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Development of Vaginal Adenosis-like Lesions and Uterine Epithelial Stratification in Mice Exposed Perinatally to Diethylstilbestrol (42224)

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Abstract. Pregnant ICR/JCL mice were given either four daily subcutaneous injections of 20–2000 μg diethylstilbestrol (DES)/day or a 4-day intravenous infusion of 20–200 μg DES/day starting on Day 15 of gestation. Female mice were injected with 0.01–50 μg DES/day for 5 days starting on the day of birth. Females given oil injections during the neonatal period and offspring of mothers given injections of an infusion of respective vehicles alone served as controls when corresponding ages were attained. Incidence of adenosis-like lesions (ADL) in the vaginal epithelium and stratification of the uterine epithelium (UST) were determined in both offspring and neonatally exposed mice at 30 days of age. Injections of 0.1–50 μg DES/day during the neonatal period induced ADL in the fornical epithelium of vagina (36–70%), but not UST. A high incidence of ADL (80–88%) was found in the fornical and upper-vaginal epithelia in offspring of mothers given injections of 200–2000 μg DES/day, and UST was encountered in 38–70% of these mice. Offspring of mothers given an infusion of 20 μg DES/day had low incidences of ADL (11%) and UST (22%), whereas offspring of mothers infused with 200 μg DES/day exhibited high incidences of ADL (71%) and UST (86%). In offspring of mothers given injections of 2000 μg DES/day, ADL appeared in the fornical and upper-vaginal epithelia as early as 15 days of age. Thus, the present study revealed that ADL and UST occur at a high incidence in infantile mice exposed prenatally to high doses of DES. Previous studies failed to detect such effects perhaps due to the strain of mice, methods, duration, and/or dose of DES used.

Reproductive tract abnormalities including cervicovaginal adenosis, clear cell adenocarcinomas, and uterine hypoplasia have been well documented in young women whose mothers received diethylstilbestrol (DES) during gestation (1, 2). Vaginas of mice exposed perinatally to high doses of sex hormones have estrogen-independent proliferation and cornification, resulting in hyperplastic downgrowths into the underlying stroma and/or transplantable tumors (3–9). In addition, adenosis-like lesions (ADL) occurred in vaginas of mice treated neonatally with natural and synthetic estrogens. In the ADL-bearing animals, columnar epithelium was found in the vaginal fornices and common cervical canal, in which epithelial downgrowths frequently formed glandular structures within the stroma (10–17). ADL appeared in mice at a high incidence following neonatal treatment with DES (12, 14), but seldom occurred in mice exposed prenatally to DES (16, 18). Abnormal uterine changes were observed in mice given injections of natural and synthetic estrogens or androgens during the neonatal period (19–21), but none of the previous studies demonstrated uterine changes at early postnatal ages in mice exposed prenatally to sex hormones. The present study, therefore, was aimed at examining the vaginal and uterine responses to prenatally and neonatally administered DES.

Materials and Methods. Female ICR/JCL mice at ages 3–4 months, purchased from Clea Company, Tokyo, were mated, and the day when the vaginal plug was found in the females was considered as Day 0 of gestation. In the first group of pregnant mice, they were given a continuous, intravenous infusion of DES (Sigma Chemical, St. Louis) at the rate of 0.1 or 0.3 ml/hr from Days 15 to 19 of gestation (96-hr infusion) by using a microinfusion instrument (Type D, Natsume Co., Tokyo). The infusion method was reported in detail previously (22). DES was dissolved in absolute ethanol, and this solution was added to a Tyrode solution containing 10 U/ml heparin sodium. The final concentration of DES was

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8.33 or 27.8 μg/ml. In the second group of pregnant mice, they were given 4 daily subcutaneous injections of 20, 200, or 2000 μg DES in 0.1 ml sesame oil starting on Day 15 of gestation. Offspring of mice given an intravenous infusion or subcutaneous injections of the respective vehicle alone served as controls. On Day 19 of gestation (expected day of delivery = 0 day of age), fetuses removed by caesarian section were foster-nursed by other normal lactating mothers. In the third group of pregnant mice, they were given subcutaneous injections of 2000 μg DES in 0.1 ml sesame oil or oil vehicle alone from Days 15 to 18 of gestation. Offspring of the mothers were killed at 0, 5, 10, 15, 20, 25, and 30 days of age. They were given a single injection of colchicine (Merck Co., Darmstadt), dissolved in a 0.9% NaCl solution, 5 hr before killing, and the mitotic rate of the vaginal epithelium was calculated as previously described (23). In another group of neonatal female mice, they were given daily injections of 0.01, 0.1, 1, 5, 10, or 50 μg DES in 0.02 ml sesame oil or vehicle alone for 5 postnatal days from 0 day of age.

Mice of all groups were killed at 30 days of age; the vaginas and uteri were fixed in Bouin's solution, embedded in paraffin, and serially sectioned parasagitally (vagina) or transversely (the middle three-fifths of uterus) at 8 μm. Sections were stained with Delafield's hematoxylin and eosin. On the vaginal sections, lengths of heterotopic columnar epithelium (HCE, refer to Forsberg and Kalland (12)) with or without adenosis-like lesions (ADL) and total epithelium from fornix to vaginal orifice were measured using a camera lucida, and occupancy extent of HCE- and/or ADL-bearing epithelium in the total epithelium was calculated. Common cervical canal, vaginal fornix, upper vagina (upper three-fifths), which are derived from Müllerian duct (2), and lower vagina (lower two-fifths) derived from urogenital sinus were examined separately.

Results. *Cervicovaginal changes in female mice exposed perinatally to DES.* At 30 days of age, both offspring of pregnant mice given injections or infusion of the respective vehicle alone and mice given injections neonatally of oil showed stratified vaginal epithelia composed of 5–10 cell layers with or without superficial cornification. No histological difference was found in vaginas among these control groups.

In 30-day-old mice given injections of 0.01 μg DES/day starting on 0 day of age, the vaginal epithelia were stratified as in the controls. In contrast, injections of 0.1–50 μg DES/day during the neonatal period induced heterotopic columnar epithelium (HCE) and/or adenosis-like lesions (ADL) in the vaginal fornices (36–70%) and common cervical canal (9–56%). In such abnormal epithelium, HCE was in direct contact with the basement membrane and interrupted the normally stratified epithelium. ADL frequently formed glandular downgrowths into the stroma. In the upper nonfornical vagina, however, neither HCE nor ADL was found in the epithelium (Table I); in the lower vagina, hypospadias appeared without epithelial changes.

A high incidence (80–88%) of HCE and/or ADL was found in the fornical and upper-vaginal epithelia in offspring of mothers given injections of 200 or 2000 μg DES/day, whereas HCE and/or ADL were not found in any region of vaginas in offspring of mothers injected with 20 μg DES/day. In 25% of mice whose mothers were given injections of 200 μg DES/day, common cervical canal exhibited ADL and/or HCE; this did not occur in those of mothers given injections of 20 μg DES/day. In contrast, intravenous DES infusion to pregnant mice induced ADL in the fornical epithelium even in offspring of mothers receiving a low dose of 20 μg DES/day, although the incidence was not high (11%). A high incidence (71%) of HCE and/or ADL was encountered in the fornix and upper vaginas of offspring of mothers receiving an infusion of 200 μg DES/day. Offspring of mothers given injections of 200–2000 μg DES/day had shallow vaginal fornices, differing from the controls which had prominent fornices.

Occupancy extents (%) of columnar epithelium, HCE and/or ADL in the total vaginal epithelia are shown in Table I. Both in offspring of DES-infused mothers and in neonatally DES-injected mice, the vaginal epithelia showed a low occupancy extent of HCE and/or ADL (0–13%) as compared with those of mothers given 200 or 2000 μg DES injections (0–53%), although the extent varied with

TABLE I. INCIDENCE OF VAGINAL AND UTERINE EPITHELIAL ABNORMALITIES IN FEMALE MICE EXPOSED PERINATALLY TO DIFFERENT DOSES OF DIETHYLSTILBESTROL (DES)

Treatments and daily dose (μg)	Age at autopsy (days)	No. of mice with vaginal abnormalities/No. of mice examined	No. of mice showing vaginal and uterine abnormalities					
			ADL[a] and/or HCE[b]					
			Common cervical canal	Vaginal fornix	Upper[c] vagina	Extent of occupancy (%)	Wolffian remnants	Proliferation and stratification of uterine epithelium
DES injections to pregnant mice								
Vehicle	0–30[d]	0/42	0[a] (0)[b]	0 (0)	0 (0)	0	0	0
20	30	0/13	0 (0)	0 (0)	0 (0)	0	13[e]	5
200	30	7/8[e]	2 (1)	6 (4)	4 (2)	11 ± 3.8[i]	8[e]	3
2000	0	0/7	0 (0)	0 (0)	0 (0)	0	7[e]	0
	5	8/8[e]	0 (8)	0 (8)	0 (8)	16 ± 2.5[g]	8[e]	0
	10	9/9[e]	0 (9)	0 (9)	0 (9)	17 ± 2.5[g]	9[e]	0
	15	7/7[e]	0 (2)	2 (6)	3 (7)	15 ± 3.0[g]	7[e]	0
	20	10/10[e]	0 (5)	9 (10)	10 (10)	27 ± 3.7[g]	9[e]	4
	25	9/9[e]	7 (7)	9 (8)	8 (9)	22 ± 4.0[g]	9[e]	3
	30	16/20[e]	1 (1)	12 (8)	16 (11)	17 ± 4.0[i]	20[e]	14[e]
DES infusion into pregnant mice								
Vehicle	30	0/7	0 (0)	0 (0)	0 (0)	0	0	0
20	30	1/9		1 (0)	0 (0)	0.3 ± 0.3	9[e]	2
200	30	5/7[f]		2 (0)	3 (0)	3 ± 1.1[i]	7[e]	6[f]
DES injections to neonatal mice								
Vehicle	30	0/7	0 (0)	0 (0)	0 (0)	0	0	0
0.01	30	0/7	0 (0)	0 (0)		0	4	
0.1	30	4/11	1 (1)	2 (4)		2 ± 1.3	11[e]	
1	30	7/10[f]	0 (0)	4 (6)		2 ± 0.4[h]	8[e]	
5	30	3/7	0 (3)	0 (3)		2 ± 1.2	4[f]	
10	30	5/9[f]	0 (6)	3 (3)		2 ± 0.7[i]	7[e]	
50	30	5/9[f]	0 (5)	3 (2)		2 ± 0.8[i]	9[e]	

[a] Adenosis-like lesion.
[b] Heterotopic columnar epithelium.
[c] Upper three-fifths of vagina.
[d] Seven mice each were examined at ages of 0, 5, 15, 20, 25, and 30 days, respectively.
[e] vs control, $P < 0.005$.
[f] vs control, $P < 0.05$ (Fisher's exact probability test).
[g] vs control, $P < 0.0005$.
[h] vs control, $P < 0.005$.
[i] vs control, $P < 0.05$ (Student's t test).

the individual, since varying lengths of stratified areas intervened frequently in the columnar epithelium.

Prominent remnants of the Wolffian duct lined by a single layer of cuboidal cells were observed in the vaginal stromata of mice exposed to DES prenatally (90–100%) or neonatally (57–100%), whereas the remnants were never found in the controls.

Uterine changes in female mice exposed perinatally to DES. In 30-day-old control mice, uteri had a single epithelial layer of tall columnar cells and well-developed glands in the endometria. In 38–70% of offspring of mothers given injections of 20–2000 μg DES/day, however, proliferated and stratified epithelia (UST) consisting of 3–6 cell layers was found in their uteri (Table I). A high incidence (86%) of UST was encountered in offspring of mothers given an intravenous infusion of 200 μg DES/day, whereas the infusion of 20 μg DES/day to mothers induced a lower incidence of UST in their offspring (22%). DES-injected neonatally failed to induce any uterine changes even when the highest dose was used (50 μg/day).

Histological changes in vaginal and uterine epithelia of mice exposed prenatally to DES. In neonatal mice whose mothers were given four daily injections of oil vehicle alone starting on Day 15 of gestation, the epithelia of the Müllerian (upper) vagina was lined with a pseudostratified columnar epithelium. By contrast, the sinus vagina was composed of a solid cord of the densely aggregated epithelial cells. At 5 days of age, the vaginal epithelia of the controls consisted of two layers of cuboidal cells except for the sinus vagina which was a solid cord. ADL and HCE were not found in vaginas of 10- to 30-day-old control mice; the epithelium was composed of three to five layers of cuboidal cells with or without superficial mucification.

The vaginal epithelia of neonatal mice whose mothers were given four daily injections of 2000 μg DES/day starting on Day 15 of gestation consisted of pseudostratified columnar cells as compared with those of the age-matched controls. In 5-day-old mice exposed to DES *in utero* (DES mice), however, nodules of polygonal cells appeared under the columnar epithelia of the Müllerian vagina (Fig. 1). The vaginal epithelia of 10-day-old DES mice were composed mostly of a single layer of columnar cells with some regions of the stratified epithelium, to the outermost layer of which a columnar cell layer was still attached. In 15- and 20-day-old DES mice, the cervicovaginal epithelia was mainly stratified, although HCE sporadically interrupted this epithelium. The fornical and upper-vaginal epithelia frequently possessed ADL (Fig. 2), whereas the common cervical canal was free from the lesions. Incidence of mice showing ADL was higher in 20-day-old DES mice than in 15-day-old DES mice (Table I). In 25-day-old DES mice, ADL was found in the epithelium of the common cervical canal, although the extent of lesion was decreased in the same area at 30 days.

The epithelium of the lower vagina was composed of 5–10 layers of cells with or without superficial cornification in DES mice at 15–30 days. Extents of occupancy of HCE and/or ADL in the total vaginal epithelium of DES mice are shown in Table I. The extent rose between 5 and 20 days, but declined at 25 days. In 20% of 30-day-old DES mice, no abnormality was found in the vaginal epithelium.

The mitotic rate of the vaginal epithelium undergoing ADL and/or HCE was similar to that of the basal cells of stratified epithelium without lesions in 15-day-old DES mice, whereas the rate of stratified epithelium (1.6 ± 0.19) was higher than that of the ADL-free columnar epithelium (0.7 ± 0.10) in DES-exposed mice at ages of 20–30 days.

Uterine epithelium of control mice aged 0–30 days was composed of a single layer of columnar cells. In contrast, the epithelium gave rise to UST (three to five cell layers) in uteri of 33–70% of DES mice at ages 20–30 days (Fig. 3), whereas in DES mice at 0–15 days, UST was not detected (Table I).

Discussion. Vaginal adenosis and cervical ectropion are commonly encountered in women exposed *in utero* to DES during the first trimester of pregnancy (1, 2). Perinatal exposure of female mice to natural and synthetic sex hormones results in vaginal abnormalities including persistent proliferation and cornification of the epithelium, hyperplastic epithelial downgrowths into the underlying stroma, and transplantable tumors in advanced ages (3–9). Adenosis-like lesions in vagina have also been reported in perinatally

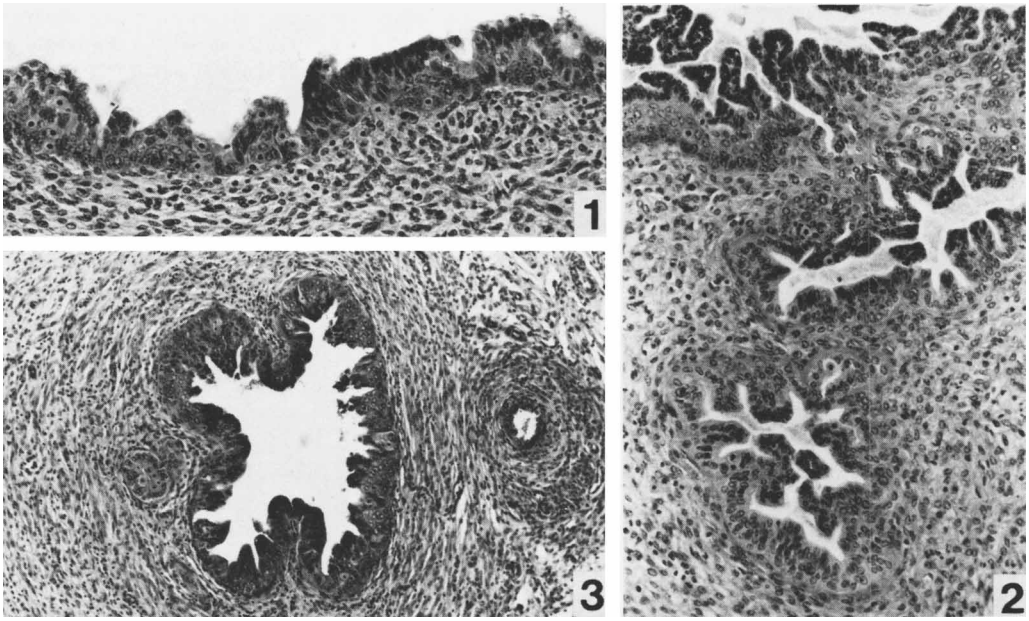

FIG. 1. Upper-vaginal epithelium of a 5-day-old mouse whose mother was given four daily subcutaneous injections of 2000 μg DES/day starting on Day 15 of gestation. Nodules are present sporadically under the columnar epithelium. Mitotic figures are seen in both nodules and columnar epithelium. ×140.

FIG. 2. Upper-vaginal epithelium of a 20-day-old mouse born of mother given DES-injections as in Fig. 1. Adenosis-like lesion (ADL) is present in the connective tissue stroma. Vaginal lumen at the top is lined with the epithelium, a part of which is proliferated and stratified. Note mitotic figures in ADL. ×140

FIG. 3. Uterus of a 20-day-old mouse born of mother given DES-injections as in Fig. 1, showing a proliferated epithelium. A uterine gland is seen on the left side. Note Wolffian remnant on the right side. ×70.

DES-exposed mice (10–17). Some investigators have reported that no ADL or only a low incidence occurs in mice exposed prenatally to DES. They gave either a single injection of a high dose of DES or eight daily injections of lower doses of DES to pregnant mice (16, 18). In the present study, however, administration of large doses of 200 or 2000 μg DES/day given to pregnant mothers starting on Day 15 of gestation resulted in a high incidence (80–88%) of heterotopic columnar epithelium (HCE) and/or ADL in the fornical and upper-vaginal epithelia of their offspring. Injections of 0.1–50 μg DES/day during the neonatal period induced such lesions in the epithelium of fornix and common cervical canal. These results are in accordance with those in previous studies (12, 14). In the upper vagina, where an occurrence of HCE and/or ADL was reported in NMRI mice treated neonatally with DES (12), the lesions were never encountered in neonatally DES-treated ICR/JCL mice used in the present study. This difference between the two strains may be due to different sensitivity of the responsive parts of vaginal and fornicocervical epithelia or due to different stage of vaginal differentiation.

Injections of large doses of DES to pregnant mothers caused HCE and/or ADL to a large extent of vaginal epithelia of their offspring. In contrast, an intravenous infusion of DES into pregnant mothers or injections of DES to neonatal mice induced the lesions only to a small extent of the epithelium. This finding suggests that injections of DES given to pregnant mice are more effective in inducing the lesions than are neonatal DES injections or DES infusion into pregnant mothers.

Forsberg and Kalland (12) reported that the primary action of estrogen on the neonatal mouse fornical epithelium is to induce inhibition of cell division and that the division-inhibited columnar epithelium represents the future site of adenosis. However, the present

results indicate that the columnar epithelial cells continue to divide in vaginas of postnatal mice exposed prenatally to DES. The progressive development of HCE into gland-like downgrowths into the stroma (ADL) has been described as a postpubertal event in neonatally estrogen-treated mice (12). In the present study, the gland-like structures were found as early as at 15 days of age in mice whose mothers were given four daily injections of DES starting on Day 15 of gestation. This finding suggests that prenatal exposure to DES accelerates the development of HCE into ADL compared with that in neonatally exposed mice.

Nodules of polygonal cells appeared sporadically under the columnar epithelium of the Müllerian vagina in 5-day-old mice exposed to DES *in utero* but not in 0-day-old mice. This noduligenesis has been considered as the first sign of development of permanent proliferation of the vaginal epithelia in mice treated neonatally with natural and synthetic estrogens. The nodules were found during or immediately after neonatal estrogen treatment (5, 23). The present study, however, demonstrated a delayed nodule formation starting on the fifth postnatal day after prenatal exposure to DES. This finding suggests a latent period of noduligenesis in fetal vaginas.

Proliferation and stratification of the uterine epithelium with or without superficial cornification have been reported in adult mice given neonatal injections of estrogens or androgens (19–21). In the present study, such uterine changes were not found in neonatally DES-treated immature mice, whereas the changes were observed in 22–86% of prenatally DES-exposed mice as early as 30 days of age. These findings suggest that prenatal exposure of mice to DES is more effective than neonatal DES-treatment in early induction of uterine stratification.

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