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# Critical Period for Neonatal Estrogen Exposure in Occurrence of Mammary Gland Abnormalities in Adult Mice[1] (41552)

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*Abstract.* There exists a critical period for the development of cervicovaginal lesions in both mice and humans exposed neonatally and antenatally to sex hormones. Mammary glands from year-old female BALB/c mice exposed neonatally to 20 μg estradiol for 5 days commencing at 1 day of age showed the most mammary abnormalities, significantly greater than in controls ($P < 0.005$). The incidence of abnormalities declined when treatment was begun after Day 1. Treatments begun after Day 3 did not result in this structural pattern. Mice ovariectomized after treatment all had inactive mammary glands with no abnormalities. There is a critical exposure period for the later occurrence of mammary gland abnormalities. However, the aberrant secretory state which accompanies these mammary gland alterations may be a consequence of permanent alteration in ovarian function or its endocrine control.

Women may develop clear cell vaginal adenocarcinoma as a result of diethylstilbestrol (DES) exposure *in utero* when the exposure has occurred within the first trimester of pregnancy (1). A critical period also exists for the development of cervicovaginal pathologies in the mouse exposed neonatally to sex hormones. If sex hormone treatment is not administered within the first 3 days after birth, there is a dramatic drop in the incidence of persistent vaginal cornification in mice of the C57BL/Ms strain (2) and of heterotopic columnar epithelium in the upper vagina in mice of the NMRI stock (3). The aim of the present study was to examine the possible existence of a critical period for exposure of the neonatal mouse mammary gland to hormones, analogous to that which exists in the reproductive tract.

**Materials and Methods.** Five groups, each consisting of about 40 female newborn BALB/cCrgl mice (mammary tumor virus-unexpressed), were given five daily subcutaneous injections of 20 μg 17β-estradiol ($E_2$) (Cal Biochem, La Jolla, Calif.) in 0.02 ml sesame oil (Hain's cold pressed) or the sesame oil vehicle alone beginning at 1, 2, 3, 5, or 10 days of age. This dose is known to induce ovary-independent changes in the female mouse vagina; owing to binding by α-fetoprotein, large amounts of estradiol must be used to be effective (cf. (4)). About one-half of the mice in each of the 10 treatment groups were ovariectomized at 40 days of age. Mice were maintained at a constant photoperiod of 12 hr light and 12 hr dark and given food (Berkeley Diet—roughly equivalent to other standard rodent chows: at least 20% protein and 7% fat) and water *ad libitum*. All mice were killed at 12 months of age.

Mammary glands attached to the skin were fixed in 10% formalin. After removal from the skin, the glands were stained with iron–hematoxylin and examined blindly as whole-mounts. Subsequently, representative areas of mammary gland were embedded in paraffin, sectioned at 7 μm and stained with hematoxylin and eosin.

**Results.** Mammary abnormalities were indicative of an aberrant secretory state. The major ducts were often dilated. Dilated portions of both the major and lateral ducts frequently had a beaded appearance and were filled with secretion (Fig. 1), which often appeared white (milk-like) in unfixed glands. The beaded ducts were incompletely segmented structures lined by flattened epithelium surrounding basophilic secretion masses (Fig. 2), histologically resembling dilated lactating

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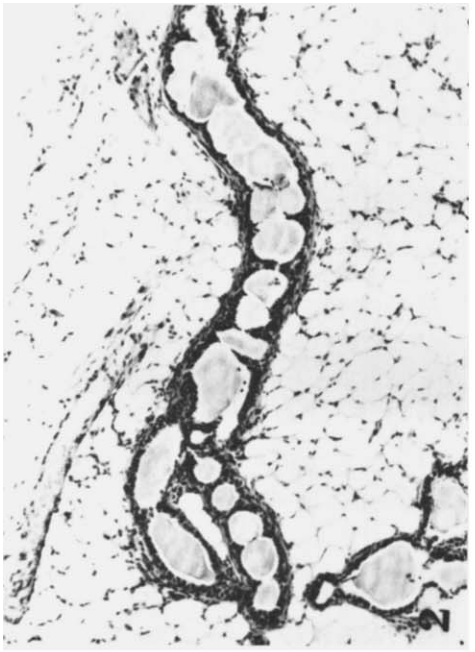

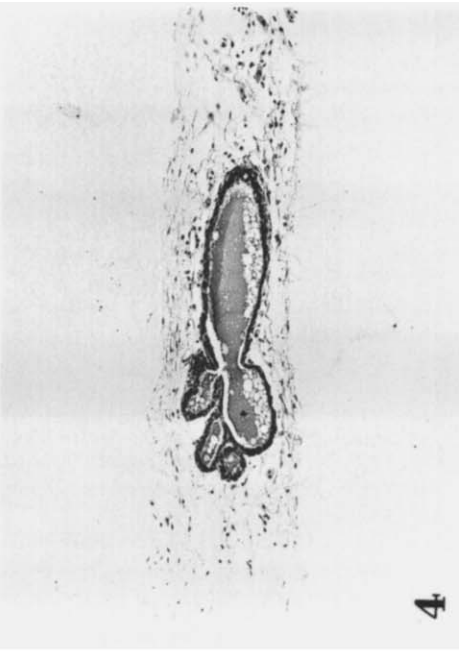

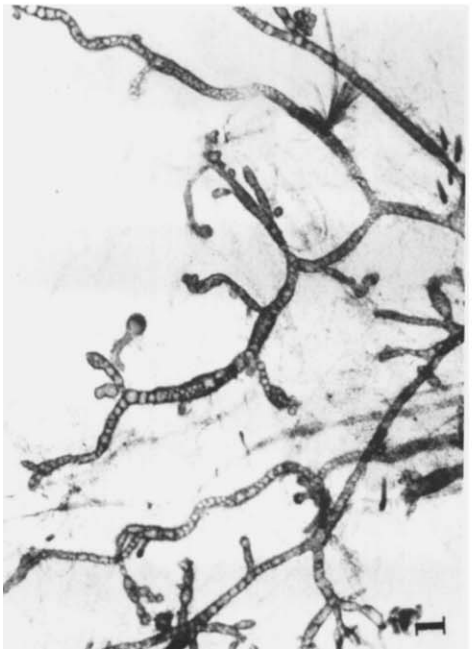

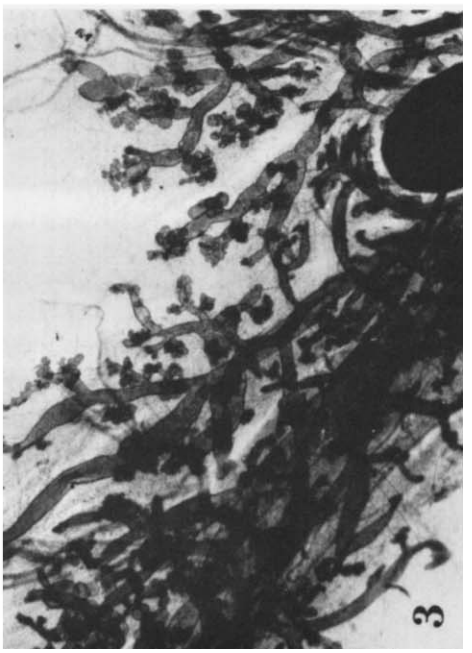

TABLE I. INCIDENCE OF MAMMARY ABNORMALITIES IN 12-MONTH-OLD INTACT FEMALE BALB/cCrgl MICE NEONATALLY TREATED FOR 5 DAYS

| Neonatal treatment period[a] (days) | No. of mice with aberrant secretory state (%) | | No. of mice with HAN[b]-like lesions (%) | |
|---|---|---|---|---|
| | Control | Estradiol | Control | Estradiol |
| 1–5 | 0/22 (0) | 6/18 (33)[c] | 0/22 (0) | 1/18 (6) |
| 2–6 | 0/23 (0) | 1/23 (4) | 2/23 (9) | 1/23 (4) |
| 3–7 | 1/23 (4) | 2/24 (8) | 0/23 (0) | 0/24 (0) |
| 5–9 | 0/21 (0) | 0/23 (0) | 0/21 (0) | 0/23 (0) |
| 10–14 | 0/20 (0) | 0/24 (0) | 0/20 (0) | 0/24 (0) |

[a] Subcutaneous injections of 20 μg 17β-estradiol daily.
[b] Hyperplastic alveolar nodule.
[c] $P < 0.005$ ($\chi^2$).

glands. Although beaded ducts and dilated ducts were often found concomitantly, beaded ducts were also found independently of dilated ducts.

A second abnormality consisted of dilated ductules and endbuds (Fig. 3). Lateral buds were also distended (Fig. 3). The ductules were lined with epithelial cells flattened by the distension of the lumen with secretion (Fig. 4). The extent of secretory abnormality varied within each treatment group from a picture of general stimulation to one of regional stimulation.

Mammary glands from intact mice treated postnatally with estradiol from Days 1–5 had the highest incidence of mammary abnormalities (6/18 or 33%; significantly greater than in controls: $P < 0.005$ by $\chi^2$ test). Treatment periods begun later than Day 1 showed a marked decline in incidence of mammary abnormalities to 8% or less. Only one control female exhibited an aberrant secretory state (Table I).

Hyperplastic alveolar-like dysplasias of unknown significance were also observed in a few animals in both treated and control groups (Table I). All ovariectomized females (15–20 per group), regardless of treatment, had poorly developed, thin ductal systems which frequently occupied only part of the mammary fat pad; no pathological changes were encountered. Accordingly, these negative data were not included in Table I.

**Discussion.** Treatment of mice with sex hormones prenatally leads to a variety of structural anomalies, such as athelia, amastia, and altered growth rates in early adult life (5–7). Our studies have been concerned with the long-term effects of neonatal estrogen exposure.

Inasmuch as the basic development of the mouse mammary gland occurs during embryonic life, neonatal exposure to exogenous estrogen had little effect on its gross morphological growth pattern. However, neonatal exposure at 1–5 days of age did result in increased incidence of abnormal secretory states (8–10). The incidence of this condition declines with delay in onset of treatment; treatments begun after Day 3 are without visible effect.

Although this experiment was not designed to distinguish between direct and indirect effects of neonatal estrogen on the mammary

FIG. 1. Wholemount of a mammary gland from a 12-month-old BALB/cCrgl intact female given estradiol neonatally on Days 1–5. Segmented or beaded ducts are present. (iron–hematoxylin, 14×)

FIG. 2. Histological section from Fig. 1. Note the segmented ducts and basophilic secretion. (hematoxylin and eosin, 80×)

FIG. 3. Whole mount of a mammary gland from a 12-month-old BALB/cCrgl intact female given estradiol neonatally on Days 1–5. Dilated ductules and end buds are present. (iron–hematoxylin, 14×)

FIG. 4. Histological section from Fig. 3. Note the flattened epithelial cells and distension of the lumen with basophilic secretion. (hematoxylin and eosin, 80×)

gland, the absence of any increase in mammary dysplasias and the atrophic condition of the gland in all ovariectomized females, regardless of treatment, support the hypothesis that the abnormal secretory state is a consequence of long-term alteration in the ovary or its endocrine control. Secondary to abnormal ovarian function, sustained prolactin secretion may occur (4, 11); changes in mammary gland sensitivity as a consequence of the direct effect of the neonatal hormonal stimulation and/or the changed endocrine milieu (4, 12) may also be involved. The critical period may begin in late embryonic life (not examined) but does not last beyond the first 3 days, if indeed beyond the first postnatal day (Table I).

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1. Herbst AL, Bern HA, eds. Developmental Effects of Diethylstilbestrol (DES) in Pregnancy. New York, Thieme-Stratton, 1981.
2. Takasugi N. Cytological basis for permanent vaginal changes in mice treated neonatally with steroid hormones. Int Rev Cytol **44**:193–224, 1976.
3. Forsberg J-G, Kalland T. Neonatal estrogen treatment and epithelial abnormalities in the cervicovaginal epithelium of adult mice. Cancer Res **41**:721–734, 1981.
4. Bern HA, Talamantes FJ Jr. Neonatal mouse models and their relation to human disease in the female. In: Herbst AL, Bern HA, eds. Developmental Effects of Diethylstilbestrol (DES) in Pregnancy. New York, Thieme-Stratton, Chap 10, p129, 1981.
5. Raynaud A. Foetal development of the mammary gland and hormonal effects on its morphogenesis. In: Falconer IR, ed. Lactation. London, Butterworths, p3, 1971.
6. Hoshino K. Hormonal teratogenesis in mammary glands of the mouse. In: Persand TVN, ed. Advances in the Study of Birth Defects, Vol 2, Teratological Testing. Lancaster, MTP Press, p139, 1979.
7. Tomooka Y, Bern HA. Growth of mouse mammary glands after neonatal sex hormone treatment. Proc Amer Assoc Cancer Res, Abstract 23, **22**:6, 1981.
8. Jones LA, Bern HA. Longterm effects of neonatal treatment with progesterone, alone and in combination with estrogen, on the mammary gland and reproductive tract of female BALB/cfC3H mice. Cancer Res **37**:67–75, 1977.
9. Jones LA, Bern HA. Cervicovaginal and mammary gland abnormalities in BALB/cCrgl mice treated neonatally with progesterone and estrogen, alone or in combination. Cancer Res **39**:3560–3567, 1979.
10. Mori T, Nagasawa H, Bern HA. Long-term effects of perinatal exposure to hormones on normal and neoplastic mammary growth in rodents: A review. J Environ Pathol Toxicol **3**:191–206, 1979.
11. Kawashima S, Bern HA, Jones LA, Mills KT. Histometric study of the pituitary in mice treated neonatally with steroids and the relationship between prolactin cells and mammary tumorigenesis. Endocrinol Japon **25**:341–348, 1978.
12. Warner MR, Yau L, Rosen JM. Longterm effects of perinatal injection of estrogen and progesterone on the morphological and biochemical development of the mammary gland. Endocrinology **106**:823–832, 1980.

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