Europ. J. Cancer Vol. 16, pp. 1583–1589

0014-2964/80/1201-1583 $02.00/0

Long-Term Effects of Progesterone or Diethylstilbestrol with or without Estrogen after Maturity on Mammary Tumorigenesis in Mice*

HIROSHI NAGASAWA,† TAKAO MORI‡ and YUKO NAKAJIMA†
†*Pharmacology Division, National Cancer Center Research Institute, Tsukiji* 5-1-1, *Chou-kuo, Tokyo* 104 *and*
‡*Zoological Institute, Faculty of Science, University of Tokyo, Hongo* 7-3-1, *Bunkyo-ku, Tokyo* 113, *Japan*

Abstract—*The long-term effects of perinatal exposure to progesterone or diethylstilbestrol (DES) with or without the additional administration of estradiol benzoate (EB) on mammary tumorigenesis were studied in virgin SLN mice bearing expressed mammary tumor virus. Spontaneous mammary tumorigenesis of female offsprings from mothers receiving single subcutaneous injections of DES* (5 μg) *on* day 12 *of pregnancy (prenatal* 12 days) *was significantly lower than that of offsprings from mothers given progesterone* (1 mg) *or oil on the same day. This difference in mammary tumorigenesis was not altered by the s.c. EB implantation during* 3 months *between* 2 *and* 5 months *of age. Single injection of progesterone at prenatal* 17 days *with or without EB after maturity also inhibited mammary tumorigenesis at advanced ages. Mammary tumorigenesis was enhanced by s.c. injection of progesterone* (100 μg) *or DES* (0.1 μg) *on the day of birth; however, it was arrested by EB after maturity. All findings indicate that long-term effects of perinatal exposure to steroids or DES on mammary tumorigenesis at advanced ages are largely dependent upon perinatal ages of the subjects and that sex hormones given after maturity can modulate these perinatal hormone effects.*

INTRODUCTION

THE BEST-KNOWN example of the long-term effects of prenatal hormone exposure is earlier and higher development of cervico-vaginal tumors of women whose mothers received diethylstilbestrol (DES) during the first trimester of pregnancy [1, 2]. This clinical situation parallels experimental works [3]. Recently, attention has also been paid to the possibility of persistent changes in mammary glands analogous those seen in genital tracts after perinatal exposure of animals to hormones [3]. Inasmuch as mammary gland is affected in experimental rodents by neonatal sex hormone exposure, the possibility exists that such effects may eventually be seen in the steroid hormone-exposed human females. From this point of view, some studies have been done on the effects of neonatal treatments of female mice with DES and other hormones on spontaneous mammary tumorigenesis; the treatments resulted in the earlier and higher incidence of mammary tumors in mammary tumor virus (MTV)-expressed mice [4]. However, no data are available on the long-term effects of these hormone treatments at different prenatal ages despite that the initial mammary gland development at fetal stages has sincere influence on its growth after puberty [5]. Moreover, it would be of much importance to examine the participation of estrogen given after maturity in this process in animals exposed perinatally to several hormones, considering the fact that most women will generally experience steroid hormones, especially through contraceptive pills.

In this paper, the long-term effects of treatment with progesterone or DES at different perinatal ages on spontaneous mammary tumorigenesis were studied in mice. Progesterone and DES were specifically selected, since these are used most commonly for prevention of abortion and for contraception.

Accepted 9 July 1980.
*This work was supported partly by the grants-in-aid for Cancer Research from the Ministry of Education, Science and Culture, Japan (Nos. 301082 and 401086).

MATERIALS AND METHODS

Animals

An inbred strain of SLN mice with MTV [6, 7] was used. Female mice were mated with males at about 70 days of age and the day when vaginal plug was found was designated as day 1 of pregnancy. Pregnant mice were given single s.c. injections of 5 μg DES (E. Merck AG, Darmstadt, Federal Republic of Germany) or 1 mg progesterone (Sigma Chem. Co., St. Louis, Missouri, U.S.A.) on day 12 or 17 of pregnancy. Each dose of hormones was dissolved in 0.1 ml olive oil. The control received vehicle only at the respective days. The daughters of these mice were designated as animals treated at prenatal 12 or 17 days. Pregnancy, parturition, lactation and growth of pups were not affected by the treatments. As neonatal treatments, female pups were given single s.c. injections of 0.1 μg DES or 100 μg progesterone dissolved in 0.02 ml olive oil on the day of birth. Pups were weaned at 20 days of age. Some mice in each group were further implanted s.c. with pellets of estradiol benzoate (EB: Sigma Chem. Co., St. Louis, Missouri, U.S.A.) mixed with cholesterol at the ratio of 1:1 and weighed about 20 mg for the limited period of 3 months between 2 and 5 months of age.

Throughout the experiments, mice were kept 6 per cage in Teflon cages (15 × 30 × 12 cm) with wood shavings, maintained in an animal room that was air-conditioned (24 ±0.5°C and 65–70% r.h.) and artificially illuminated (14 hr light from 5 a.m. to 7 p.m.) and provided with a commercial diet (CA-1: CLEA Japan Inc., Tokyo, Japan) and tap water *ad libitum*.

Vaginal opening and estrous cycle

The age of vaginal opening and the body weight on that day were recorded. Estrous cycles were estimated by checking vaginal smears every morning in 12–18 mice of each group for 30–40 days around 2–3 months and 9–10 months of age.

Mammary tumorigenesis

Each mouse was checked for palpable mammary tumors every 7 days until 3 weeks after the first tumor appearance or it became moribund. Mammary tumor incidence was examined until the month when all mice came to develop mammary tumors or died. Cumulative mammary tumor incidence at each age was calculated by the modified life-table method of Varma [8] by which the possibility of tumor development of dead mice without tumors was taken into consideration at respective ages on the assumption that these mice were still alive. The number and size of tumors expressed in terms of geometric mean of the major two diameters were recorded.

Ovarian histology

After 3 weeks of the first tumor appearance, mice in each group were checked by vaginal smears, bled from vena cava under light ether anesthesia and killed by decapitation. Ovaries were fixed in Bouin's fluid, sectioned at 7 μm and stained with hematoxylin and eosin.

Plasma prolactin level

Plasma was frozen and kept at −20°C and prolactin level was assayed by radioimmunoassay using the kit donated by Dr. W. P. VanderLaan, La Jolla, California, U.S.A. There was little difference in plasma prolactin level between estrous stages in all groups and the data were pooled as in Results.

Statistics

Statistical significance of difference in mammary tumorigenesis was evaluated by the multiple classification (two-way experiment) method in analysis of variance [9]. By this method, the difference between groups could be checked considering simultaneously both the incidence and the onset age of mammary tumors. The differences of other parameters were evaluated by Duncan's multiple range test.

RESULTS

Age and body weight at vaginal opening

There were little differences between groups of any perinatal treatment in either age or body weight at vaginal opening.

Estrous cycles

Each prenatal and neonatal treatments with progesterone or DES did not affect the estrous cycles at both 2–3 months and 9–10 months of age. Mice in all groups showed 4–5-day cycles as seen previously [10].

On the other hand, after 4 months of EB pellet removal, mice given EB pellet implan-

tation between 2–5 months of age showed continued diestrous vaginal smears interrupted occasionally by estrus, irrespective of the different perinatal treatments. This pattern appeared to continue over further several months, since, in all animals, only diestrous vaginal smears were observed at autopsy.

Mammary tumorigenesis

Mammary tumor incidence increased with the advance of age in mice receiving progesterone or oil at prenatal 12 days. On the other hand, in mice given DES, the first tumor developed at 15 months of age, which was 4 months later than the control given oil and the incidence was stopped at about 45% after 20 months (Fig. 1A). Thus, mammary tumorigenesis of DES-group was significantly lower than the other two groups when evaluated by analysis of variance.

Mammary tumor incidence in mice treated with progesterone or oil at prenatal 12 days was much stimulated by the additional implantation with EB after maturity. While the incidence of DES-group was also promoted by EB, it was much lower than those of the other groups at most ages and the difference was again statistically significant (Fig. 2A).

In treatment at prenatal 17 days, mammary tumor incidence of progesterone-group was much retarded between 13 and 18 months of age when compared to that of DES- or oil-group and mammary tumorigenesis of this group was significantly lower than the other two groups (Fig. 1B). Even after EB treatment, similar retardation of the incidence in the progesterone-group was retained between 8 and 11 months of age and the difference in mammary tumorigenesis between groups was statistically significant (Fig. 2B).

Mice given DES neonatally were much more marked than mice receiving progesterone or oil in mammary tumorigenesis. The incidence in the DES-group was 90% at 17 months of age when those in the progesterone- and oil-groups were about 60 and 20%, respectively. Progesterone-group was also higher than oil-group in the incidence except for 23 and 24 months of age (Fig. 1C). Meanwhile, these differences in the incidence due to neonatal treatments were nullified by the treatment with EB after maturity (Fig. 2C).

Ovarian histology

Irrespective of the time of treatment, the administration of progesterone or DES perinatally only influenced little the ovarian structures; ovaries in each experimental and control groups consisted of both follicles and corpora lutea at various stages of development.

On the other hand, s.c. EB implantation after maturity resulted in an increase of a number of mice with ovaries lacking corpora lutea in all groups receiving different perinatal treatments.

Plasma prolactin level

No difference was observed in plasma prolactin levels at autopsy between the three groups given treatments at different perinatal ages with or without EB after maturity.

DISCUSSION

It has been reported that treatment with estrogen inhibited most effectively fetal mammary gland development when applied between 12 and 14 days of pregnancy. The inhibitory effects of estrogen was slight if given after 15 days or before 11 days of pregnancy [11]. In the present study, mammary tumorigenesis was significantly suppressed by the administration of DES at prenatal 12 days. Meanwhile, the parameters indicating pituitary and ovarian secretion of mammotropic hormones, i.e., the age and body weight at vaginal opening, the pattern of estrous cycles, ovarian structures and plasma prolactin level, were little affected by the treatment. Therefore, lower mammary tumorigenesis in DES-group would primarily be ascribed to the long-term inhibitory effects of DES on fetal mammary glands. This effect was not arrested by EB treatment after maturity; mammary tumorigenesis in mice given DES at prenatal 12 days and EB after maturity was still significantly lower than those of the other two groups.

The treatment with progesterone at prenatal 17 days with or without EB after maturity resulted in the significant suppression of mammary tumorigenesis associated with no difference from DES and the control groups in all parameters mentioned above as the indices of pituitary and ovarian secretion of mammotropic hormones. These suggest that progesterone would also act inhibitorily on the fetal mammary gland development with the critical period around prenatal 17 days and, in its turn, on spontaneous mammary tumorigenesis at advanced ages.

Promotion by neonatal treatment with estrogen and progesterone of mammary tumorigenesis in mice has been reported by some

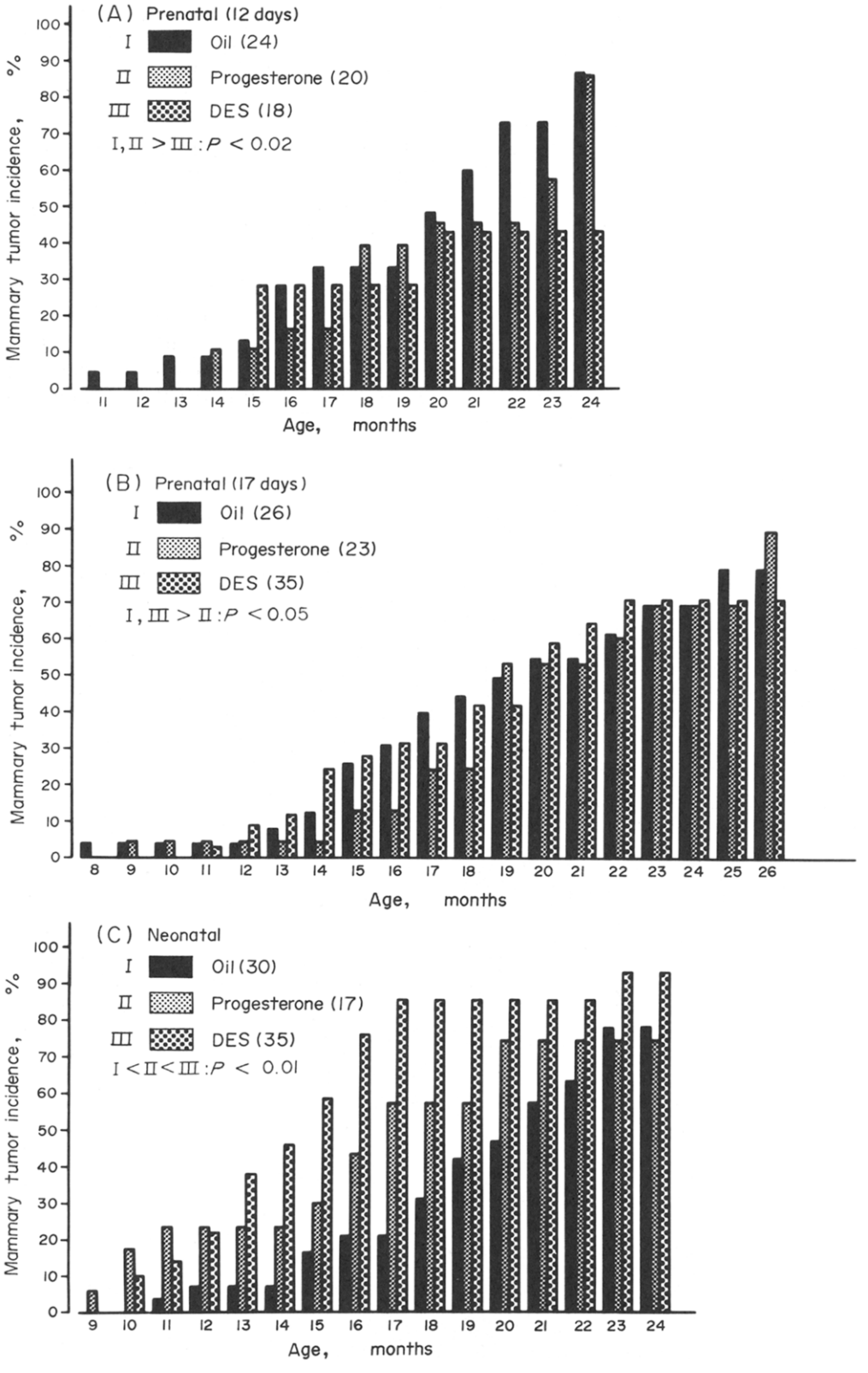

Fig. 1. *Cumulative mammary tumor incidence in virgin SLN mice whose mothers received single s.c. injections of* 1 mg *progesterone,* 5 μg *diethylstilbestrol* (*DES*) *or oil on* day 12 *or* 17 *of pregnancy* (*prenatal* 12 *or* 17 days) *and in mice injected with* 100 μg *progesterone,* 0.1 μg *DES or oil on the day of birth* (*neonatal*). *Number of mice examined is in the parentheses. Cumulative mammary tumor incidence at each age was calculated by the modified life-table method of Varma* [8] *by which the possibility of tumor development of dead mice without mammary tumors was taken into consideration on the assumption that these mice were still alive. Statistical significance of differences between groups in mammary tumorigenesis was evaluated by the multiple classification* (*two-way experiment*) *method in analysis of variance* [9].

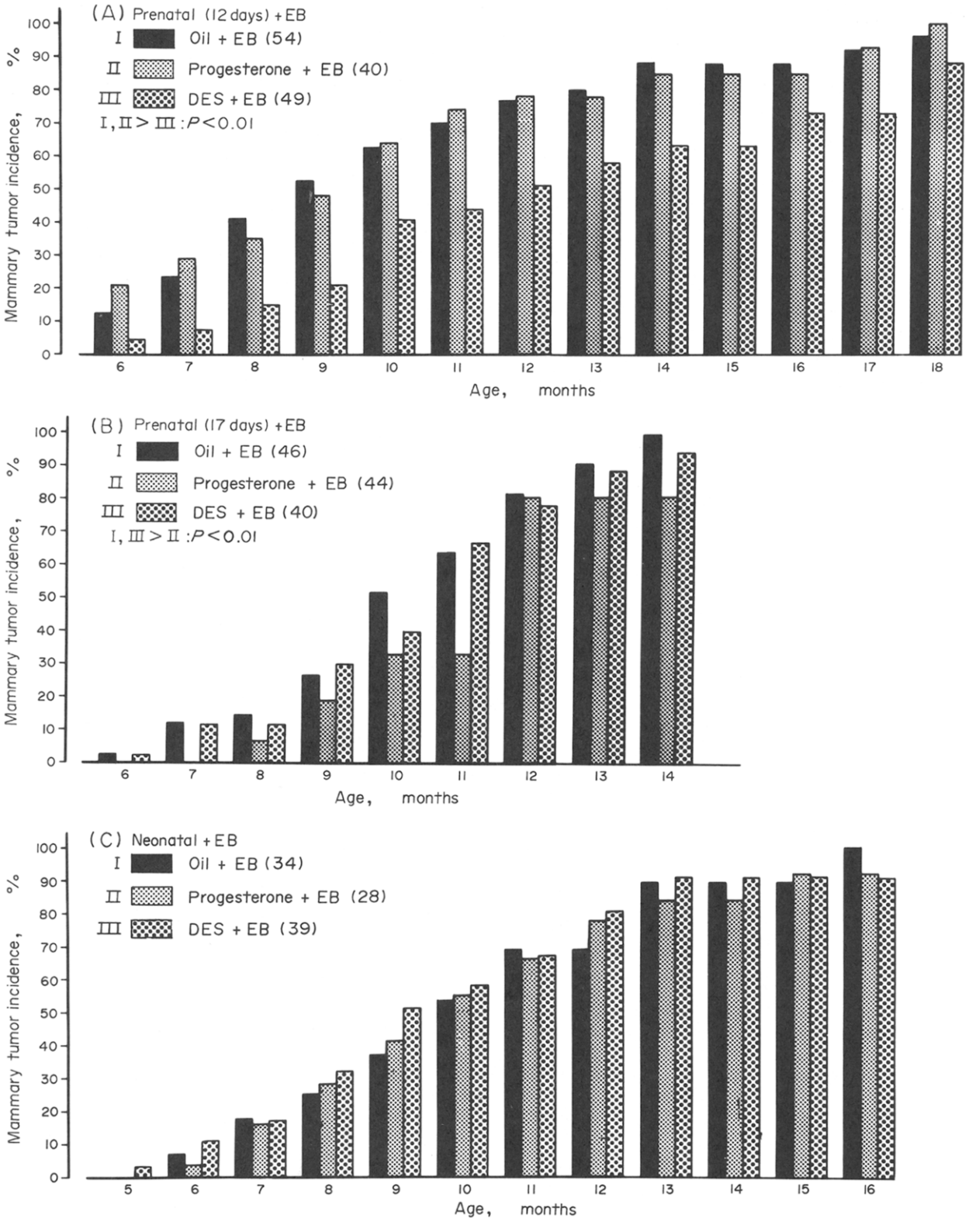

Fig. 2. Cumulative mammary tumor incidence in virgin SLN mice whose mothers received single s.c. injections of 1 mg *progesterone,* 5 μg *diethylstilbestrol* (*DES*) *or oil on* day 12 *or* 17 *of pregnancy* (*prenatal* 12 *or* 17 days) *and in mice injected with* 100 μg *progesterone,* 0.1 μg *DES or oil on the day of birth* (*neonatal*); *all mice were further given s.c. implantation of estradiol benzoate* (*EB*) *mixed with cholesterol* (1:1) *for* 3 months *between* 2 *and* 5 months *of age. Number of mice is in the parentheses. See Fig.* 1 *for details of mammary tumor incidence and statistical significance of differences.*

workers [4]. In these studies, the hormone treated mice showed constantly estrous vaginal smears [12, 13], their ovaries consisted only of follicles and lacked corpora lutea [14, 15] and plasma prolactin levels in these animals were higher than those of the control [15, 16]. On the other hand, in the present study, mice receiving neonatally progesterone or DES were little different from the control in any of age and body weight at vaginal opening, the estrous cycles, the ovarian structures and plasma prolactin level. This was principally due to the lower doses of hormones applied in this study than previously reported (about 1/5 for progesterone and 1/250–1/1000 for DES). Nevertheless, neonatal treatments with these doses of hormones induced marked stimulation of mammary tumorigenesis. While the stimulation of mammary tumorigenesis observed in this study would principally result from the irreversible alteration of hypothalamus–pituitary–ovarian function induced by hormones given neonatally as already evidenced [4], the present results indicate that mammary tumorigenesis is enhanced by the neonatal hormone treatment even at dose levels which induce no alteration in the parameters as the indices of pitutary and ovarian secretion of mammotropic hormones. The results suggest that the administration of sex hormones to pregnant women should be cautioned repeatedly.

All findings obtained in the present study indicate that the long-term effects of perinatal exposure to steroids or DES on mammary tumorigenesis are largely dependent upon the prenatal or neonatal ages of the subjects. They further suggest that steroids given after maturity can modulate these long-term effects of perinatal hormone treatment.

The responsiveness of vaginal epithelium to steroids was much decreased by high doses of estrogen [17]. This may partially account for the continued diestrous vaginal smears in any group of mice given EB pellets after maturity.

Acknowledgements—We thank Dr. Reiko Yanai for her interest. Technical help by H. Taniguchi is also acknowledged.

REFERENCES

1. A. L. Herbst, R. E. Scully and S. J. Robboy, Prenatal diethylstilbestrol exposure and human genital tract abnormalities. *Nat. Cancer Inst. Monogr.* **51,** 25 (1979).
2. S. Shapiro and D. Slone, The effects of exogenous female hormones of the fetus. *Epidemiol. Rev.* **1,** 110 (1979).
3. H. A. Bern, L. A. Jones, K. T. Mills, A. Kohrman and T. Mori, Use of the neonatal mouse in studying longterm effects of early exposure to hormones and other agents. *J. Toxicol. environ. Hlth Suppl.* **1,** 103 (1976).
4. T. Mori, H. Nagasawa and H. A. Bern, Long-term effects of perinatal exposure to hormones on normal and neoplastic mammary growth in rodents: a review. *J. environ. Path. Toxicol.* **3,** 191 (1979).
5. A. Raynaud, Foetal development of the mammary gland and hormonal effects on its morphogenesis. In *Lactation.* (Edited by I. R. Falconer) p. 3. Butterworths, London (1971).
6. H. Nagasawa, R. Yanai, H. Taniguchi, R. Tokuzen and W. Nakahara, Two-way selection of a basal stock of swiss albino mice for mammary tumorigenesis: establishment of two new strains (SHN and SLN). *J. nat. Cancer Inst.* **57,** 425 (1976).
7. J. Staats, Standardized nomenclature for inbred strains of mice: sixth listing. *Cancer Res.* **36,** 4333 (1976).
8. A. A. Varma, Life table procedure in toxicological studies. *Drug Inform. Bull.* **69,** 134 (1969).
9. C. W. Snedecor, *Statistical Methods,* 5th edn, p. 241. Iowa State Univ. Press, Ames (1966).
10. H. Nagasawa, R. Y. Yanai, H. Taniguchi and S. Hayashi, Mammary tumor incidence in relation to the pattern of oestrous cycle in mice. *Horm. Res.* **10,** 123 (1979).
11. K. Kratochewil, Experimental analysis of the prenatal development of mammary gland: a review. In *Milk and Lactation.* (Edited by N. Kretchmer, E. Rossi and F. Sereni) p. 1. S. Karger, Basel (1975).
12. L. A. Jones and H. A. Bern, Long-term effects of neonatal treatment with progesterone, alone and in combination with estrogen, on mammary gland and reproductive tract of female BALB/cfC3H mice. *Cancer Res.* **37,** 67 (1977).

13. L. A. JONES and H. A. BERN, Cervicovaginal and mammary gland abnormalities in BALB/cCrgl mice treated neonatally with progesterone and estrogen, alone or in combination. *Cancer Res.* **39,** 2560 (1979).
14. T. MORI, H. A. BERN, K. T. MILLS and P. N. YOUNG, Long-term effects of neonatal steroid exposure on mammary gland development and tumorigenesis. *J. nat. Cancer Inst.* **57,** 1057 (1976).
15. H. NAGASAWA, T. MORI, R. YANAI, H. A. BERN and K. T. MILLS, Long-term effects of neonatal hormonal treatments on plasma prolactin levels in female BALB/cfC3H and BALB/c mice. *Cancer Res.* **38,** 942 (1978).
16. H. NAGASAWA, R. YANAI, L. A. JONES, H. A. BERN and K. T. MILLS, Ovarian dependence of the stimulating effect of neonatal hormone treatment on plasma levels of prolactin in female mice. *J. Endocr.* **79,** 391 (1978).
17. B. PECKHAM, J. LADINSKY and W. KIEKHOFER, Autoradiographic investigation of estrogen response mechanisms in rat vaginal epithelium. *Amer. J. Obst. Gynec.* **87,** 710 (1963).