Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 673–676. Pergamon Press. Printed in Great Britain

EXPOSURE OF NEONATAL MICE TO STEROIDS: LONGTERM EFFECTS ON THE MAMMARY GLAND AND OTHER REPRODUCTIVE STRUCTURES

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SUMMARY

Mice exposed perinatally to various steroid hormones, individually or in combination with other hormones, are being used as an experimental model relevant to clinical observations on the occurrence of vaginal cancer in the human female exposed during fetal life to diethylstilbestrol administered to the mother. Responses of the vagina and mammary gland of female BALB/cfC3H/Crgl mice to neonatal treatments with estradiol, testosterone, progesterone, and estradiol-progesterone combinations are being compared. The steroids result in an earlier age of onset and a higher incidence of mammary tumors in these mammary tumor virus-bearing mice. If ovariectomy is performed at 40 days of age, no mammary tumors develop, thus indicating that ovary-independent alterations are not induced in the mammary gland, as they are in the vagina, by high doses of estrogen or androgen administered neonatally. Progesterone (100 μg per day for the first five days of life) does not induce ovary-independent vaginal cornification and reduces the occurrence of this phenomenon when given simultaneously with estradiol.

INTRODUCTION

Although there has been an interest in the permanent effects of the exposure of neonatal rodents to steroid hormones since the 1930's (cf. [1]), it was not until the early 1960's that such exposure was related to preneoplastic and neoplastic changes. Then, in the early 1970's several clinical studies, beginning with that of Herbst and Scully[2], were published indicating that treatment of mothers with diethylstilbestrol for threatened abortion in the first trimester was correlated with early appearance of vaginal cancer in their daughters [3–9]. These reports underlined the potential seriousness for man of fetal and neonatal exposure to steroids in regard to longterm consequences. The possible relevance of the experimental studies in mice to human problems, including neoplasia, was indicated by several authors ([10, 11]; see also [12–13] on rats). Communication between experimental laboratory and clinic is regrettably so limited in both directions that the purpose of this paper is to call attention again to the experimental findings which may have bearing on the *longterm* consequences of antenatal and neonatal exposure of developing human organisms to hormones, especially but not only steroids, drugs, and other agents (cf. [14]). Diethylstilbestrol, which is only now being examined in our laboratory for the longterm effects on mice of brief neonatal exposure, is only one agent which may give rise to serious consequences. Forsberg[15] has reported early changes prognostic of later cancer development in neonatally diethylstilbestrol-treated mice. Natural estrogens, androgens and even progestins, alone and in combination, should be considered. For example, in the human female, one can well afford to be concerned with the longterm consequences for her offspring, of continued taking of the "pill" during the first months of an unrecognized pregnancy.

The neonatal rodent provides an admirable model system for the study of the longterm effects of short-term exposure to perinatal hormonal stimuli or deprivations. The relative developmental immaturity of the mouse and rat reproductive systems at the time of birth insures a degree of lability in response to a particular hormonal milieu. Persistent vaginal cornification (persistent "estrus") was first observed in female rats after neonatal transplantation of testes by Pfeiffer[16] and after perinatal estrogenic steroid administration by Greene and Burrill[17] and Turner[18]. Studies on development of comparable changes in the mouse vaginal epithelium treated neonatally with steroids, which can be irreversible (ovary-independent), have been summarized by Takasugi, Kimura and Mori[19] in a lucid review that is unfortunately not readily available.

LONGTERM EFFECTS OF NEONATAL EXPOSURE OF MICE TO ESTROGEN AND ANDROGEN

Figure 1 summarizes the known longterm effects of neonatal treatment of mice with steroid hormones. Effects on the hypothalamo-pituitary complex have occupied much attention, and the physiological and behavioral consequences and concomitants are many (cf. [20]). Low doses of estrogen and androgen administered neonatally both cause persistent vaginal cornification, apparently equally efficaciously. However, ovariectomy abolishes this response, indicating that the hypothalamo-hypophysio-ovarian axis has been fundamentally affected. However, higher doses result in ovary-independent cornification, which may

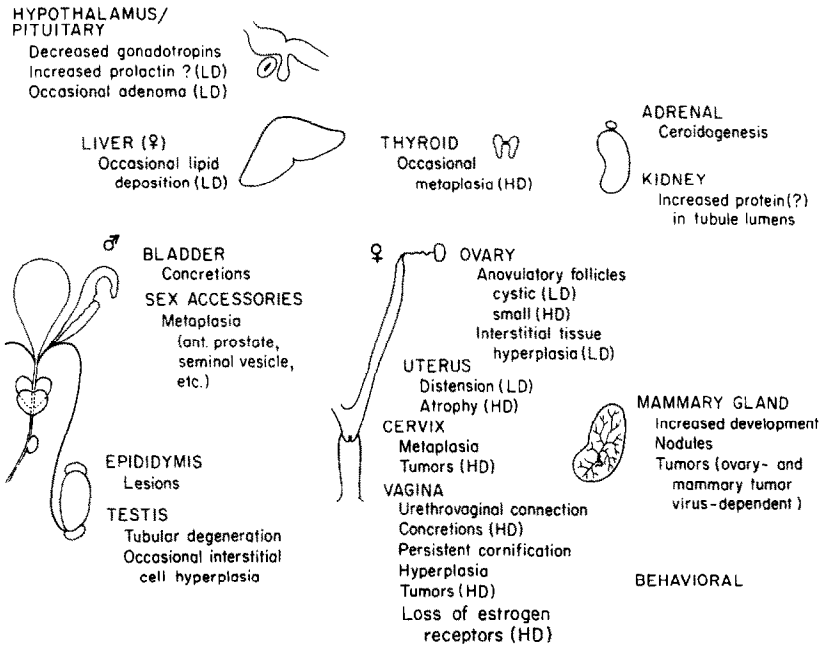

Fig. 1. Summary of longterm consequences of neonatal exposure of mice to estrogen and androgen. LD, low dosage (<5 μg daily for days 1–5); HD, high dosage (*ca.* 20 μg daily for days 1–5).

progress at later ages to the formation of hyperplastic and neoplastic lesions [11, 21]. Takasugi [22–24] has offered a cogent explanation for the histogenesis of the ovary-independent, permanently cornified vaginal lining, which appears to result from the selection of a cell population under steroid influence during a critical period of postnatal development. After three days of age, the cells capable of giving rise to the ovary-independent cornifying epithelium ordinarily degenerate. Adequate amounts of neonatal estrogen preserve this cell line, which replaces the normal epithelium and later gives rise to the dyscrasias associated with the development of vaginal cancer. Forsberg[15] has offered a similar histogenetic explanation for the development of abnormal vaginal epithelium in neonatally estrogenized mice, but also recognizes changes reminiscent of those accompanying vaginal adenosis and clear-cell carcinoma in women [15].

Testosterone has the same effect as estradiol probably owing to its conversion to estrogen, a metabolic process which would appear to be remarkably efficient in the neonate. Neonatal vaginae exposed *in vitro* to testosterone do not respond with persistent cornification, unlike the response to similar exposure to estradiol [25]. The higher neonatal doses of estradiol and testosterone also abolish estrogen receptors in the vagina [26]. Indeed, there is a consistent correlation between ovary-independent cornification and the loss of vaginal estrogen receptors.

In this paper, we shall discuss only some aspects of the vaginal response and of the mammary gland response of mice to neonatal exposure to sex steroids. The recent studies in our laboratory have been conducted on BALB/cCrgl mice, both uninfected with the mammary tumor virus and bearing the virus (BALB/cfC3H/Crgl).

A. *Vaginal changes*

In a new series of experiments (Jones and Bern, unpublished), we have determined the influence of progesterone alone or in combination with estradiol on the neonatal vagina. Persistent cornification in the presence of considerable numbers of nucleated epithelial cells and leucocytes occurs after treatment with 100 μg progesterone alone daily for five days. Ovariectomy at day 40 eliminates this response. These results are in essential agreement with those of Takasugi[27]. Progesterone given with 5 μg or 20 μg estradiol daily for the first five days of life reduces the incidence of persistent vaginal cornification in both intact and ovariectomized mice. Progesterone also seems to reduce the proliferation of the epithelium which results from estradiol treatment alone. In general, progesterone alone at the 100 μg daily dose does not have the same effect as estrogen or androgen and appears to nullify partially the effect of estrogen.

One additional aspect of the vaginal response needs comment here. Vaginal concretions (stones) occur in several strains of mice after neonatal estrogen or androgen treatment. Stone formation is related to abnormalities in the separation of the urethral and vaginal canals and is correlated with the occurrence of vaginal downgrowths and neoplasms [19]. A possible "promoter" or "co-carcinogenic" contribution of these obviously irritating deposits in the vagina to neoplastic development must be considered in evaluating neonatal hormonal effects.

B. *Mammary changes*

Neonatal exposure to steroids has been claimed to have some effects on the mammary gland, although in earlier studies the gland was found almost always to be inactive in appearance, with no nodules or tumors in evidence [11]. Mori[28, 29] reported acceleration of mammary tumorigenesis after neonatal estrogenization of C3H/MS mice. The predisposition to mammary dysplasias in rats given dimethylbenzanthracene was increased by neonatal estrogenization [30]. Our experiments were designed to see if a phenomenon similar to that seen in the development of vaginal cancer in mice following neonatal sex steroid treatment, could also be demonstrated in the mammary gland.

The effects of perinatal exposure to sex steroids upon subsequent development of mammary tumors in BALB/c mice are difficult to assess definitively. Two points are evident: any effect is dependent upon (1) the presence of the mammary tumor virus (BALB/cfC3H mice show tumors and the virus-free BALB/c do not) and (2) the presence of the ovary (ovariectomy at 40 days of age completely eliminates any mammary dysplasias even in the presence of the virus). In our first experimental series reported earlier [31], neonatal exposure of female BALB/cfC3H mice to androgen resulted in a mean tumor incidence of 86% (42/49), with a mean tumor age of $8{\cdot}5 \pm 0{\cdot}5$ months, and to estrogen in an incidence of 23% (8/35), with a mean tumor age of $11 \pm 0{\cdot}5$ months. None of the control (not steroid-treated) groups ($n = 40$) showed tumors. Androgen was significantly more potent than estrogen ($P < 0{\cdot}001$).

In a second experimental series, intended to be a repeat of the first series, the sesame oil- or saline-injected control mice showed a 19% tumor incidence (4/21) (the virgin tumor incidence in our BALB/cfC3H strain has been increasing). Neonatal treatment with 5 μg and 20 μg estradiol-17β daily for the first five days of life resulted in tumor incidences of 81% (13/16) and 60% (6/10), significant at the 0·001 and 0·05 levels, respectively. All the estrogen-treated groups showed a mean tumor incidence of 50% (32/64) and the androgen-treated groups 63% (22/35), compared with the overall control (not steroid-treated) incidence of 21% (9/43), significantly higher ($P < 0{\cdot}005$) at the 0·01 and 0·05 levels, respectively. The significant difference between androgen- and estrogen-treated groups seen in the first series was not evident here. Again the mean tumor age was lowered from 10–12 months in the control groups to 7–9·5 months in the steroid-treated groups. The mice were all killed by 12 months of age.

In a third series, the influence of progesterone alone (100 μg daily for five days) and in combination with estradiol-17β (5 μg and 20 μg daily for five days) was examined. Incomplete data indicate that the low dose of estrogen resulted in significantly more tumors than in the controls, as did progesterone alone and progesterone with both levels of estrogen. The mean tumor age of the steroid-treated mice was 2–3 months less than in the controls, significantly lower in all but the high estrogen group.

In all three experiments, it appears that neonatal sex steroid treatment results in higher tumor incidence, or at least in an earlier appearance of tumors, so that by one year of age, steroid-treated mice show a higher incidence. Whether this is a direct effect by changing the sensitivity of the mammary gland, or by changing the level of ovarian function, or by changing the output of pituitary hormones has yet to be determined.

Inasmuch as ovariectomy at day 40 completely eliminates tumor incidence regardless of neonatal treatment, no evidence of a permanent selective influence, as occurs in the vagina, is evident in the mammary gland. It is possible, of course, that the critical period for the mammary gland occurs earlier, in antenatal life.

The mammary gland development associated with the neonatal steroid treatment is often characterized by extensive development of lobules of large alveoli and dilatation of the duct system, suggesting secretory stimulation. Higher levels of the lactogenic hormones: corticoid and/or prolactin, may prove to be involved in this response. Basal plasma corticoid levels have not as yet proven to be significantly different in our neonatally treated mice (Hawkins, unpublished data); however, the sensitivity of the adrenal cortex to administered ACTH is significantly greater after neonatal estrogenization of male CF1 mice [32]. With Dr. H. Nagasawa, we are presently determining plasma prolactin levels by radioimmunassay, as well as undertaking a cytological analysis of the neonatally steroid-treated pituitary (with Dr. S. Kawashima). A "prolonged stimulative effect" on prolactin synthesis and release has been indicated after neonatal estradiol injection in rats [33] and a significant increase in circulating prolactin levels has also been reported [34]. An effect of neonatal testosterone in increasing prolactin secretion in rats has been shown by several workers [35–37]. Changes in the hypothalamic aminergic system, visualized as retarded development of fluorescence in the median eminence, have been recently observed in our laboratory by Swanson and Nishioka in female mice given high doses of estradiol neonatally. Such changes may prove to be related to changes in the secretion of prolactin, as well as that of other pituitary hormones.

PERSPECTIVES

By use of the neonatal mouse as a model system for studying the longterm effects of perinatal exposure to various stimuli, it should be possible to gain information regarding the possible dangers in later life of such early exposures of the developing human organism. Factors to be considered include not only hormones but also drugs, nutrition, viruses, stress, radiation, chemical carcinogens, immunosuppressive agents, etc. To date, the consequences of neonatal administration of only a few steroids: estradiol, testosterone and progesterone, have been studied in any

detail in regard to obvious target organs such as the gonads, uterus, vagina, male sex accessories, and mammary apparatus. Experiments with corticoids and with other hormones such as prolactin (cf. [26, 38] have been limited. The effects of estrogen-progesterone combinations mimicking the "pill" should be studied [39] and the longterm consequences of neonatal hormone deficiencies need to be considered. For example, hypothyroidism is thought to increase mammary gland sensitivity to hormonal stimuli, especially prolactin [40]; neonatal hypothyroidism could conceivably prove to be a predisposing factor in the development of breast cancer. Despite the prolonged and tedious nature of the experimental approach, much broader investigation of factors potentially predisposing to the development of neoplasia seems called for. The studies with steroids to date have established the value of the neonatal rodent as a system both for screening perinatal stimuli and for the elucidation of the mechanisms involved in the later induction of cancerous changes by such stimuli.

Acknowledgements—Various portions of our laboratories' studies in this area have been aided by NIH grants CA-05388 and CA-05045, by NSF grants GB 35239X and GF-39781, and by the Japan Society for the Promotion of Science. LAJ is a National Fellow. We are indebted to Professor Noboru Takasugi for suggestions in regard to the figure, to Emily Reid for its preparation, to Sue Handa and Karen Mills for assistance with the experiments, to Walter Jackson and Ann Mos for microtechnical assistance and to William Young for animal care.

REFERENCES

1. Takewaki K.: *Experientia* **18** (1962) 1–6.
2. Herbst A. L. and Scully R. E.: *Cancer* **25** (1970) 745–757.
3. Greenwald P., Barlow J. J., Nasca P. C. and Burnett W. S.: *New Engl. J. Med.* **285** (1971) 390–392.
4. Greenwald, P., Nasca P. C., Burnett W. S. and Polan A.: *Cancer* **31** (1973) 568–572.
5. Henderson B. E., Benton B. D. A., Weaver P. T., Linden G. and Noland J. F.: *New Engl. J. Med.* **288** (1973) 354.
6. Herbst A. L., Kurman R. J. and Scully R. E.: *Obstet. Gynaecol.* **40** (1972) 287–298.
7. Herbst A. L., Kurman R. J., Scully R. E. and Poskanzer D. C.: *New Engl. J. Med.* **287** (1972) 1259–1264.
8. Herbst A. L., Ulfelder H. and Poskanzer D. C.: *New Engl. J. Med.* **284** (1971) 878–881.
9. Tsukada Y., Hewett W. J., Barlow J. J. and Pickren J. W.: *Cancer* **29** (1972) 1208–1214.
10. Dunn T. B. and Green A. W.: *J. natn. Cancer Inst.* **31** (1963) 425–455.
11. Takasugi N. and Bern H. A.: *J. natn. Cancer Inst.* **33** (1964) 855–865.
12. Jacobsohn D.: *Acta endocr., Copenh.* **45** (1964) 402–414.
13. Jacobsohn, D.: *Acta Universitatis Lundensis* **17** (1965) 3–19.
14. Fraumeni J. F. Jr. and Miller R. W.: *J. natn. Cancer Inst.* **48** (1972) 1267–1270.
15. Forsberg J.: *Am. J. obstet. Gynecol.* **115** (1973) 1025–1043; **121** (1975) 101–104; *Adv. Biosci.* **13** (1974) 139–151.
16. Pfeiffer C. A.: *Am. J. Anat.* **58** (1936) 195–225.
17. Greene R. R. and Burrill M. W.: *Am. J. Physiol.* **133** (1941) 302–303.
18. Turner C. D.: *Am. J. Physiol.* **133** (1941) 471–472.
19. Takasugi N., Kimura T. and Mori T.: In *The Post-Natal Development of Phenotype.* Butterworths, London (1970) pp. 229–251.
20. Bern H. A., Gorski R. A. and Kawashima S.: *Science* **181** (1973) 189–190.
21. Kimura T. and Nandi S.: *J. natn. Cancer Inst.* **39** (1967) 75–93.
22. Takasugi N.: *Proc. Japan Acad.* **47** (1971) 193–198.
23. Takasugi N.: *Intl. Rev. Cytol.* (1975) In the press.
24. Takasugi N. and Kamishima Y.: *Dev. Growth and Differentiation* **15** (1973) 127–139.
25. Kimura T., Basu S. L. and Nandi S.: *J. exp. Zool.* **165** (1967) 497–504.
26. Shyamala G., Mori T. and Bern H. A.: *J. Endocr.* **63** (1974) 275–284.
27. Takasugi N.: *J. Fac. Sci.*, Univ. of Tokyo IV, **10** (1964) 397–401.
28. Mori T.: *Annot. Zool. jap.* **41** (1968) 43–52.
29. Mori T.: *Annot. Zool. jap.* **41** (1968) 85–94.
30. Warner M. R. and Warner R. L.: *Proc. Am. assoc. Cancer Res.* **15** (1974) 135.
31. Bern H. A., Mori T. and Young P. N.: *VIII Meeting on Mammary Cancer in Exp. Animals and Man*, Airlie House, Va. (1973) p. 12 (U.S. Natl. Cancer Program).
32. Simmons J. E., DiClementi D. and Maxted G.: *J. exp. Zool.* **186** (1973) 123–126.
33. Pantić V. and Genbačev O.: *Z. Zellforsch.* **126** (1972) 41–52.
34. Nagasawa H., Yanai R., Kikuyama S. and Mori J.: *J. Endocr.* **59** (1973) 599–604.
35. Christakos S., Sinha D. and Dao T. L.: *Proc. Am. assoc. Cancer Res.* **14** (1973) 67.
36. Mallampati R. S. and Johnson D. C.: *Neuroendocr.* **11** (1973) 46–56.
37. Shellabarger C. J. and Soo V. A.: *Cancer Res.* **33** (1973) 1567–1569.
38. Mori T. and Bern H. A.: *Proc. Japan Acad.* **48** (1972) 698–702.
39. Browning H. C., Larke G. A. and Talamantes F.: *Proc. Soc. exp. Biol. Med.* **128** (1968) 1199–1202; Talamantes F. and Browning H. C.: *Texas Rep. Biol. Med.* **30** (1972) 361–369.
40. Mittra I.: *Nature* **248** (1974) 525–526.