J. steroid Biochem. Vol. 23, No. 6A, pp. 1037–1041, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

AGE-DEPENDENT CHANGE IN SENSITIVITY OF OESTROGEN-INDUCED UTERINE CELL PROLIFERATION OF MICE, ESTIMATED BY INCORPORATION OF [^{125}I]IODODEOXYURIDINE*

N. TERADA†‖, T. YAMANE‡, Y. OGASAWARA‡, K. MATSUMOTO‡ and Y. KITAMURA§

†Division of Experimental Pathology, The Center for Adult Diseases, Higashi-Ku, Osaka, 537, ‡Department of Pathology and §Institute for Cancer Research, Osaka University Medical School, Nakanoshima 4-3-57, Kita-Ku, Osaka, 530, Japan

(*Received* 13 *May* 1985)

Summary—The stimulative effects of both oestradiol-17β (E_2) and diethylstilbesterol (DES) injections on the proliferation of uterine cells of mice were investigated by using 5-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IdUrd) incorporation as an index. Female mice of (WB × C57BL/6)F_1 were neonatally castrated, and the [^{125}I]IdUrd uptake by the whole uterus was determined on days 1, 5, 10, 20, 30 and 40 after birth. The relative minimal dose (a minimal dose expressed per body weight, μg/g b.wt) of E_2 necessary for the maximal [^{125}I]IdUrd uptake was much higher than the relative minimal dose of DES on days 1, 5, and 10, when the serum concentration of α-fetoprotein was relatively high. However, the relative minimal dose of E_2 was comparable to that of DES on days 30 and 40, when the concentration of α-fetoprotein was negligible. The difference between the effect of injected E_2 and DES during neonatal and suckling periods seems to be attributable to the presence of α-fetoprotein in serum, which binds to E_2 but not to DES with high affinity. When the relative minimal dose of DES necessary for the maximal [^{125}I]IdUrd uptake was examined in detail, the value was lower on days 10 and 20 of age than on days 30 and 40 of age. This might represent the increased sensitivity of uterine cells to oestrogen on days 10 and 20.

INTRODUCTION

Neonatal castration resulted in very small or no difference in the growth of the uterus of rats [1, 2] and mice [3] that were up to 20 days old, but after this period of ovarian independence, further uterine growth depends on the presence of ovaries. Although some authors [4–7] suggested that the high levels of serum oestrogen found in 5–20 days of age originated mainly from adrenals, the adrenalectomy did not influence the growth of uterus in suckling mice [3]. The independence of uterine growth on the ovary does not necessarily imply that the uterine cells themselves are not sensitive to oestrogens in neonatal and suckling periods. In fact, the injection of a large amount of oestradiol-17β (E_2) induces the increase of weight and DNA synthesis in the uterus of neonatal and suckling rats and mice [3, 8, 9]. The requirement of the high dose of E_2 is considered to be due to the presence of α-fetoprotein, which is known to bind to E_2 with high affinity [10–14]. Since the affinity of α-fetoprotein for synthetic oestrogens such as diethylstilbesterol (DES) and R2858 is much lower than the affinity for E_2 [10–12, 14], the effect of DES or R2858 provides a useful information about the sensitivity of uterine cells themselves to oestrogens. Stack and Gorski[9] reported that the dose of DES to induce DNA synthesis was much lower than the dose of E_2 in neonatal rats. However, there are few reports in which the sensitivity of uterine cell proliferation to DES was examined in detail during neonatal and suckling periods. Recently, we showed that the uptake of 5-[^{125}I]-iodo-2′-deoxyuridine ([^{125}I]IdUrd) by the whole uterus of mice was a sensitive and convenient method for evaluating the DNA synthesis of uterine cells in mice [3]. By using this method, we carried out a detailed study about the sensitivity of murine uterine cells to both E_2 and DES in neonatal and suckling periods. The sensitivity of uterine cells was evaluated by determining the minimal dose of E_2 or DES (per body weight, b.wt) which is necessary to induce the maximal uptake of [^{125}I]IdUrd. The present results indicate that the sensitivity of uterine cells during neonatal and suckling periods are greater than that in the adult and that a high concentration of α-fetoprotein demands a high dose of E_2 to induce the uterine cell proliferation.

*This study was supported by grants from the Ministry of Education, Science and Culture, the Ministry of Health and Welfare, the Princess Takamatsu Cancer Research Fund, and the Japanese Foundation for Multidisciplinary Treatment of Cancer.

‖Correspondence should be addressed to: Nobuyuki Terada, M.D., Division of Experimental Pathology, The Center for Adult Diseases, Higashi-Ku, Osaka 537 Japan.

EXPERIMENTAL

Mice

Female (WB × C57BL/6)F_1 hybrid mice were raised in our laboratory from parental strains de-

scribed previously [15]. The mice were castrated within 24 h after birth. The castration was done via the dorsal route under the dissection microscope. The mice were weaned on day 25 after birth.

Oestrogen injection

E_2 or DES (Sigma Chemical Co., St Louis, MO, U.S.A.) was suspended in the steroid solution (0.9% NaCl, 0.4% Polysorbate 80, 0.5% carboxymethylcellulose, and 0.9% benzylalchol) and injected s.c. The control mice were injected with only the steroid solution.

[^{125}I]IdUrd uptake by the uterus

The uptake of [^{125}I]IdUrd by the whole uterus was estimated according to the method described previously in detail [16]. Briefly, the mice were given i.p. injection of 8 nmol/g b.wt of fluordeoxyuridine (Carbiochem, San Diego, Calif., U.S.A.) to inhibit endogenous thymidine synthesis [17]. After 1 h, 0.2 μCi/g b.wt of [^{125}I]IdUrd (New England Nuclear Corp., Boston, Mass., U.S.A.; sp. act. > 2000 Ci/mmol) was injected i.p. and the mice were killed 3 h later. The whole uterus was removed, weighed and incubated in 10% phosphate (0.01 M) buffered formalin (pH 7.2) for at least 3 days, with daily changes of buffered formalin. The ^{125}I-radioactivity retained in the whole uterus was measured in an autowell γ-counter. The uptake of [^{125}I]IdUrd by the whole uterus was expressed as percentage of the retained radioactivity to the injected radioactivity. We demonstrated that there was a close and positive co-relationship between the mitotic index and the [^{125}I]IdUrd uptake by the whole uterus [3]. Moreover, we confirmed that the retained ^{125}I-radioactivity is incorporated into DNA [16].

E_2 or DES was injected daily and the [^{125}I]IdUrd uptake by the whole uterus was determined 24 h after the last injection of E_2 or DES. Our previous result showed that the [^{125}I]IdUrd uptake by the uterus of mice reached the maximal level after the single injection of E_2 in mice that were 1 and 10 days of age and that it reached the maximal level after the second injection of E_2 in mice that were 20 and 40 days of age [3]. Thus, the [^{125}I]IdUrd uptake was determined after the single injection of E_2 or DES in mice that were 1, 5, and 10 days of age, and after the second injection of E_2 or DES in mice that were 20, 30, and 40 days of age.

Concentration of serum α-fetoprotein

The concentration of serum α-fetoprotein was measured by the single radical immunodiffusion method described by Mancini *et al.*[18]. A rabbit immunoglobulin against α-fetoprotein of mice and purified α-fetoprotein of mice were generously supplied by Dr Shinzo Nishi of Hokkaido University.

Retention of radioactivity after the injection of [^{3}H]DES

On days 20 and 40 after birth, neonatally castrated mice were divided into two groups. In one group, mice were injected with 2 μCi/g b.wt of [^{3}H]DES alone ([^{3}H]DES, Amersham International plc, Buckinghamshire, England; sp. act., 105 Ci/mmol). In another group, mice were injected with both unlabeled DES (10 μg/g b.wt) and [^{3}H]DES (2 μCi/g b.wt). Unlabeled DES was suspended in 0.1 ml of steroid solution and injected 1 and 2 h before the injection of [^{3}H]DES. [^{3}H]DES was dissolved in 0.9% NaCl containing 10% ethanol, and the injection volume was 0.1 ml. The mice were killed 30 min, 3, and 9 h after the injection of [^{3}H]DES; the uterus, spleen and blood were collected. The uterus and spleen were weighed and homogenized in 0.9% saline. The blood was centrifuged at 1000 g for 10 min to obtain serum. Radioactivity in the homogenates and sera were extracted 3 times with 10 ml of ether. The ether fractions were combined and washed twice with 2 ml of water. Then, the radioactivity in the ether fraction was measured as described previously [19]. The radioactivity retained in tissues and serum in mice injected with [^{3}H]DES alone was considered as the total retention and that in mice injected with both [^{3}H]DES and unlabelled DES was considered as the nonspecific retention. The value of the specific retention was obtained by subtracting the mean value of the nonspecific retention from the value of the total retention.

RESULTS

Neonatally castrated female mice were daily injected with various doses of E_2 or DES on days 1, 5, 10, 20, 30, and 40 after birth. The [^{125}I]IdUrd uptake by the whole uterus was determined 24 h after a single injection of E_2 or DES in mice that were 1, 5, and 10 days of age, and it was determined 24 h after the second injection of E_2 or DES in mice that were 20, 30, and 40 days of age (i.e. 2 days after the initiation of oestrogen injection). The uterine [^{125}I]IdUrd uptake values of vehicle-injected control animals were relatively high (0.10–0.13%) when the experiment was started on days 1, 5, and 10 after birth, but the values were very low (0.01–0.02%) when the experiment was started on days 20, 30 and 40 after birth (Fig. 1). In spite of this difference, injection of either E_2 or DES increased the uterine [^{125}I]IdUrd uptake at all ages examined. As shown in Fig. 1, the minimal dose (μg/mouse) of E_2 that was necessary to induce the maximal [^{125}I]IdUrd uptake was much higher than that of DES in mice that were 1, 5, and 10 days of age. In contrast, the minimal dose (μg/mouse) of E_2 was comparable to that of DES in mice that were 30 and 40 days of age (Fig. 1). The minimal dose (μg/mouse) of E_2 or DES necessary for the maximal response was plotted against the age of mice (Fig.

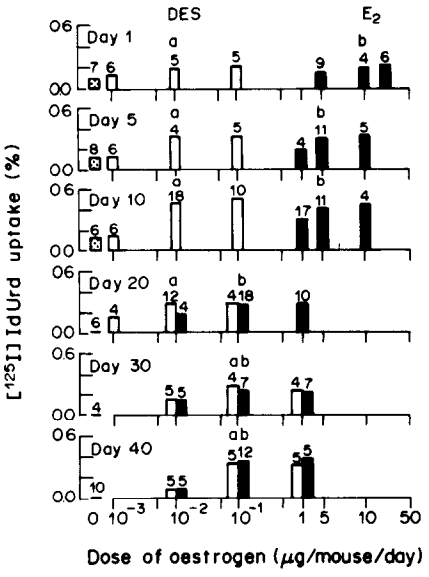

Fig. 1. Effect of E_2 and DES injections on [^{125}I]IdUrd uptake by the whole uterus in neonatally castrated mice at different ages. The height of each bar represents the mean value of mice which were injected with vehicle alone (hatched bar), DES (open bar), or E_2 (closed bar). Number of mice is shown above the top of each bar. $^{a}P < 0.05$, when compared to the value of mice which were injected with the lower dose of DES. $^{b}P < 0.05$, when compared to the value of mice which were injected with the lower dose of E_2. The dose of E_2 or DES indicated by a letter, a or b, is the minimal dose (μg/mouse) necessary for the maximal [^{125}I]IdUrd uptake.

2A). Since the weight of the mice increased with age (Fig. 2B), the minimal doses (μg/mouse) of E_2 and DES were divided by the weight of the mice (Figs 2C and 2D). The decrease of the minimal dose per body weight (relative minimal dose, μg/g b.wt) indicated an increase in sensitivity to injected oestrogens. Thus, we considered the relative minimal dose (μg/g b.wt) as an index that showed the refractory of uterine cells to injected oestrogens.

The serum concentrations of α-fetoprotein are shown in Fig. 3. They were roughly parallel to the relative minimal dose (μg/g b.wt) of E_2 necessary for induction of maximal uterine [^{125}I]IdUrd uptake as shown in Fig. 2C. In other words, the refractiveness of uterine cells to injected E_2 was proportional to the concentration of serum α-fetoprotein.

The scale of the ordinate of Fig. 2C was magnified to show the age-dependent change in sensitivity of uterine cells to DES (Fig. 2D). The relative minimal dose (μg/g b.wt) for the maximal uterine [^{125}I]IdUrd uptake (i.e. refractiveness of uterine cells to DES) was lower in mice that were 10 and 20 days of age than in mice that were 1, 5, 30 and 40 days of age (Fig. 2D). Since the sensitivity of uterine cells to DES was greater in mice that were 20 days of age than in mice that were 40 days of age, we determined the retention of radioactivity after the injection of [^{3}H]DES in mice that were 20 and 40 days of age. The radioactivity in the serum was high 30 min after the injection of [^{3}H]DES, but it decreased thereafter (Table 1). There was no significant differences in the serum radioactivity between mice that were 20 and 40 days of age after the injection of [^{3}H]DES. In the uterus, but not in the spleen, we observed specific retention of radioactivity (i.e. difference between the total and nonspecific radioactivity after [^{3}H]DES injection). The values (mean $\pm$ SE) of the specific retention in the uterus of mice that were 20 and 40 days of age were 1087 ± 425 *vs* 1293 ± 131, 2126 ± 478 *vs* 1489 ± 102, and 900 ± 229 *vs* 1014 ± 45 ($\times 10^3$ dpm/uterus) at 30 min, 1 h, and 9 h, re-

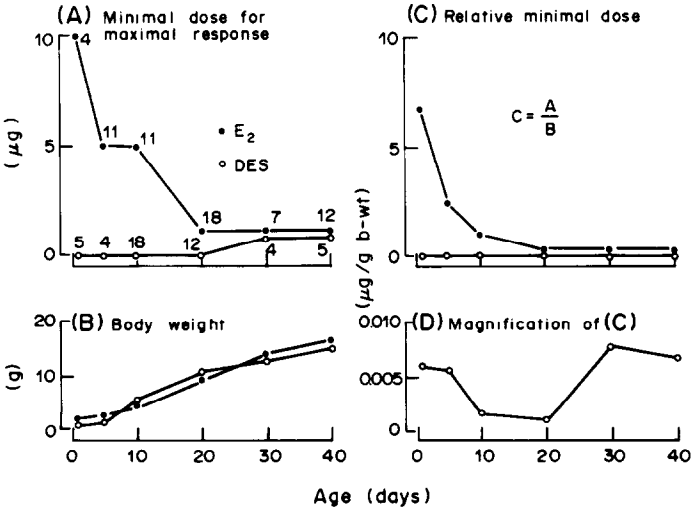

Fig. 2. A. The minimal dose (μg/mouse) of E_2 and DES necessary for induction of maximal [^{125}I]IdUrd uptake by the whole uterus on different days after birth. Number of mice is shown around each point. B. The body weight of mice on different days after birth. C. The relative minimal dose (μg/g b.wt), which was obtained by dividing the minimal dose (μg/mouse) of E_2 and DES (shown in A) by the weight of the body (shown in B). D. The relative minimal dose (μg/g b.wt) of DES; the ordinate is magnified.

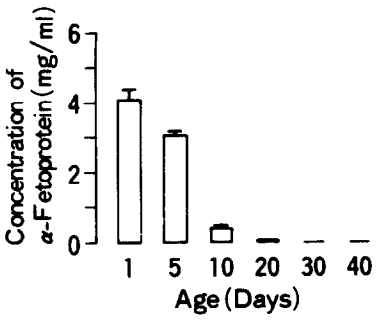

Fig. 3. The concentration of α-fetoprotein in sera of neonatally castrated mice at different ages. The height of each bar represents the mean ± SE of 5 mice.

spectively. No significant difference ($P > 0.1$) in the specific retention values in the uterus was found between mice that were 20 and 40 days of age.

DISCUSSION

The minimal dose of E_2 necessary to induce the maximal [^{125}I]IdUrd uptake by the whole uterus was much higher than that of DES in neonatal and suckling mice, whereas both values were comparable in adult mice. The α-fetoprotein of mice as well as that of rats binds E_2 with high affinity [10, 12, 14], and the affinity of murine α-fetoprotein to DES was about 10% of the affinity to E_2 [14]. In fact, we demonstrated that the concentration of serum α-fetoprotein was inversely proportional to the sensitivity of the uterine cells of mice to injected E_2. Thus, the difference between the effects of injected E_2 and DES during neonatal and suckling periods seems to be attributable to the binding of E_2 with α-fetoprotein. Stack and Gorski[9] also reported that DES induces the proliferation of rat uterine cells at lower doses than E_2 during the neonatal and suckling periods. However, we investigated the detailed relationship between the dose of injected hormones and the magnitude of uterine cell proliferation, since the simplicity of the present method made it possible for us to examine a lot more animals than in the method used by Stack and Gorski[9].

When the minimal dose (μg/mouse) of DES necessary to induce the maximal [^{125}I]IdUrd uptake was divided by the body weight, the calculated value (relative minimal dose, μg/g b.wt) was lower in mice that were 10 and 20 days of age than in mice that were 1, 5, 30, and 40 days of age. Since α-fetoprotein binds DES with a low but significant affinity [14], there is a possibility that the relative minimal dose estimated in the present study may be greater than the true value on days 1 and 5 after birth. It has been reported that the immature state in female rats is characterized by an increase in sensitivity to the inhibitory effect of oestrogen on gonadotropin secretion [20, 21]. The present result of the increased sensitivity to oestrogen in the uterus of immature mice is consistent with the reported findings about the pituitary and hypothalamus of rats. On the other hand, no significant differences were detected in the serum concentration of radioactivity and the specific retention value in the uterus after injection of [^{3}H]DES between mice that were 20 and 40 days of age. Further studies are necessary to clarify the above-mentioned age-dependent change observed in the sensitivity of uterine cells to DES.

The present results showed that the measurement of [^{125}I]IdUrd uptake by the whole uterus was a useful and simple method to investigate the physiology of hormone-induced uterine cell proliferation.

Acknowledgement—We wish to thank Dr Shinzo Nishi of Hokkaido University for giving us a rabbit immunoglobulin against α-fetoprotein of mice and purified α-fetoprotein of mice.

REFERENCES

1. Clark J. H. and Gorski J.: Ontogeny of the estrogen receptor during early uterine development. *Science* **169** (1970) 76–78.
2. Döcke F., Rohde W., Badell J., Geier T. and Dörner G.: Influence of neonatal ovariectomy on the devel-

Table 1. The radioactivity retained in the serum, spleen and uterus of mice of 20 and 40 days of age after the injection of [^{3}H]DES

Tissue	Age of mice	Injection of nonradioactive DES	Radioactivity (×10^3 dpm)* at each time after injection of [^{3}H]DES 30 min	3 h	9 h
Serum (1 ml)	20	No	470 ± 73 (4)	25 ± 6 (5)	21 ± 3 (5)
		Yes	423 ± 78 (5)	42 ± 9 (4)	20 ± 4 (5)
	40	No	590 ± 60 (4)	67 ± 2 (5)	47 ± 7 (4)
		Yes	665 ± 82 (4)	69 ± 6 (4)	37 ± 2 (4)
Spleen (1 g)	20	No	599 ± 84 (4)	58 ± 10 (5)	20 ± 5 (5)
		Yes	741 ± 160 (5)	105 ± 24 (4)	41 ± 11 (5)
	40	No	663 ± 62 (4)	97 ± 7 (5)	34 ± 4 (4)
		Yes	530 ± 71 (4)	78 ± 21 (4)	24 ± 5 (4)
Uterus (1 g)	20	No	1462 ± 425 (4)†	2317 ± 477 (5)†	927 ± 228 (5)†
		Yes	375 ± 70 (5)	191 ± 10 (4)	27 ± 11 (5)
	40	No	1556 ± 130 (4)†	1520 ± 101 (5)†	1034 ± 45 (4)†
		Yes	263 ± 52 (4)	31 ± 3 (4)	20 ± 3 (4)

*Mean ± SE, number of mice is shown in parenthesis.
†$P < 0.01$, when compared to the value of mice which were injected with both radioactive and nonradioactive DES.

opmental patterns of serum gonadotropins, hypothalamic LH-RH concentration and organ weights in female rats. *Endokrinologie* **77** (1981) 273–285.
3. Ogasawara Y., Okamoto S., Kitamura Y. and Matsumoto K.: Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice, assayed by incorporation of [^{125}I]iododeoxyuridine. *Endocrinology* **113** (1983) 582–587.
4. Weisz J. and Gunsalus P.: Estrogen levels in immature female rats: true or spurious-ovarian or adrenal? *Endocrinology* **93** (1973) 1057–1065.
5. Ojeda S. R., Kalra P. S. and McCann S. M.: Further studies on the maturation of the estrogen negative feedback on gonadotropin release in the female rats. *Neuroendocrinology* **18** (1975) 242–255.
6. Rabii J. and Ganong W. F.: Responses of plasma estradiol and plasma LH to ovariectomy, ovariectomy plus adrenalectomy, and estrogen injection at various ages. *Neuroendocrinology* **20** (1976) 270–281.
7. Morera A., Audi L., Bertrand J. and Saez J. M.: Estrogen-like compounds and progesterone in male and female rats before puberty. I. Pattern and origin. *J. steroid Biochem.* **9** (1978) 623–629.
8. Sheehan D. M., Branham W. S., Medlock K. L., Olson M. E. and Zehr D. R.: Uterine responses to estradiol in the neonatal rat. *Endocrinology* **109** (1981) 76–82.
9. Stack G. and Gorski J.: The ontogeny of estrogen responsiveness reexamined: the differential effectiveness of diethylstilbesterol and estradiol on uterine deoxyribonucleic acid synthesis in neonatal rats. *Endocrinology* **112** (1983) 2142–2146.
10. Raynaud J. P., Mercier-Bodard C. and Baulieu E. E.: Rat estradiol binding plasma protein (EBP). *Steroids* **18** (1971) 767–788.
11. Raynaud J. P.: Influence of rat estradiol binding plasma protein (EBP) on uterotropic activity. *Steroids* **21** (1973) 249–262.
12. Soloff M. S., Morrison M. J. and Swartz T. L.: A comparison of the estrone-estradiol-binding proteins in the plasmas of prepubertal and pregnant rats. *Steroids* **20** (1972) 597–608.
13. Germain B. J., Campbell P. S. and Anderson J. N.: Role of the serum estradiol-binding protein in the control of tissue estradiol levels during postnatal development of the female rat. *Endocrinology* **103** (1978) 1401–1410.
14. Savu L., Benassayag C., Genevieve V., Nevena C. and Emmanuel N.: Mouse α_1-fetoprotein and albumin. *J. biol. Chem.* **25** (1981) 9414–9418.
15. Go S., Kitamura Y. and Nishimura M.: Effect of *W* and W^v alleles on production of tissue mast cells in mice. *J. Hered.* **71** (1980) 41–44.
16. Okamoto S., Ogasawara Y., Yamane T., Kitamura Y. and Matsumoto K.: Proliferative response of mouse seminal vesicle epithelium to androgen and estrogen, assayed by incorporation of [^{125}I]iododeoxyuridine. *Endocrinology* **110** (1982) 1796–1803.
17. Bennett M.: Graft-versus-host reaction in mice. I. Kinetic and immunogenetic studies on alloantigen-sensitive units of lymphoid tissue. *Transplantation* **11** (1971) 158–169.
18. Mancini G., Carbonara A. O. and Heremans J. F.: Immunochemical quantitation of antigens by single radical immunodiffusion. *Immunochemistry* **2** (1965) 235–254.
19. Terada N., Kuroda H., Namiki M., Kitamura Y. and Matsumoto K.: Augmentation of aromatose activity by FSH in ovaries of fetal and neonatal mice in organ culture. *J. steroid Biochem.* **20** (1984) 741–745.
20. Ramirez V. D. and McCan S. M.: Comparison of the regulation of lutenizing hormone (LH) secretion in immature and adult rats. *Endocrinology* **72** (1963) 452–464.
21. Steele R. E. and Weisz J.: Changes in sensitivity of the estradiol-LH feedback system with puberty in the female rat. *Endocrinology* **95** (1974) 513–520.