Carcinogenicity of Diethylstilbestrol in the Wistar Rat: Effect of Postnatal Oral Contraceptive Steroids

Raymond B. Baggs, Richard K. Miller, and Charles L. Odoroff

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ABSTRACT

Diethylstilbestrol (DES) has been associated with vaginal neoplasia and malformations in humans. We have studied a test population of 504 female Wistar rats given diethylstilbestrol at from 0.0 to 0.5 mg/kg maternal body weight on days 18, 19, and 20 of gestation. Animals were euthanized in extremis, or at 2 years of age. The incidence of vaginal epithelial tumors was dose related. The types of epithelial tumors of the vagina were adenocarcinoma, squamous cell carcinoma, and mixed carcinoma, containing discrete adenomatous and squamous components. The incidence of vaginal epithelial tumors was determined to be dose related: rats exposed to 0 mg DES/kg maternal weight had an incidence of 0.6% (1 of 167 rats); 0.1 mg/kg, 4.1%; and 0.5 mg/kg, 4.3% (6 of 140); 25 mg/kg, 1.6% (1 of 63); and 50 mg/kg, 11.5% (3 of 26). Tumors of other reproductive tissues (mammary gland, ovary, oviduct, cervix, or uterus) demonstrated no discernible DES dose-response relationship. There was no oncogenic effect of postnatal administration of oral contraceptives (0 oral contraceptives, 31.25 μg/kg diet ethynylestradiol, and 31.25 μg/kg diet norethindrone or 104 μg/kg diet ethynylestradiol and 31.25 μg/kg diet norethindrone). Thus, vaginal tumors can be induced in a dose-related manner in the rat following in utero DES exposure. Oral contraceptive treatment did not increase the risk of neoplasia.

INTRODUCTION

Transplacental carcinogenesis, one of the most ominous of all tumor-inducing processes, predisposes an unborn child to the latent development of life-threatening neoplasia. DES is one agent that is associated with both vaginal adenocarcinoma and malformations (e.g., adenosis, T-shaped uterus, cervical hoods, and ridges) in the human female exposed in utero (1). In mice, the long-term effects of neonatal exposure to estrogen include hyperplastic vaginal lesions and cervicovaginal cancer. In experiments with appropriate controls, Dunn and Green (2) reported a vaginal tumor incidence of approximately 8% (our calculations) for female mice given s.c. injections at birth of 2 mg/kg of DES in saline. These tumors were epithelial in origin and were observed between 20 and 23 months, but the precise tumor type was not identified. There were no such tumors reported in the four controls. McLachlan et al. (3, 4) reported the occurrence of 3 vaginal adenocarcinomas in mice following in utero exposure to DES (1 of 29 at 0.005 mg/kg/day; 1 of 57 at 0.01 mg/kg/day; 1 of 46 at 0.1 mg/kg/day). Their subsequent studies (5) using postnatal DES exposure in mice have demonstrated a very high (90%) incidence of uterine adenocarcinoma, but no additional vaginal adenocarcinomas. Walker (6) examined mice exposed in utero to DES. Although none of the 15 animals exposed in utero was reported to have had macroscopically detectable tumors, of 40 offspring, 10 had uterine adenocarcinoma and 5 had ovarian cystadenocarcinoma.

The rat has proven to be highly susceptible to the abortifacient and teratogenic effects of DES (7–9). These reproductive effects are dependent upon when the exposure period occurs during development. Before day 18 of gestation, a high incidence of pregnancy loss is observed. Between 18 and 20 days, female hypospadias, uncoiled oviducts, and vaginal uroliths can be produced in a dose-related manner (10). Postnatal exposure to DES did not produce these effects, but rather an increased incidence of adenosis. Branhm et al. (11, 12), studying the effect of early postnatal DES on uterine cell populations in rats, found that decreased uterine growth was a consequence of combined hypothyroidism and hypoplasia in all cell types except longitudinal muscle.

Besides the issue of in utero exposure to diethylstilbestrol and induction of vaginal tumors and malformations, there has been increasing interest in postnatal confounding factors. Among these confounding postnatal factors is the use of oral contraceptives by women who were exposed in utero to diethylstilbestrol. Therefore, this rat model was evaluated to determine the incidence of prenatal DES exposure when the müllerian duct is developing (18–20 days). We then used this in utero exposed DES population to determine risk for carcinogenicity with postnatal exposure to oral contraceptives.

MATERIALS AND METHODS

Virgin female Wistar [Crl:(WI)BR] rats (Charles River Laboratory, Wilmington, MA) were treated with DES (Sigma Chemical Co., St. Louis, MO) in corn oil s.c. on days 18–20 of gestation (presence of vaginal plugs in the animal or in the cage debris, or sperm in the vaginal smear used as the index of a positive pregnancy). The determination of pregnancy was considered day 0 of pregnancy. The doses of DES used in this experiment are quite analogous to those used in the human experience (13). The daily dose of DES received by women ranged from 5 mg/day (7–8 weeks pregnant) to 150 mg/day (34–35 weeks pregnant); thus the dose for a 50-kg woman ranged from 0.1 to 3 mg/kg. The rats were given DES at 0.0 to 0.5 mg/kg maternal body weight on days 18, 19, and 20 of gestation and at 25 and 50 mg/kg on days 19 and 20 of gestation (Table 1).

Beginning at 10 weeks of age, two groups of animals were investigated, DES and non-DES exposed. Using a computer-generated random assignment of animals, female offspring were fed OC in diet for 5 days/week at doses of control (no OC), low (31.25-μg/kg feed of EE, 31.25-μg/kg feed of NE) or high (104-μg/kg feed of EE, 31.25-μg/kg feed of NE). The rats weighed 0.25–0.45 kg and ate between 10 and 15 g of diet/day; the p.o. oral dose was thus 1–1.2 μg/kg/day EE, 1–1.2 μg/kg/day NE for the low dose OC group and 3–4 μg/kg/day EE, 1–1.2 μg/kg/day NE for the high dose OC group. The EE doses are comparable to those used in women for contraception (0.03 mg EE/day for a 50-kg individual, or 0.6 μg/kg/day). The NE doses are low by comparison with modern "low dose" formulation, in which women would receive 10 μg/kg/day (14). The oral contraceptives were dissolved in corn oil, diluted in 95% ethanol, and mixed with the powdered diet (Purina 5001). The ethanol was allowed to evaporate thoroughly at

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2 To whom requests for reprints should be addressed, at Division of Laboratory Animal Medicine, Box 674, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642.
3 The late Professor Odoroff was responsible for the statistical component of this research.
4 The abbreviations used are: DES, diethylstilbestrol; OC, oral contraceptives; EE, ethynylestradiol; NE, norethindrone.

3311
Diethylstilbestrol (mg/kg maternal wt)

<table>
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<th>Litters</th>
<th>0.0</th>
<th>0.1</th>
<th>0.5</th>
<th>25</th>
<th>50</th>
</tr>
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<tbody>
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<td>37</td>
<td>14</td>
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<td>21</td>
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<td>208</td>
<td>184</td>
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Male and female rats were each dosed orally 0.0 DE in utero, 0.1 mg/kg maternal body weight days 18, 19, and 20 of gestation; 0.5 mg/kg maternal body weight days 18, 19, and 20 of gestation; No OC, no oral contraceptive. 104 Mg/kg diet ethynylestradiol and 31.25 Mg/kg diet norethindrone; Hi OC, 31.25 wg/kg diet ethynylestradiol and 31.25 Mg/kg diet norethindrone; Lo OC, 0.1 mg/kg maternal body weight days 18, 19, and 20 of gestation; No OC, no oral contraceptive; Hi OC, 31.25 wg/kg diet ethynylestradiol and 31.25 Mg/kg diet norethindrone; Hi OC, 31.25 wg/kg diet ethynylestradiol and 31.25 Mg/kg diet norethindrone; Vag., vaginal; SCC, squamous cell carcinoma; AdenoCA, adenocarcinoma.

RESULTS

Table 1 reports the observations in this study of 948 offspring born, of which 350 female progeny are studied. The highest DES dose group (50 mg/kg) had the highest incidence of preweaning mortality (62% as compared to 10 to 15% for the other treatments). Mortality prior to 120 days of age was associated with reproductive tract malformations, such as hypospadias, cleft phallus, vaginal calculi, vaginal hyperkeratosis, and adenosis (15). The female progeny were entered onto the study at weaning.

Because of the early mortality in only the DES-exposed population, the tumor data are expressed in two forms. “Rate” reflects the number of tumors per 100,000 days at risk. This value includes all animals placed on trial. A second calculation was needed to better reflect the latency period for tumor development. Since the earliest appearance of a mammary or vaginal tumor was 6.5 months, “percentage surviving” is the percentage of female animals that survived for more than 120 days.

Prenatal administration of DES on days 18, 19, and 20 of gestation to rat dams resulted in the production of reproductive tract tumors in the female progeny (Table 1). The majority of the tumors were detectable as small masses between 1 and 2 cm in diameter. At 0.1 and 0.5 mg/kg DES, five adenocarcinomas (Fig. 1) and one mixed carcinoma, containing discrete adenomatous and squamous components (Fig. 2), were observed. At high doses only squamous cell carcinomas (Fig. 3) were present. Using the corn oil control groups as a standard, the relative odds of observing a tumor at each of the doses were:

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With the exception of the 0.1-mg/kg dose, these relative odds are all significant (P < 0.05; nonsimultaneous comparisons). It should be noted, however, that the two tumors in the corn oil controls were nonepithelial in origin (leiomysarcoma and leiomyoma) and may not be directly comparable to vaginal epithelial tumors. Rat days at risk for all vaginal tumors were dose related, increasing from 6.7 to 55.3 tumors/100,000 days at risk.

Of 165 animals given only corn oil in utero, there was one animal with a vaginal epithelial neoplasm. Histologically (Fig. 4), this was an adenocarcinoma. The animal was euthanized in extremis at 419 days of age. Grossly, the uterus and vagina were incarcerated in an irregular 2.5- x 2.0- x 1.9-cm firm tan mass.

Table 2 Vaginal tumor type and incidence by treatment

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The severely invasive characteristics of the tumor obscured the precise tissue of origin, with local extension to uterine serosa and peritoneum and metastasis to lung. Including this animal, the incidence of vaginal epithelial tumors was determined to be dose related: rats exposed to 0 mg DES/kg maternal weight had an incidence of 0.6% (1 of 167 rats); 0.1 mg/kg, 4.1%; and 0.5 mg/kg, 4.3% (6 of 140), 25 mg/kg, 1.6% (1 of 63), and 50 mg/kg, 11.5% (3 of 26).

When the data for all vaginal tumors were analyzed by the method of Laird and Oliver (16), a significant dose response was observed ($\chi^2 = 19.31, P = 0.001$). The function increased from 0 to 0.5 mg/kg, dropped at 25 mg/kg, and then rose sharply. In view of the different tumor types observed, this may suggest different induction processes at each dose level.

The sarcomas, unlike vaginal epithelial tumors, did not show an obvious relationship to treatment. There was no litter effect in the tumor incidence. Tumors of other reproductive tissues (mammary gland, ovary, oviduct, or uterus) demonstrated no discernible dose-response relationship for DES (Table 1). The female offspring treated in utero with 0, 0.1, or 0.5 mg DES/kg were fed oral contraceptives at doses up to 104 µg/kg feed of ethynylestradiol; 31.25 µg/kg of norethindrone for 5 days per week and demonstrated no significant effects of the oral contraceptive steroids on the incidence of vaginal tumors (Table 2), or in any other reproductive tissue following 2 years of exposure.
DISCUSSION

Many similarities among species are reported concerning perinatal exposure to diethylstilbestrol (17, 18). Takasugi and Bern (19) demonstrate that persistent estrus in rodents is an alteration in the hypothalamo-hypophyseal system. Although there are dramatic alterations in the morphology of the vaginal and uterine epithelium, extrapolations are not successful because of the low incidence of vaginal tumors. Both the teratogenic and carcinogenic aspects of DES indicate the importance of the epithelial/stromal components of the reproductive tract (20). Different cell types assumed to be exposed in these studies undergo growth and differentiation at different gestational ages. Thus it is important to correlate the development of the reproductive tract, in particular the vagina, with the particular experimental model used.

A variety of regimens have been used to induce vaginal epithelial changes in mice. The highest incidence of adenosis occurred following postnatal exposure, whereas the only descriptions of vaginal adenocarcinomas associated with DES have occurred following prenatal exposure. Following prenatal exposure to DES on days 9–16, three vaginal tumors had been induced in mice; however, no dose-response relationship had as yet been demonstrated (4). Besides these vaginal tumors, a high incidence of ovarian lesions was noted. No such ovarian lesions were noted in the present rat study. These differences may not be species dependent but rather may be due to the timing of the exposure. The current rat study specifically emphasized the

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Fig. 3. Squamous cell carcinoma in vaginal wall from rat treated with DES in utero. V, vaginal lumen; S, neoplastic epithelial cells with squamous differentiation. Bar, 50 μm.

Fig. 4. Adenocarcinoma in rat treated with corn oil vehicle only. V, vaginal lumen; A, neoplastic epithelial cells with adenomatous differentiation. Bar, 50 μm.
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period of vaginal and uterine tract development, while in the mouse studies, earlier treatment was utilized. The immune system may be affected by DES. Kalland and Holmdahl (21) reviewed the complex literature of murine models and concluded that neonatally DES-exposed mice do have impaired natural killer lymphocyte function. In that study, transplantation of target tissue from DES-treated to untreated thymectomized mice did not increase the frequency of neoplasia (22); T-cell function is apparently irrelevant.

It is important to note that the development of the rodent (rat and mouse) reproductive tract is truly perinatal. Thus, to document teratogenesis/carcinogenesis of the reproductive tract, exposure only until day 14 of gestation in the rodent is not appropriate. Vorherr et al. (23) reported, in ten Sprague-Dawley rats exposed to five different pre- and postnatal regimens of DES, four reproductive tract tumors and associated malformations, but no vaginal adenocarcinomas. Unfortunately, no controls were reported. Boylan (24) observed abnormal nipple development, ovarian cysts, and reproductive tract malformations in the rat, following prenatal exposure to DES. No tumors were reported, however. It should be noted that the current study indicates that the lack of tumors in the Boylan study may have been due to the short postnatal observation period. We observed similar precocious development of the nipples in the current rat study; however, this precocious neonatal mammary response did not result in an increase in mammary tumors (Table 1). The Wistar rat studies identified a definite “window” during gestation when DES induced vaginal epithelial changes, including adenocarcinomas. Administration prior to day 15 produced abortion; administration after day 20 induced lactation suppression in the dam.

The presence of epithelial tumors of the vagina in the Wistar rat and their appearance in a dose-related manner add further support to the general association in the human between DES and vaginal tumors (1) as well as with the dose-related incidence of reproductive tract malformations in the rat.

In conclusion, vaginal tumors of epithelial origin (adenocarcinomas and squamous cell carcinomas) can be induced in Wistar rat offspring in a dose-related manner by DES administration between days 18 and 20 of gestation. Later administration of oral contraceptive steroids to those offspring did not enhance the risk of neoplasia due to DES in this model.

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