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Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol

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Prenatal exposure to diethylstilbestrol (DES) has been associated with the subsequent development of reproductive tract abnormalities, including poor reproductive outcome and neoplasia, in experimental animals and humans. Experimental animal studies with chemical carcinogens have raised the possibility that adverse effects of DES may be transmitted to succeeding generations. To evaluate this possibility and to determine if there is a sensitive window of developmental exposure, outbred CD-1 mice were treated with DES during three stages of development: group 1 was treated on days 9–16 of gestation (2.5, 5 or 10 μg/kg maternal body wt), the time of major organogenesis; group II was treated once on day 18 of gestation (1000 μg/kg maternal body wt) just prior to birth; group III was treated on days 1–5 of neonatal life (0.002 μg/pup/day). Female mice (F1) in each group were raised to sexual maturity and bred to control males. As previously reported, fertility of the F1 DES-exposed females was decreased in all groups. Female offspring (DES lineage or F2) from these matings were raised to maturity and housed with control males for 20 weeks. The fertility of these DES lineage female mice was not affected by DES exposure of their 'grandmothers'. DES lineage mice were killed at 17–19 and 22–24 months of age. An increased incidence of malignant reproductive tract tumors, including uterine adenocarcinoma, was seen in DES lineage mice but not in corresponding controls; the range and prevalence of tumors increased with age. Because uterine adenocarcinomas were seen in all three DES groups, all developmental exposure periods were considered susceptible to the adverse effects of DES. These data suggest that the reduced fertility observed in the DES F1 female mice was not transmitted to their descendants; however, increased susceptibility to tumor formation is apparently transmitted to subsequent generations.

Abbreviations: DES, diethylstilbestrol; PPL, progressive proliferative lesions.

Introduction

Concerns have been raised regarding the role of chemicals in the environment that possess estrogenic and/or endocrine-disrupting activity and their possible contributions to an increased incidence of various diseases in hormone target tissues (1–3). It has been hypothesized that these endocrine-active compounds, especially those that bioaccumulate, are related to increases in breast cancer, endometriosis, fibroids and uterine adenocarcinoma in females, and decreased sperm quality and quantity, benign prostatic hyperplasia, prostatic and testicular cancer and developmental abnormalities in males (1–4). It remains to be determined whether exposure to these chemicals at ambient environmental levels is related to human disease; however, ample evidence exists in both humans and experimental animals to link developmental exposure to pharmacological levels of the synthetic estrogenic compound diethylstilbestrol (DES), with poor reproductive outcome and tumors later in life (5). Furthermore, experimental studies from two laboratories suggest the possibility of a transgenerational carcinogenic effect of DES since prenatal treatment of mice with the compound was followed by an increased incidence of uterine and ovarian tumors in their second-generation descendants (6–10). In spite of these studies, the transmission of DES-induced lesions to subsequent generations is still a controversial idea. In fact, studies from another laboratory (11) report no adverse second generation effects of DES. In this study, however, the determination of long-term abnormalities, including cancer, were not evaluated because the oldest animals in the study were only 8–12 weeks of age (11).

For many years, research in our laboratory has centered on studying the effects of DES and other estrogens on differentiating reproductive tract tissues. Using the CD-1 outbred mouse, we have shown that benign and malignant changes in the developmentally DES-exposed murine genital tract closely parallel the human situation (12–23). In fact, this DES animal model has both duplicated and predicted lesions observed in similarly exposed humans (24). The etiology of these various DES-induced abnormalities has remained unclear. While many of the alterations are thought to be teratogenic changes (20–23), the pathogenesis of some of the lesions, especially those with neoplastic potential, is more difficult to discern (19). DES and other estrogens are known to be carcinogenic in humans and rodents (5), but the mechanisms by which these hormones induce cancer are only partially understood. Stimulation of cell proliferation and gene expression by binding to the estrogen receptor have been reported to be important mechanisms in hormonal carcinogenesis (25). The significance of these mechanisms is supported by our recent study showing increased DES-induced tumor prevalence and time to tumor formation in the uteri of transgenic mice that overexpress the estrogen receptor (26). However, binding to the estrogen receptor and cell proliferation alone are not sufficient to explain the carcinogenic activity of estrogens because not all estrogens are carcinogenic. Estrogens are not mutagenic in many assays,

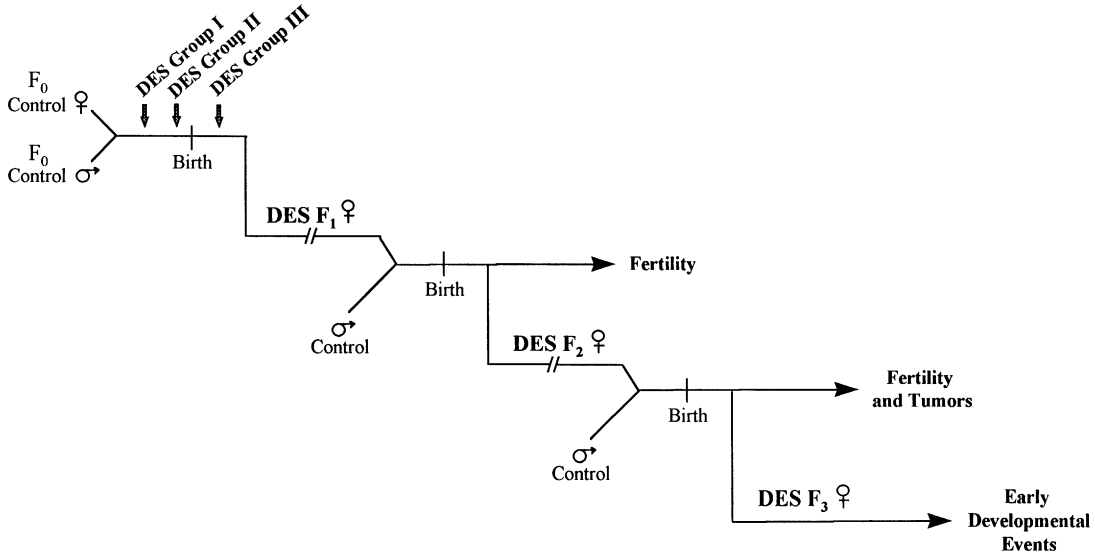

Fig. 1. Generation of DES lineage mice. Details are described in Materials and methods. Group I, prenatal DES treatment at a dose of 2.5, 5 or 10 μg/kg maternal body wt administered by s.c. injection on days 9–16 of gestation; group II, prenatal DES treatment at a dose of 1000 μg/kg maternal body wt s.c. injected on day 18 of gestation; group III, neonatal DES at a dose of 0.002 μg/pup/day injected s.c. on days 1–5.

but have been shown to exhibit specific types of genotoxic activity under certain conditions (for a review see ref. 27). In cell culture, DES and 17β-estradiol and their metabolites have been reported to induce morphological and neoplastic transformation of Syrian hamster embryo cells that express no measurable levels of estrogen receptor; Syrian hamster embryo cell transformation rates correlate with aneuploidy induction and DNA damage caused by DNA adduct formation (27). Further evidence of genetic/epigenetic effects associated with estrogen treatment have been described in our studies of developmentally DES-exposed mice (28,29) and humans (30), and studies from other laboratories (31–33). Together, these data raise the possibility that changes seen following developmental exposure to DES may be transmitted to subsequent generations.

Thus, to confirm if these abnormalities are passed on to subsequent generations and to identify potential mechanisms involved, we studied breeding performance and tumor incidence in DES lineage mice. We included three windows of developmental exposure in this study to determine if a critical stage of differentiation for the DES-exposed mouse (F1) was essential in transmitting adverse effects: (i) DES exposure on days 9–16 of gestation, the period of major organogenesis in the mouse and a time we have shown to be sensitive to DES adverse effects (13–15); (ii) DES exposure on day 18 only (the day preceding birth), an exposure time reported by Walker (6) to be associated with multi-generational effects; and (iii) DES exposure on days 1–5 of neonatal life, previously reported to result in an increased incidence of uterine adenocarcinoma in the F1 generation (19,20), although the tumorigenic dose used previously was 1000 times higher than that used in the current study. In the present study, a rare cancer seen in developmentally DES-exposed mice was transmitted to subsequent generations.

Materials and methods

F0 generation

As previously described (14), adult CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same strain in the breeding facility at the National Institute of Environmental Health Sciences (Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. On day 9 of gestation, pregnant female mice were individually housed in cages with hardwood chip bedding and a cotton fiber nesting block. Pregnant mice were housed under controlled lighting (12 h light/12 h dark) and controlled temperature (21–22°C) conditions. NIH laboratory mouse chow and fresh water were supplied *ad libitum.*

F1 generation

Group I. DES (Sigma, St Louis, MO) dissolved in corn oil or corn oil alone (control) was administered as an s.c. injection to the pregnant dam on days 9–16 of gestation at a daily dose of 2.5, 5 or 10 μg/kg maternal body wt (prenatal DES 2.5, prenatal DES 5 and prenatal DES 10) as previously described (14). Pregnant mice delivered their young and litters were standardized to eight pups each.

Group II. DES dissolved in corn oil or corn oil alone (control) was administered as a single s.c. injection to the pregnant dam on day 18 of gestation at a dose of 1000 μg/kg maternal body wt (prenatal DES day 18) as described (34). Pregnant mice delivered their young and litters were standardized to eight pups.

Group III. Untreated pregnant mice delivered their young and litters were standardized to eight female pups. Pups were s.c. injected once daily with DES dissolved in corn oil (0.002 μg DES/pup/day, the weight of pups ranged from 1 g on day 1 to 3.5 g on day 5) or corn oil alone (control) on days 1–5 of life (neonatal DES) as described (19,20). The dose of 2 μg DES/pup/day used in previous studies (19,20) was not compatible with fertility; thus, to generate a second generation for this study, a lower dose was used.

All mice were weaned at 3 weeks of age and housed five per cage. These mice are referred to as the F1 generation. Figure 1 is a schematic diagram of the experimental design for the generation of DES lineage mice.

F1 breeding

According to a previously described protocol (15), 8–12-week-old F1 female mice (group I, 42 prenatal DES 2.5, 42 prenatal DES 5, 39 prenatal DES 10 and 25 control; group II, 99 prenatal DES day 18 and 25 control; group III, 42 neonatal DES and 25 control) were bred to proven untreated male mice of the same strain (4 females/male). (The number of animals in group II, prenatal DES day 18 was larger than the other groups so that sufficient numbers of F2 animals could be generated for the study.) Females observed to be pregnant were removed and housed individually until delivery. When F1 female mice delivered their young, pups were counted and litters were standardized to 8 pups/litter. The offspring of the F1 mice are referred to as second-generation (F2) or DES lineage mice. F2 mice were weaned at 3 weeks of age and held five per cage for further study. Because the controls for all three groups were similar, they were combined and the data are presented as a single set.

F2 breeding

DES lineage (F2) female mice (75 control; group I, 25 prenatal DES 2.5, 25 prenatal DES 5 and 7 prenatal DES 10; group II, 25 prenatal DES day 18;

Table I. Comparison of fertility between F1 and F2 DES-treated female mice[a]

Treatment	No. bred	No. pups in first litter[b]	Days to first litter[b]	Females with litter (%)
Fertility of F1 female mice[c]				
Control	75	11.1 ± 0.16	27.5 ± 0.31	75/75 (100)
Group I				
Prenatal DES 2.5	42	9.3 ± 0.54	30.5 ± 1.07	42/42 (100)
Prenatal DES 5	42	6.9 ± 0.63[d]	30.0 ± 1.66	35/42[d] (83)
Prenatal DES 10	39	1.9 ± 0.47[d]	38.5 ± 3.14[d]	11/39[d] (28)
Group II				
Prenatal DES day 18	99	7.0 ± 0.92[d]	35.3 ± 2.26[d]	26/99[d] (26)
Group III				
Neonatal DES	42	9.4 ± 0.86	29.3 ± 1.51	34/42[d] (81)
Fertility of F2 female mice[e]				
Control	75	12.5 ± 0.22	21.6 ± 0.18	75/75 (100)
Group I				
Prenatal DES 2.5	25	12.1 ± 0.42	23.0 ± 0.63	25/25 (100)
Prenatal DES 5	25	13.8 ± 0.49	22.6 ± 0.89	25/25 (100)
Prenatal DES 10	7	14.1 ± 0.94	23.0 ± 1.41	7/7 (100)
Group II				
Prenatal DES day 18	25	12.3 ± 0.49	22.1 ± 0.37	25/25 (100)
Group III				
Neonatal DES	25	13.2 ± 0.33	21.4 ± 0.20	25/25 (100)

[a]DES-exposed female mice were mated with untreated control male mice of the same strain.
[b]Values shown are means ± SE.
[c]F1 female mice were exposed to DES either prenatally or neonatally as described in Materials and methods.
[d]$P < 0.01$ versus controls (Dunnett's test for count data, Fisher's exact test for proportion data).
[e]Female mice are the offspring (DES lineage) of F1 DES-treated female mice that were mated with control male mice of the same strain.

group III, 25 neonatal DES) were bred at 8–12 weeks of age to proven untreated control males. Four female mice identified by ear notches, each from a different F1 treatment group, were randomly assigned to a breeding cage (4 females/male).

When an F2 female mouse appeared pregnant, she was removed from the breeding cage, weighed and individually housed. When the female mouse delivered, pups (F3) were counted, weighed and examined for gross abnormalities; the female mouse was then returned to the breeding cage. At the end of 20 weeks, the breeding study was discontinued. Male mice were removed and pregnant females delivered their pups (F3). F2 female mice were held until killing at 17–19 or 22–24 months of age for tumor incidence determinations. For some groups, additional F2 animals were generated to supplement the number for long-term tumor studies.

F2 tumor incidence

At necropsy, animals were weighed and observed for any gross abnormalities. Reproductive tract tissues were quickly removed and fixed in 10% neutral buffered formalin; ovaries and oviducts were fixed in Bouin's solution. Other tissues, including liver, lung, kidneys, adrenal glands, heart and para-aortic lymph nodes, were also removed and fixed in 10% neutral buffered formalin. Tissues were processed, embedded in paraffin and sectioned at 6 μm. Tissue sections were stained with hematoxylin and eosin and evaluated by light microscopy. Additional serial sections were made for some lesions to include the entire area of pathological change.

Results

As we reported earlier (15), the reproductive outcome of prenatally DES-exposed female offspring (F1) was found to be decreased as compared with control females. Factors that indicated poor reproductive outcome included lower numbers of live pups per litter, an increase in the time in days to first litter and a decrease in the percent of females with litters (Table I). Across all DES-treated groups studied, group I, prenatal DES 10 was the most severely affected (Table I).

On the other hand, the fertility of the DES lineage mice (F2 females) showed no adverse effects over a 20 week continuous breeding period (Figure 2). The number of live pups per litter, time in days to first litter and percent of females with litters were not different from control corn oil-treated females (Table I). Further, there were no noted malformed neonates in the F3 litters from the F2 generation. Table II summarizes body weights in the control and DES groups for the F1, F2 and F3 generations. While some statistically significant differences in body weights were detected in the F1 and F2 generations, these differences were generally <15%. For the F3 generation, there was no biologically significant difference in body weights between DES and control groups. In addition, there was no consistent difference in age at puberty, which was determined by vaginal opening in F2 or F3 female mice (data not presented).

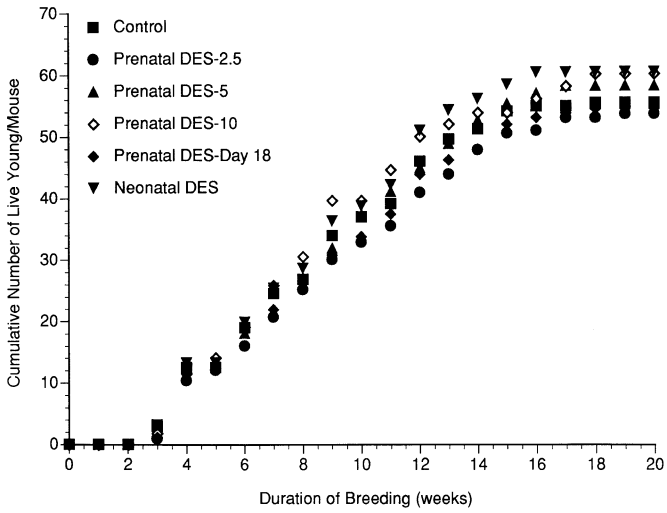

Fig. 2. Total reproductive capacity of DES lineage female mice (F2) whose mothers were exposed prenatally or neonatally to DES. Mice were the CD-1 female offspring (F2) obtained from mating control male mice and females (F1) exposed prenatally to DES on days 9–16 of gestation (prenatal DES 2.5, 5 or 10) or on day 18 of gestation (prenatal DES day 18) or exposed neonatally on days 1–5 (neonatal DES). Fertility of F2 female mice was determined by a continuous breeding protocol and expressed as the cumulative number of live young born per mouse over a 20 week interval.

Table II. Body weight over time among DES lineage female mice

Treatment	Day 8		Day 15		Day 22		Day 29 (F1, F2), days 29–30 (F3)		Days 33–36 (F1), days 34–35 (F2), days 32–33 (F3)	
	n	Body wt	*n*	Body wt	*n*	Body wt	*n*	Body wt	*n*	Body wt
F1 generation										
Control	47	5.6 ± 0.1	71	9.1 ± 0.1	71	13.2 ± 0.2	71	19.9 ± 0.2	46	22.9 ± 0.1
Group I										
Prenatal DES 2.5	24	5.1 ± 0.1[a]	24	9.2 ± 0.2	24	14.3 ± 0.3[a]	24	21.3 ± 0.4[a]	24	23.6 ± 0.4
Prenatal DES 5	16	5.0 ± 0.1[a]	16	7.5 ± 0.2[a]	16	12.1 ± 0.4[a]	16	19.6 ± 0.6	16	23.7 ± 0.5
Prenatal DES 10	19	4.9 ± 0.1[a]	19	8.1 ± 0.2[a]	19	11.9 ± 0.2[a]	19	18.5 ± 0.4[a]	19	23.3 ± 0.5
Group II										
Prenatal DES day 18	23	5.3 ± 0.1	23	8.3 ± 0.1[a]	23	12.0 ± 0.2[a]	23	19.4 ± 0.3	23	24.0 ± 0.3
Group III										
Neonatal DES	24	5.3 ± 0.1	24	8.2 ± 0.2[a]	24	11.7 ± 0.2[a]	24	18.5 ± 0.3[a]	24	23.2 ± 0.3
F2 generation										
Control	56	5.9 ± 0.04	64	9.6 ± 0.1	61	14.3 ± 0.1	56	21.3 ± 0.2	21	23.6 ± 0.2
Group I										
Prenatal DES 2.5	21	5.8 ± 0.1	21	9.9 ± 0.2	21	14.9 ± 0.2	21	20.6 ± 0.3	21	23.8 ± 0.5
Prenatal DES 5	11	5.3 ± 0.2[a]	11	9.7 ± 0.6	11	13.4 ± 0.5	11	19.9 ± 0.6	10	22.8 ± 0.8
Prenatal DES 10	4	6.0 ± 0.7	4	11.6 ± 1.1[a]	4	16.2 ± 1.5[a]	4	21.2 ± 1.8		
Group II										
Prenatal DES day 18	22	5.0 ± 0.1[a]	16	8.8 ± 0.1[a]	22	12.6 ± 0.2[a]	22	18.6 ± 0.3[a]	11	22.1 ± 0.4
Group III										
Neonatal DES	19	6.0 ± 0.1	19	10.0 ± 0.2	19	14.5 ± 0.2	19	20.2 ± 0.3	19	22.6 ± 0.4
F3 generation										
Control	71	5.4 ± 0.1	69	9.5 ± 0.1	69	14.4 ± 0.1	69	21.1 ± 0.2	46	23.5 ± 0.2
Group I										
Prenatal DES 2.5	24	5.3 ± 0.1	24	9.1 ± 0.1	24	14.1 ± 0.2	24	21.2 ± 0.3	24	23.3 ± 0.3
Prenatal DES 5	19	5.4 ± 0.1	19	9.9 ± 0.2	19	15.4 ± 0.2[a]	19	22.0 ± 0.3	19	23.2 ± 0.3
Prenatal DES 10	24	5.5 ± 0.1	24	9.8 ± 0.2	16	14.0 ± 0.3	24	20.7 ± 0.2	16	23.3 ± 0.4
Group II										
Prenatal DES day 18	23	5.4 ± 0.1	23	9.8 ± 0.2	23	13.9 ± 0.2	23	21.7 ± 0.3	16	24.8 ± 0.5[a]
Group III										
Neonatal DES	23	5.5 ± 0.1	22	9.9 ± 0.2	22	14.8 ± 0.3	22	22.0 ± 0.4	22	24.7 ± 0.4

Values shown are means ± SE.
[a]$P < 0.01$ versus controls (Dunnett's test).

In our previous studies, we showed an increase in vaginal (14,20) and uterine (19) adenocarcinomas in mice exposed to DES either prenatally or neonatally. To determine if these rare genital tract cancers associated with DES were transmitted to another generation, histological changes in the genital tracts of the DES lineage (F2) mice were evaluated later in life, at 17–19 or 22–24 months of age; data showed an increased incidence in reproductive tract tumors in DES lineage mice as compared with control mice. Abnormalities observed in the DES lineage mice at 17–19 months of age are listed in Table III. The incidence of lesions in the ovary and oviduct of F2 animals at this age did not appear to be significantly different from control animals, except for group I, prenatal DES 10, which had progressive proliferative lesions (PPL) of the oviduct (7/16, 44%). However, the occurrence of preneoplastic and neoplastic tumors in the uterus was of particular significance. In group I, uterine adenocarcinomas were seen in the prenatal DES 2.5 group (2/29, 7%) and in the prenatal DES 5 group (2/35, 6%); atypical hyperplasia was seen in the prenatal DES 5 group (1/35, 3%) and in the prenatal DES 10 group (2/16, 13%). In group II, prenatal DES day 18, no malignant tumors were observed at this age. In group III, neonatal DES, atypical uterine hyperplasia (1/29, 3%) and uterine adenocarcinoma (1/29, 3%) were seen; this group also had one stromal cell sarcoma (1/29, 3%).

The range and prevalence of histological abnormalities increased with age. In mice at 22–24 months of age (Table IV), we observed preneoplastic and neoplastic lesions in reproductive tract tissues in the DES lineage (F2) mice. In group I, atypical uterine hyperplasia was seen in the prenatal DES 2.5 group (1/35, 3%; Figure 3), in the prenatal DES 5 group (2/37, 5%) and in the prenatal DES 10 group (4/24, 17%); uterine adenocarcinomas were found in the prenatal DES 2.5 group (3/35, 9%; Figure 4) and in the prenatal DES 5 group (6/37, 16%); one vaginal carcinoma *in situ* (Figure 5), one vaginal adenocarcinoma (Figure 6) and one clitoral gland adenocarcinoma were found in the prenatal DES 5 group; one cervical carcinoma (1/24, 4%) was observed in the prenatal DES 10 group. In group II, prenatal DES day 18, we observed atypical hyperplasia (1/15, 7%) and uterine adenocarcinoma (1/15, 7%). In group III, neonatal DES, atypical hyperplasia (3/36, 8%), uterine adenocarcinoma (4/36, 11%), stromal cell sarcoma (1/36, 3%) and vaginal carcinoma *in situ* (1/36, 3%; Figure 7) were observed. No similar reproductive tract lesions were observed in the uterus and vagina of corresponding control animals in this study; the atypical hyperplasia observed in the uterus of a control animal (1/23, 4%) was only a focal area of change. Lesions observed in the ovary of F2 animals included cysts, cystadenomas and gonadal stromal tumors. PPL of the oviduct was also observed at this

Table III. Abnormalities in female DES lineage (F2) mice (17–19 months)

Developmental dose regime	F_1 DES treatment[a]	Ovary/oviduct	Reproductive tract
Control	Corn oil	13/32 Cystic (41)[b] 2/32 Cystadenoma (6) 1/32 Hemangioma (3)	3/32 CEH (9)[c] 7/32 Adenomyosis (22) 2/32 Endometrial polyp (6) 1/32 Deciduoma (3)
Group I	Prenatal DES 2.5	18/29 Cystic (62) 1/29 Cystadenoma (3)	4/29 CEH (14) 3/29 Adenomyosis (10) 2/29 Deciduoma (7) 2/29 Uterine adenocarcinoma (7)
	Prenatal DES 5	14/35 Cystic (40) 1/35 Cystadenoma (3)	4/35 CEH (11) 1/35 Adenomyosis (3) 3/35 Endometrial polyp (9) 1/35 Hemangioma (3) 1/35 Papillary metaplasia (3) 1/35 Atypical hyperplasia (3) 2/35 Uterine adenocarcinoma (6) 1/35 Papilloma of the vaginal opening (3)
	Prenatal DES 10	9/16 Cystic (56) 1/16 Cystadenoma (6) 1/16 Hemangioma (6) 1/16 Granulosa cell tumor (6) 7/16 PPL (44)[d]	4/16 CEH (25) 1/16 Adenomyosis (6) 5/16 Endometrial polyp (31) 2/16 Atypical hyperplasia (13)
Group II	Prenatal DES day 18	18/33 Cystic (56) 6/33 Cystadenoma (18)	1/33 CEH (3) 1/33 Adenomyosis (3) 3/33 Endometrial polyp (9) 1/33 Hydrometra (3) 1/33 Hemangioma (3)
Group III	Neonatal DES	19/29 Cystic (66) 1/29 Cystadenoma (3)	5/29 CEH (14) 2/29 Leiomyoma (7) 1/29 Atypical hyperplasia (3) 1/29 Uterine adenocarcinoma (3) 1/29 Stromal cell sarcoma (3)

[a]F1 female mice were exposed either prenatally or neonatally to DES as described in Materials and methods.
[b]Numbers in parentheses are percentages.
[c]CEH, cystic endometrial hyperplasia.
[d]PPL, progressive proliferative lesion of the oviduct.

age. A summary of the incidence of uterine adenocarcinoma in DES lineage mice at 17–19 and 22–24 months of age is presented in Table V.

Other organs from DES lineage mice were also screened for any histological abnormalities. The incidence of tumors of the liver, lung or other organs examined in this study was not significantly different from the incidence of these lesions observed in control animals.

Discussion

Data described in this study show that F1 mice that are developmentally exposed to DES (at different gestational periods or as neonates) are subfertile. This is in agreement with our previously published report; the prenatal DES 2.5, DES 5 and DES 10 doses (group I) were associated with a dose-related decrease in fertility (15). These particular doses were chosen for this study because they represented a dose range that was compatible with fertility but still demonstrated an adverse effect on reproduction. Group II, prenatal DES day 18, was chosen because it corresponded to the dose and time reported by Walker (6,7) to result in neoplasia in DES lineage mice. The F1 females from this group were also subfertile. In group III, the neonatal DES dose (0.002 μg/pup/day) also caused subfertility in the F1 mice; it decreased the number of pups per litter and increased time to the first litter as in the group I, prenatal DES 2.5 group; it also resulted in less females with a litter, whereas the prenatal DES 2.5 dose did not. This particular neonatal DES dose was used in this study because it was the highest neonatal DES dose that was compatible with subsequent pregnancy in DES-exposed animals (19).

In contrast to the subfertility seen in F1 DES-exposed mice, their offspring (F2, DES lineage) exhibited normal fertility when evaluated early in life. Parameters measured included cumulative number of pups per mouse over a 20 week breeding period, number of pups per litter, number of days to first litter and the percent of females with litters. No significant changes were observed in any of these parameters when compared with corresponding control mice. Likewise, there were no noted malformed neonates (F3) in the study and no biologically significant differences between DES and control groups in prepubertal growth rates for the F3 generation. While some statistically significant differences in growth rates were detected in the F1 and F2 generations (Table II), these differences were generally $<15\%$ and were considered to be of little biological significance. Furthermore, no consistent changes in time of puberty were observed in either the F2 or F3 female animals. We found no adverse effect on fertility in the second generation DES-exposed mice, as defined by the measurements in this study; this is consistent with a previously published report by Forsberg and Halling (11).

Table IV. Abnormalities in female DES lineage (F2) mice (22–24 months)

Developmental dose regime	F_1 DES treatment[a]	Ovary/oviduct	Reproductive tract
Control	Corn oil	16/23 Cystic (70)[b] 3/23 Cystadenoma (13) 1/23 Gonadal stromal tumor (4) 3/20 PPL (15)[d]	19/23 CEH (83)[c] 3/23 Adenomyosis (13) 4/23 Endometrial polyp (17) 1/23 Focal atypical hyperplasia (4)
Group I	Prenatal DES 2.5	29/35 Cystic (83) 2/35 Hemangioma (6) 4/35 Cystadenoma (11) 1/35 Gonadal stromal tumor (3) 10/28 PPL (36)	20/25 CEH (57) 1/35 Adenomyosis (3) 7/35 Endometrial polyp (20) 2/35 Uterine hemangioma (6) 5/35 Uterine squamous metaplasia (14) 1/35 Atypical hyperplasia (3) 1/35 Deciduoma (3) 5/35 Leiomyoma (14) 3/35 Uterine adenocarcinoma (9)
	Prenatal DES 5	25/36 Cystic (69) 3/36 Cystadenoma (8) 1/36 Gonadal stromal tumor (3) 5/32 PPL (16)	20/37 CEH (54) 1/37 Endometrial hyperplasia (3) 1/37 Adenomyosis (3) 9/37 Endometrial polyp (24) 3/37 Uterine hemangioma (8) 2/37 Atypical hyperplasia (5) 1/37 Deciduoma (3) 6/37 Uterine adenocarcinoma (16) 1/37 Vaginal CIS (3)[e] 1/37 Vaginal adenocarcinoma (3) 1/37 Clitoral gland adenocarcinoma (3)
	Prenatal DES 10	16/24 Cystic (66) 4/24 Cystadenoma (17) 1/24 Gonadal stromal tumor (4) 1/24 Leiomyoma (4) 3/22 PPL (14)	16/24 CEH (67) 1/24 Adenomyosis (4) 5/24 Endometrial polyp (21) 2/24 Uterine hemangioma (8) 4/24 Atypical hyperplasia (17) 1/24 Squamous metaplasia (4) 1/24 Leiomyoma (4) 1/24 Cervical carcinoma (4)
Group II	Prenatal DES day 18	12/15 Cystic (80) 1/15 Cystadenoma (7) 5/15 PPL (33)	10/15 CEH (67) 1/15 Endometrial hyperplasia (7) 3/15 Adenomyosis (20) 3/15 Squamous metaplasia (20) 1/15 Atypical hyperplasia (7) 1/15 Uterine adenocarcinoma (7)
Group III	Neonatal DES	30/34 Cystic (88) 3/34 Cystadenoma (9) 7/34 PPL (21)	25/36 CEH (69) 5/36 Endometrial polyp (14) 3/36 Atypical hyperplasia (8) 2/36 Leiomyoma (6) 4/36 Uterine adenocarcinoma (11) 1/36 Stromal cell sarcoma (3) 1/36 Vaginal CIS (3) 3/36 Vaginal adenosis (8) 1/36 Cervical adenosis (3) 1/36 Cervical leiomyoma (3)

[a]F1 female mice were exposed either prenatally or neonatally to DES as described in Materials and methods.
[b]Numbers in parentheses are percentages.
[c]CEH, cystic endometrial hyperplasia.
[d]PPL, progressive proliferative lesion of the oviduct.
[e]CIS, carcinoma *in situ*.

Histological abnormalities in the genital tracts of the DES lineage mice evaluated later in life suggested an increased susceptibility to tumor occurrence, in particular, in reproductive tract tissues. These data support and confirm data from other laboratories (6–10) reporting the transgenerational effects of DES. While there is ample evidence that transplacental exposure of the fetus to DES and other chemicals results in tumors later in life (5,10,35), there is increasing evidence that exposure to many chemical carcinogens may result in increased incidences of tumors in more than one generation of their 'untreated' descendants (36).

The multi-generational effects reported in this study are striking, even though they are not as great as effects reported in F1 animals that were developmentally exposed to DES (14,19). The highest rate of uterine adenocarcinoma in the current study was in group I, prenatal DES 5, with 6/37 (16%) of the F2 females having tumors at 22–24 months of age. Of note was the lack of uterine adenocarcinomas at the prenatal DES 10 dose; because the F1 females of this group were so severely affected, the survivors that were able to breed probably represented a selected population. In group III, neonatal DES, 4/46 (11%) of the F2 animals had uterine adenocarcinomas at

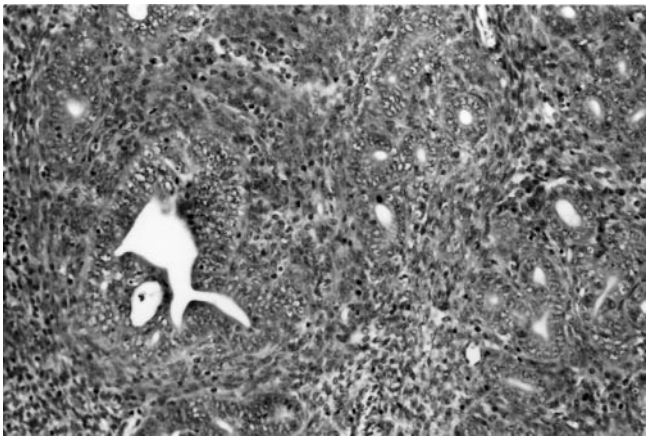

Fig. 3. Photomicrograph of atypical hyperplasia in the uterus of an F2 prenatal DES 2.5 mouse. In some endometrial glands nuclei are piled up and there is little intervening stroma between glandular structures. Hematoxylin and eosin, magnification ×50.

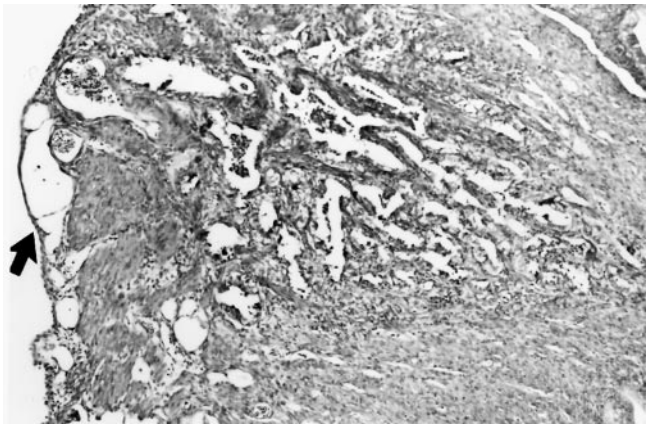

Fig. 4. Photomicrograph of uterine adenocarcinoma in an F2 prenatal DES 2.5 mouse. There is extension of uterine glands through the myometrium to the serosal surface (arrow). This lesion had metastasized to the para-aortic lymph nodes. Hematoxylin and eosin, magnification ×25.

Fig. 5. Photomicrograph of carcinoma *in situ* in the vagina of an F2 prenatal DES 5 mouse. The cells of the thickened vaginal mucosa have pleomorphic nuclei and a variable amount of cytoplasm. Small keratin pearls appear in one area of the lesion (arrow). Hematoxylin and eosin, magnification ×50.

22–24 months of age; in earlier studies (19,20), 90% of the F1 DES-exposed animals had similar tumors, but the dose in the current study is 1000 times lower. The incidence of

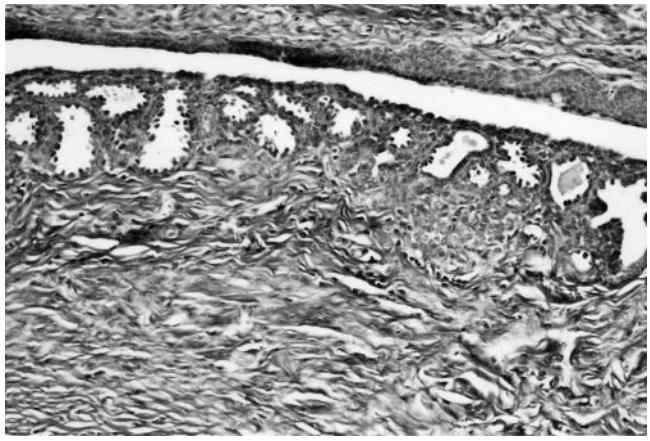

Fig. 6. Photomicrograph of vaginal adenocarcinoma in an F2 prenatal DES 5 mouse. Gland-like spaces are lined with hobnail cells in the vagina. There are solid nests of cells below some of the glands. Hematoxylin and eosin, magnification ×50.

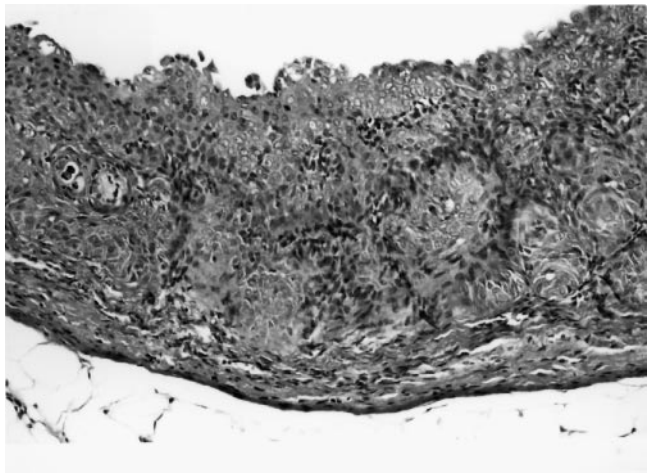

Fig. 7. Photomicrograph of carcinoma *in situ* of the vagina in an F2 neonatal DES mouse. Pleomorphic nuclei are seen. Hematoxylin and eosin, magnification ×50.

spontaneously occurring uterine adenocarcinoma in this strain of mouse was reported by Charles River Breeding Laboratories to be 0.4% (2/482) (37) and by Englelhardt *et al*. (38) to be <1%.

Another remarkable finding in this study was a vaginal adenocarcinoma in a 22–24-month-old DES lineage mouse in group I, prenatal DES 5. This is a rare and unique lesion that we have only observed in prenatally DES-treated animals (14); it has not been reported in control animals at any age and has only been seen in a total of three other prenatally exposed DES animals (F1) in all the treated animals observed in our laboratory to date (unpublished data).

The mechanisms involved in these transgenerational events are unknown; however, the data in this study suggest that they are probably maternal and germ cell related. While we did not look at transmission of DES-induced lesions along the male line, another transgenerational study showed increased cancer susceptibility transmitted via the DES-exposed male (8). Because DES has been reported to have genetic/epigenetic effects (27–33), damage to the germ cell is a possibility. If DES-induced damage was carried from generation to generation by a simple dominant gene mutation, we would expect a significant reduction in tumor incidence between generations because

Table V. Incidence of uterine adenocarcinoma in DES lineage (F2) female mice

	17–19 months	22–24 months	Total	Statistical significance	
				Versus concurrent controls[a]	Versus historical controls[b]
Control	0/32 (0)[c]	0/23 (0)	0/55 (0)	–	NS
Group I					
Prenatal DES 2.5	2/29 (7)	3/35 (9)	5/64 (8)	$P < 0.05$	$P < 0.001$
Prenatal DES 5	2/35 (6)	6/37 (16)[d]	8/72 (11)	$P < 0.01$	$P < 0.001$
Prenatal DES 10	0/16 (0)	0/24 (0)[e]	0/40 (0)	NS	NS
Group II					
Prenatal DES day 18	0/33 (0)	1/15 (7)	1/48 (2)	NS	NS
Group III					
Neonatal DES	1/29 (3)[f]	4/36 (11)[g]	5/65 (8)	$P < 0.05$	$P < 0.001$

NS, not significant ($P > 0.05$).
[a]Relative to concurrent control rate of 0/55.
[b]Relative to historical control rate of 0.4% (2/482) in 21–24-month-old female Charles River CD-1 mice (38).
[c]Numbers in parentheses are percentages.
[d]One vaginal adenocarcinoma and one vaginal carcinoma *in situ* also observed in this group.
[e]One cervical carcinoma also observed in this group.
[f]One stromal cell sarcoma also observed in this group.
[g]One stromal cell sarcoma and one vaginal carcinoma *in situ* also observed in this group.

DES lineage females were mated to control males. This awaits an answer because tumor incidence studies in the F3 generation are currently incomplete, but underway.

Another possible explanation of the transgenerational effects is that the cancer effect is being transmitted by imprinting (39). Two tumors cited as examples of abnormal genomic imprinting in human cancer are hydatidiform mole and ovarian teratoma (40). Lesions in the ovary have been observed in mice that were developmentally exposed to DES (15,16), thus abnormal imprinting is a possible hypothesis to explain the transgenerational carcinogenicity of DES. In fact, a recent report from our laboratory describes imprinting of abnormal methylation patterns in estrogen-responsive genes following developmental DES exposure (29); this alteration is being studied in the F3 mice as a possible explanation for the observed adverse DES lineage effects.

Another hypothesis involves microsatellite instability. Molecular genetic analysis of DES-induced vaginal and cervical adenocarcinoma in humans has revealed a high incidence of microsatellite instability (30). Such defects can occur through both somatic and germ cell mutations (41). This offers a possible explanation for how the same type of tumor can occur in both developmentally DES-exposed mice and DES lineage mice. Specifically, DES could alter both the somatic cells of the exposed fetus and its germ cells. Studies are underway to examine microsatellite instability in animals prior to tumor appearance.

While the occurrence of reproductive tract tumors in DES lineage mice does not predict a similar outcome in DES-exposed humans, continued close surveillance of the prenatally DES-exposed cohort and their offspring is warranted. Using the animal model, we can now systematically analyze the genetic/epigenetic changes caused by DES, which will aid in the comparison of similarities and differences between the mouse and human. Ongoing mechanistic studies with the experimental DES-exposed animal model may thus prove useful in identifying specific genetic/epigenetic changes that lead to tumor development and thereby provide markers for early detection and prevention of human disease.

In summary, this report describes irreversible changes in the female genital tract that are transmitted to other generations. These results indicate that the cascade of events that lead to the appearance of tumors may well begin before birth and perhaps before conception. Additional studies on prenatal and developmental exposures are essential for an accurate assessment of risks that can be attributed to specific environmental agents. However, these experimental studies will contribute to our understanding of some of the mechanisms underlying the genetic predisposition to cancer.

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