Transplacental Action of Diethylstilbestrol on Mammary Carcinogenesis in Female Rats Given One or Two Doses of 7,12-Dimethylbenz(a)anthracene

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ABSTRACT

Aspects of the development, morphology, and estrogen binding capacity of mammary tumors in rats exposed prenatally to the synthetic estrogen, diethylstilbestrol (DES), and treated postnatally with 7,12-dimethylbenz(a)anthracene (DMBA) were analyzed as part of a project aimed at understanding the effects of transplacental exposure to DES on estrogen-sensitive tissues. Pregnant Sprague-Dawley rats were given injections of DES (total dose, 1.2 μg) or vehicle alone on Days 15 and 18 of gestation. All female offspring were given gastric intubations of DMBA, either a single 10-mg dose on Day 50 or two doses (10 mg each) on Days 50 and 57. Among rats treated postnatally with 10 mg of DMBA, the DES-exposed group had a significantly greater incidence of palpable mammary tumors than did the vehicle-exposed controls. In addition, there was an earlier time of appearance of palpable tumors in the DES-exposed group. When the data from rats treated postnatally with two 10-mg doses of DMBA were analyzed, there were no significant differences in palpable mammary tumor incidence or tumor latency between the DES-exposed and vehicle-exposed groups. When the pathology of the mammary tumors produced in rats treated with 10 mg of DMBA was analyzed, the DES-exposed group had a significantly higher proportion of benign tumors (fibroadenoma, adenoma, lobular hyperplasia) than adenocarcinoma compared to vehicle-exposed controls. Both exposure groups had similar numbers of nonpalpable mammary lesions discovered at necropsy. Estrogen binding capacities of representative adenocarcinoma did not differ significantly between the two prenatal exposure groups treated postnatally with 10 mg of DMBA. These results demonstrate the importance of the dose of the challenge carcinogen in revealing the effects of transplacental drug exposure and may have special significance for women who were exposed to DES in utero.

INTRODUCTION

The anatomical development of the mammary gland and the incidence of mammary tumors in rodents are known to be influenced by exogenous estrogenic hormones administered in the perinatal period. We have shown previously that prenatal exposure to the synthetic estrogen DES1 had teratogenic effects on nipple structure in neonatal rats (3) and altered incorporation of [3H]uridine in the primary mammary duct epithelium of 5-day-old pups (2). Others have observed nipple malformations in fetuses or newborn rats exposed to large doses of estradiol dipropionate (10, 16) or DES (9). Using mice, Jean (14) found persistent nipple and mammary gland abnormalities in DES-exposed offspring examined at 80 days of age; Raynaud (25) described teratogenic effects on nipple structure of mouse fetuses when estradiol dipropionate was injected directly into 12- to 15-day embryos; and Yasuda et al. (37) demonstrated a hypertrrophic effect of ethinyl estradiol (2 mg/kg) on the nipples of female offspring. Mammary gland branching was inhibited by prenatal exposure to estradiol dipropionate in mice examined as neonates; this effect was overcome with time, since no effect was observed when similarly treated mice were examined at older ages (15). The effect of prenatal exposure to DES on mammary tumorigenesis has been studied in several rodent systems. Exposure of rat fetuses to DES (1 mg/kg) resulted in an incidence of mammary fibroadenomata of 4 of 18 DES-exposed rats versus 3 of 34 control rats at an average age of approximately 2 years (23). Increased mammary tumor multiplicity was reported in hamster offspring exposed prenatally to DES and treated postnatally with DMBA (28). However, mammary tumorigenesis in the mouse was suppressed by prenatal exposure to DES on Day 12 of gestation; exposure on Day 17 had no effect (21).

Compared to the results when estrogens were used prenatally, opposite effects on mammary tumorigenesis have been reported when rats or mice were treated neonatally with estrogens. Thus, in rats where prenatal exposure to DES increased mammary tumor incidence, 17β-estradiol or estradiol benzoate on Days 2 or 5 after birth resulted in suppression of mammary tumors induced with DMBA (30, 38). Furthermore, treatment of rats with estradiol by injections on Days 1 to 30 or by an intrahypothalamic implant inhibited or delayed mammary tumor appearance (13, 22). In mice, where early prenatal exposure to DES suppressed mammary tumorigenesis, neonatal treatment with 17β-estradiol on Days 1 to 5 generally resulted in the stimulation of mammary gland growth, differentiation and hormonal sensitivity, and an increase in mammary dysplasias in older animals (17, 18, 32–36); however, neonatal DES treatment inhibited duct branching in 6- and 33-day-old mice (32). Thus, mice and rats appear to differ in the period when perinatal hormone exposure influences the later development of proliferative abnormalities in the mammary gland. In addition, some of the apparently contradictory reports in the literature may have resulted from the use of both natural and synthetic estrogens which differ in their affinity for serum binding proteins, such as α-fetoprotein (8, 29). For further review of the literature on perinatal hormone treatment and...
mammary tumorigenesis in rodents, see Mori et al. (20).

Previously, we reported increased mammary tumor multiplicity in rats exposed prenatally to DES and treated postnatally with 2 doses of 10 mg of DMBA (4). However, a subsequent experiment* where offspring were treated with a single dose of 15 mg of DMBA failed to confirm these results, with the DES-exposed and vehicle-exposed groups showing indistinguishable tumor incidences (3.5 versus 3.6 tumors per rat) and percentages of tumor-bearing rats (68 versus 60%). Here, we present results of an experiment which directly tested the effect of the dose of carcinogen used after transplacental exposure to DES on various aspects of mammary tumor development. Data reported here support the position that prenatal exposure to DES has a stimulatory effect on mammary tumorigenesis when a low dose of DMBA is used. Use of the high dose of DMBA masked the effect of DES. Further analysis of these data in relation to our earlier paper is presented in the "Discussion." Animals whose mammary tumors are described here were subjected to necropsy; data on their reproductive and endocrine organs and serum hormone levels are presented in a companion paper (6).

MATERIALS AND METHODS

Prenatal Exposure. Pregnant CD rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were assigned randomly to 2 treatment groups: 18 rats were given injections s.c. of DES (Sigma Chemical Co., St. Louis, Mo.) dissolved in sesame oil on Days 15 and 18 (2 injections of 0.6 μg in 0.3 ml of sesame oil = 1.2 μg of DES, total dose); 10 rats received 2 injections of vehicle only. Animals were allowed to deliver and raise their offspring to weaning. Large litters were reduced to no more than 10 pups by removing male offspring. After weaning, female offspring were housed 5 per cage. Details regarding animal care can be found in a companion paper (6).

Postnatal Treatment. One-half of the 64 DES-exposed females and one-half of the 59 vehicle-exposed females received one dose of DMBA (Eastman Organic Chemicals, Rochester, N. Y.; 10 mg in 1 ml of sesame oil) at 50 days of age, while the other half of each exposure group received 2 doses of DMBA (10 mg each) on Days 50 and 57. DMBA was dissolved in oil, stirred in the dark overnight to achieve complete solubilization, and used the following day. A repeating syringe fitted with an 18-gauge intubation needle was used to perform the gastric intubations. The average weight of the rats was approximately 175 g; therefore, each 10-mg dose of DMBA was the equivalent of about 60 mg of DMBA per kg of body weight. Beginning 5 weeks after the first intubation with DMBA and continuing until the animals reached 9 months of age, palpations were performed weekly to determine the time of appearance of each mammary tumor. Animals which were clearly moribund before the end of the experiment were sacrificed so that tissues could be obtained. At sacrifice, all palpable and nonpalpable mammary tumors were removed and weighed. Where possible, a portion of each tumor was frozen in liquid nitrogen and placed in a Revco—80°C freezer (Revco, Inc., West Columbia, S. C.) until assayed for estrogen binding capacity. The remaining tissue was fixed in neutral buffered formalin, dehydrated in Cellosolve (ethylene glycol monomethyl ether) and toluene, embedded in paraffin, and stained with hematoxylin and eosin.

Tumor Pathology. Palpable and nonpalpable mammary tumors were categorized according to the guidelines of Young and Hallowes (39). Tumors the morphology of which could not be readily classified were examined by a veterinary pathologist.

Assay for Estrogen Binding Capacity. Cytosols were prepared from thawed mammary tumors using a Brinkmann (Westbury, N. Y.) polytron homogenizer and a Beckman (Palo Alto, Calif.) L5-65 ultracentrifuge with a type 40 rotor. Binding capacity was measured using the dextran-coated charcoal procedure exactly according to the directions and with the reagents provided in the New England Nuclear (Boston, Mass.) estrogen receptor assay kit.

Statistical Methods. In all cases, the experimental unit was the litter. All statistical tests, where litters were nested in treatments, were conducted using a modified version of the methods proposed by Peto (24) for nonincidental tumors. These methods permitted the calculation of expected numbers of tumors per litter, which compensated for unequal numbers of rats per litter and also for rats which died during the course of the experiments. The first modification involved the assumption of independence of tumors within litters. It has been reported (1, 19) that metastases have not been observed in rats bearing DMBA-induced mammary tumors. Therefore, we made the statistical assumption that, within litters, mammary tumors occurred randomly and independently, and hence, all palpable mammary tumors were included in the analysis. The second modification was to substitute the log likelihood ratio test for χ². This statistic is distributed as χ² and appears to follow the probability density function more closely than other computational procedures (31). The cumulative incidence of tumors in the DES-exposed litters was compared to that for litters exposed to vehicle only; rats receiving 10 mg of DMBA and two 10-mg doses of DMBA were analyzed separately. The cumulative tumor incidences for the intervals of 1 to 10, 11 to 20, and 21 to 29 weeks were partitioned and analyzed independently.

Analysis of time of appearance of mammary tumors required a further modification of the analytical methods. Here, the observed and expected numbers of tumors were reduced to two 29 x 2 matrices and used to test for the independence of time of appearance and prenatal treatment. In any week in which no new tumors were detected, 1 d.f. was subtracted from the total; i.e., weeks with expected values of less than one were pooled.

Both the incidence of death and the time of death were analyzed using the Peto method for incidental events. This permitted testing hypotheses for differences in relative number of deaths between DES and vehicle-exposed litters, as well as the independence of time of death and prenatal treatment.

Tumors which had been sectioned were classified as either adenocarcinoma or fibroadenoma and adenoma. Expected values for adenocarcinoma and fibroadenoma and adenoma were calculated independently; the log likelihood ratio statistics were calculated separately and then pooled. Therefore, the independence of pathology versus prenatal treatment could be tested with 2 d.f.

RESULTS

Tumor Incidence and Time of Appearance. Chart A presents data on the cumulative incidence of palpable mammary tumors from rats exposed prenatally to DES or vehicle, then treated postnatally with 10 mg of DMBA, while Chart B shows data on comparable groups of rats treated postnatally with two 10-mg doses of DMBA. It is evident that differences associated with prenatal exposure to DES could be observed only when the lower dose of DMBA was used. In Table 1, the increments in palpable mammary tumor incidence from 1 to 10, 11 to 20, and 21 to 29 weeks following DMBA treatment are presented and analyzed. When 10 mg of DMBA were used, statistically significant differences in the observed number of tumors between DES-exposed and vehicle-exposed animals were found in the intervals of 1 to 10 and 11 to 20 weeks post-DMBA. No significant difference was found when tumors appearing during the 21- to 29-week interval were analyzed. At sacrifice or death, the mean number of palpable mammary tumors per rat was 4.3 ± 2.9 (S.D.) in the DES-exposed group, while the controls had only 2.8 ± 2.2 tumors/rat. The distribution of the number of tumors

* E. Boylan and R. Calhoon, unpublished data.
per rat for the DES-exposed versus vehicle-exposed groups treated with 10 mg of DMBA is illustrated in Chart 2. The shift in distribution from predominantly 0 and 1 tumor per rat in the controls to 4 and 5 tumors per rat in the DES-exposed group is evident and is reflected in the difference between mean number of tumors per rat described above. Although the DES-exposed group contained more tumor-bearing rats (30 of 32, 94%) than did the vehicle-exposed group (22 of 30, 73%), the difference was not statistically significant when the analysis compensated for the nesting relationship of rats within litters.

When rats were treated with 2 doses of DMBA (10 mg each, 1 week apart), the cumulative incidence of tumors was substan-
tially greater than after a single dose of DMBA (cf. Chart 1, A and B); and the total tumor incidence in Weeks 1 to 29 post-DMBA was elevated significantly in groups which had received two 10-mg doses of DMBA compared to the single-dose groups (G = 79.41, p < 0.01). The mean number of tumors per rat at sacrifice was 5.7 ± 3.9 for the DES-exposed rats and 5.9 ± 3.7 for the vehicle-exposed rats, and all rats in both groups had at least one tumor. When the increments in tumor incidence were analyzed for both exposure groups treated with 10-mg doses of DMBA (Table 1), no significant differences were found in the 1- to 10-, 11- to 20-, or 21- to 29-week intervals.

Prenatal exposure to DES accelerated the time of appearance of palpable mammary tumors in rats treated with 10 mg of DMBA but not in rats treated with two 10-mg doses of DMBA (Table 2). The difference in mean time of appearance between the DES-exposed and control groups was 2.3 weeks when 10 mg of DMBA were used and 1.1 weeks when two 10-mg doses of DMBA were given. Overall, tumors appeared 2.1 weeks earlier in groups treated with two 10-mg doses of DMBA than in groups treated with 10 mg of DMBA.

Some rats from all groups died before the end of the experiment. Irrespective of the dose of DMBA, DES-exposed groups exhibited greater mortality prior to termination of the experiment, but the differences were not statistically significant. Treatment with two 10-mg doses of DMBA more than doubled the death rate compared to treatment with a single dose (for DES, 10 of 32 or 31% at 10 mg of DMBA versus 21 of 32 or 66% at two 10-mg doses of DMBA; for vehicle, 4 of 30 or 13% at 10 mg of DMBA versus 14 of 29 or 48% at two 10-mg doses of DMBA).

Tumor Pathology and Estrogen Binding Capacity. In both exposure groups treated postnata!y with 10 mg of DMBA, palpable and nonpalpable mammary tumors were examined microscopically. When the effect of prenatal exposure to DES on the pathology of palpable mammary tumors was analyzed, significant differences were found (Table 3). The number of palpable adenocarcinomata in the DES-exposed group was significantly greater than in the vehicle-exposed group (G = 6.78, p ≤ 0.01), as was the number of palpable fibroadenomata and adenomata (G = 14.57, p ≤ 0.01). Among the palpable mammary tumors, the DES-exposed group had a lower ratio of adenocarcinomata to fibroadenomata plus adenomata compared to the vehicle-exposed group. In terms of the nonpalpable mammary tumors, both exposure groups had similar numbers of these small lesions, and the proportion of tumors of adenocarcinomata to fibroadenomata plus adenomata did not differ (data not shown).

Specific binding of 17β-[3H]estradiol in the cytosol of representative mammary tumors did not differ significantly between exposure groups. The mean binding capacity of 15 adenocarcinomata from 7 DES-exposed litters was 36.2 ± 3.2 fmol/mg cytosol protein (range, 7.8 to 138.2), while in the 4 vehicle-exposed litters, specific estrogen binding in 16 adenocarcinomata was 28.9 ± 3.0 fmol/mg cytosol protein (range, 8.8 to 102.7). Dissociation constants varied between 1.0 x 10⁻¹⁰ to 6.7 x 10⁻¹¹ M⁻¹.

DISCUSSION

When the 10-mg dose of DMBA was administered to 50-day-old rats, effects of transplacental exposure to DES were expressed in the offspring as an increased incidence of palpable mammary tumors, detectable by the end of the tenth week following carcinogen treatment. During the following 10 weeks, the new tumors discovered in the DES-exposed group also exceeded those found in the control group. However, for the last 9 weeks of palpation, there was no detectable difference in the rate at which palpable tumors appeared in the 2 exposure groups. Thus, the effect of prenatal exposure to DES is to accelerate the appearance of mammary tumors; rats which escape the effect of DES on this early tumor induction phase develop palpable mammary tumors at a similar rate and comparable incidence to vehicle-exposed rats treated with 10 mg of DMBA.

This sensitizing effect of DES on the induction of tumors by DMBA was not observed when the dose of carcinogen was doubled. Although the higher dose was more efficient in tumor induction, use of this dose prevented the increase in tumor incidence related to DES exposure from being detected. Therefore, to elicit a response of an increased incidence of palpable mammary tumors in DES-exposed rats, the amount of DMBA used must be below the level which results in 100% tumor-bearing animals. Our earlier report (4) describing an increased tumor multiplicity in DES-exposed, DMBA-treated rats used a 2-dose, 10-mg regimen of DMBA. However, there were several groups of animals involved which were treated with DMBA over
Table 3

Pathology of palpable mammary tumors in rats exposed prenatally to DES or vehicle and treated postnatally with 10 mg of DMBA

<table>
<thead>
<tr>
<th>Prenatal exposure</th>
<th>No. of palpable mammary tumors</th>
<th>Ratio of observed adenocarcinoma to observed fibroadenoma and adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>Fibroadenoma and adenoma</td>
</tr>
<tr>
<td>DES (n = 32)</td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>82.3</td>
</tr>
<tr>
<td>Vehicle (n = 30)</td>
<td>67</td>
<td>83.7</td>
</tr>
<tr>
<td>Statistic</td>
<td>$G = 6.78$</td>
<td>$G = 14.57$</td>
</tr>
</tbody>
</table>

* Includes all palpable tumors found prior to and after sacrifice at 30 weeks post-DMBA.
* Also includes lobular hyperplasia, hyperplasia with duct ectasia.
* $G$ statistic is distributed as $\chi^2$ and exhibits better continuity than other calculating procedures. See "Statistical Methods."
* "Statistical Methods."
* "Sum of the independently derived statistics for the 2 pathology categories ($G = 6.78 + 14.57$).
* "Significantly different from vehicle-exposed control, $p \leq 0.01$.

Mammary Tumors in DES-exposed, DMBA-treated Rats

a period of weeks, and the DMBA was not made up just prior to each intubation, as in the present experiment. (In both cases, however, the solution was kept in a foil-wrapped container to prevent photodegradation.) Thus, we propose that the apparent discrepancy in results between the earlier published report and data presented here is related to a lower effective dose of DMBA present in the solution used in the first experiment. The experiment using a single 15-mg dose of DMBA which resulted in no difference in tumor incidence between the DES-exposed and vehicle-exposed groups (see "Introduction") can also be viewed as support for the necessity of lowering the dose of carcinogen used to challenge animals exposed transplacentally to drugs previously. It should also be noted here that the 2 studies on rats which combined neonatal treatment with sex steroids and subsequent DMBA intubations used a single 20-mg dose of DMBA (30, 38). Since these experiments showed a reduction in the percentage of tumor-bearing rats (30, 38) and number of mammary tumors per rat (38) after neonatal treatment with estradiol benzoate or 17β-estradiol, it would be of interest to determine whether a lower dose of DMBA confirms the inhibitory action of neonatal estradiol treatment on chemically induced mammary tumorigenesis.

When the histological type of the palpable mammary tumors was considered, prenatal exposure to DES altered the ratio of adenocarcinomata to benign mammary tumors. While, in absolute terms, more adenocarcinomata were induced in the DES-exposed group, they comprised only 68% of all palpable mammary tumors. The higher proportion of benign lesions in the DES-exposed group may be due, in part, to the fact that more DES-exposed rats died prior to termination of the experiment. If some of the tumors classified as adenomata in the DES-exposed group would have progressed to become adenocarcinomata given additional time, the opportunity for these tumors to develop into adenocarcinomata was lost when the host died before the progression was complete. It is interesting that Shellabarger and Soo (30) also found a similar disturbed proportion of adenocarcinomata to fibroadenomata in rats treated neonatally with estradiol benzoate followed by DMBA compared to the proportion in the DMBA-treated controls; this is in spite of the fact that the absolute numbers of mammary tumors per group did not differ significantly in this experiment.

These dramatic effects associated with the transplacental action of DES on mammary tumorigenesis should be examined in light of DES-related effects on other estrogen target tissues. The morphology of the reproductive organs, the pituitary and adrenal glands, and nontumorous mammary gland tissue was examined thoroughly in these animals; these data are presented in a separate paper (6). By comparison to effects observed on mammary tumorigenesis, DES-associated alterations of the structure and function of reproductive and endocrine organs were relatively minor. The most unexpected finding was probably that, at the age when DMBA was given, DES-exposed rats had lower mean serum prolactin levels than did vehicle-exposed controls. From reports which linked DMBA susceptibility of various rat strains to the presence of elevated serum prolactin levels (7, 12), one might have predicted that DES-exposed rats would have increased levels of prolactin in their serum.

It was also surprising that significant differences in mammary gland morphology could not be demonstrated between exposure groups at 2 months of age, whether the tissue was prepared and analyzed as whole mounts or in histological sections. Russo and coworkers (26, 27) demonstrated that DMBA has its greatest tumor-initiating capacity when the mammary gland tissue still has a high density of terminal end buds prior to differentiation into alveolar buds and lobules, a state which normally occurs when an animal is 7 to 8 weeks old. Since the DES-exposed rats developed many more palpable mammary tumors after treatment with 10 mg of DMBA than did the vehicle-exposed controls, it seemed reasonable to expect that differences in mammary gland morphology would be evident at the stage when DMBA was administered, but this was not the case.

Thus, we can conclude that there is an effect on the mammary gland associated with transplacental exposure to DES, which results in the appearance of more mammary tumors when rats are treated postnatally with 10 mg of DMBA; however, the mechanism by which DES acts on the mammary gland is still to be determined. It is essential to bear in mind that these DES-exposed animals were normal with respect to fertility and ovarian-vaginal cyclicity (3, 6) and that DES exposure alone was not associated with an increased incidence of mammary tumors in rats up to 14 months of age (4). We also have demonstrated that the growth of DMBA-induced tumors in DES-exposed rats was as likely to be ovarian dependent as that in DMBA-treated controls, although tumors in the DES-exposed group tended to
enter a new phase of growth following ovariectomy-induced regression (5).

These findings may have special significance for women who were exposed to DES in utero. Although there is no evidence to date of an increased incidence of breast cancer among DES-exposed women (11), these young women have yet to reach the age range when the risk for breast cancer among American women is highest. American women in general have a chance of 1 of 11 of developing breast cancer and are exposed to many known carcinogens and tumor promoters at low but persistent levels in the American diet and environment. In light of such epidemiological findings and these data on DES-exposed rodents, DES-exposed women may be at increased risk for breast cancer.

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