	0	20	N	0 <sup>d</sup>
T	6	EPAPrGIT <sub>4</sub>	16	0	44	0	44	D	60
N	9	EPAPrGIT <sub>4</sub>	82	0	2	0	4	N	40 <sup>d</sup>
T	9	EPAPrGIT <sub>4</sub>	36	0	5	0	5	Th	4

<sup>a</sup> N, normal; D, dilated; I, insulin; T<sub>4</sub>, thyroxine; Pr, prolactin; G, growth hormone; Th, thin; E, 17β-estradiol; P, progesterone; A, aldosterone.

<sup>b</sup> *In vivo* pretreatment controls.

<sup>c</sup> 0, no lobules; +, few small alveoli or lobules in less than one-half of the explant; ++, alveoli or lobules in one-half of the explant; +++, one-half or more of explant has lactating alveoli or lobules.

<sup>d</sup>  $p < 0.05$  for N < T duct, or  $p < 0.05$  for alveolar or lobular development (Mann-Whitney *U* test).

<sup>e</sup>  $p < 0.05$  for N > T duct (Mann-Whitney *U* test).

was mixed after 6 days of pretreatment. Ducts of T explants were thinner after culture with insulin and thyroxine; they were similar to explants from uninjected controls after culture with estradiol, progesterone, aldosterone, insulin, and thyroxine; and they were more dilated than in explants from uninjected controls after culture with all hormones (estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin and thyroxine). After 9 days pretreatment, ducts of most T explants were thinner than those of controls after culture with either insulin and thyroxine, or prolactin, growth hormone, insulin and thyroxine. Fig. 5 shows typical duct and lobular configurations for a T explant after 9 days of pretreatment followed by culture with estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin, and thyroxine. Ducts of T explants were more often dilated than those of uninjected controls at 9 days of pretreatment and after culture with estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin, and thyroxine. Fig. 6 shows a normal duct in a T explant.

## DISCUSSION

These experiments show that mammary tissues of control mice given injections of testosterone as newborns resemble mammary tissues of control mice given injections of progesterone as newborns (26). In a previous study, I showed that perinatal injection of progesterone does not uniformly influence mammary lobular development *in vitro* (26). No significant ductal responses were seen in perinatally progesterone-injected mice. Perinatal injection of  $17\beta$ -estradiol resulted in enhanced alveolar and lobular development in E mice over uninjected controls after 6 or 9 days of pretreatment followed by culture with differentiation-inducing combinations of hormones (25, 28). Ductal responses were not described *in vitro* in E or perinatally progesterone-injected mice.

Mammary glands of T mice lack the uniformly enhanced lobular responsiveness to mammotropic hormones *in vitro* that is characteristic of mice given injections of  $17\beta$ -estradiol as newborns (25, 28). One incidence of alveolar lobular deficit which fits the classic expectation of testosterone suppression of mammary differentiated function (17) was observed in the present study. Fewer lobules may have developed in T explants than in uninjected controls after 6 days of pretreatment followed by culture in optimum medium (estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin, and thyroxine) because 6 days of pretreatment with 1  $\mu$ g of estradiol plus 1 mg of progesterone is the minimum time found by Wood *et al.* (29) for subsequent full *in vitro* induction of lobules. In the present study, this minimum threshold for lobule induction (6 days of *in vivo* pretreatment) may have been sensitive to suppression by an effect of perinatal testosterone, whereas the full (prolonged 9-day course) *in vivo* pretreatment could have overwhelmed this postulated threshold effect in mammary glands of perinatally androgenized females. In a previous series of experiments (26), newborns given injections of progesterone showed enhanced mammary lobular responses after culture with optimal media following 6 days of pretreatment. Prolongation of pretreatment past the 6-day threshold also obscured the effect of perinatal progesterone.

The ductal portion of the mammary glands of mice, rats, women, and female dogs represents an important site of neoplasia (5, 6, 11, 13). Primarily, fluid balance (cystic), rather than proliferative, changes were seen in ducts in the present series. The differences in duct responses between explants from controls and explants from androgenized donors were marked. Present *in vitro* findings of enhanced cystic responses of the mammary ducts of perinatally androgenized female mice agree with the *in vivo* observations of Nagasawa *et al.* (15) in androgenized female mice. My *in vitro* experiments reveal duct effects at an earlier age (5.5 weeks *versus* 2 to 7 months for mice studied by Nagasawa *et al.*). Differences between *in vivo* and *in vitro* findings may reflect an enhancing effect of pretreatment hormones on duct responses in my mice, may be due to strain differences in the animals studied, or may show a particular ability of the *in vitro* situation to reveal differences in duct sensitivity to hormones earlier in life. Determination of the hormonal basis for differential duct responses after perinatal injection of testosterone should contribute to understanding of both mammary physiology and mammary pathology.

Previously reported enhanced mammary lobular responses *in vitro* in both BALB/c and C57BL strains after perinatal estradiol (25, 28) were not observed after perinatal testosterone in the present study. Mice in the present study lacked the mammary tumor virus. Mori *et al.* (14) have observed in mice that the presence of both mammary tumor virus and intact ovaries is necessary for expression of differences in hormone response and neoplastic potential of mammary tissues. When newborn BALB/cfC3H mice which express mammary tumor virus are given injections of  $17\beta$ -estradiol, progesterone, or testosterone, they develop more mammary tumors at an earlier age (3, 4, 8, 14). Mammary tissues of BALB/c mice which do not express virus and which were given injections of  $17\beta$ -estradiol as newborns also develop more duct dysplasias after administration of chemical carcinogens (27). As early as the first or second month of life, mammary tissues of C57BL or BALB/c mice are more sensitive to both endogenous hormones (24) and exogenous hormones in organ culture (25, 26, 28).

The present experiments provide further evidence that one of the early effects of perinatal testosterone is on mammary duct responses (15). Present experiments also suggest that mammary effects of perinatal androgenization of mice may be more like the action of perinatal progesterone than the action of perinatal estrogen because there may be an interaction with systemic, viral, or genetic factors which could involve several steps subsequent to the initial perinatal exposure to testosterone.

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Fig. 1. Four-day pretreatment, control group tissue, after culture with basic medium supplemented with insulin and thyroxine. Note regional cystic dilation of duct (arrow), presence of ductal buds and a few alveoli, but not of lobules. DB, ductal buds; LN, lymph node. Iron hematoxylin,  $\times 20$ .

Fig. 2. Four-day pretreatment, perinatal testosterone group tissue after culture with basic medium supplemented with insulin and thyroxine. Note the extreme cystic dilation of ducts and the absence of other morphological structures in comparison with Fig. 1. Iron hematoxylin,  $\times 20$ .

Fig. 3. Histological section of explant shown in Fig. 2. Duct lumina are dilated. Their lining epithelial cells have not proliferated. Masson Trichrome,  $\times 160$ .

Fig. 4. Four-day pretreatment, perinatal testosterone group tissue after culture with basic medium supplemented with estradiol, progesterone, aldosterone, growth hormone, prolactin, insulin, and thyroxine (all hormones). Dilated ducts, alveoli, and a few lobules, but no end buds, are present. Dilation was less in normal controls. L, lobule; LN, lymph node. Iron hematoxylin,  $\times 20$ .

Fig. 5. Nine-day pretreatment, perinatal testosterone group tissue after culture with basic medium supplemented with estradiol, progesterone, aldosterone, insulin, and thyroxine. Alveoli, end buds (arrow), and ducts are all dilated. Dilation was not characteristic of control group tissues. LN, lymph node. Iron hematoxylin,  $\times 20$ .

Fig. 6. Nine-day pretreatment, perinatal testosterone group tissue after culture with all hormones. Alveoli and lobules were well developed, and ducts are of normal diameter. Control group tissues were similar. Iron hematoxylin,  $\times 20$ .

