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UTERINE ABNORMALITIES IN RATS EXPOSED NEONATALLY TO DIETHYLSTILBESTROL, ETHYNYLESTRADIOL, OR CLOMIPHENE CITRATE

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SUMMARY

The toxicity of the synthetic estrogens diethylstilbestrol (DES), and ethynylestradiol (EE), and the antiestrogen clomiphene citrate (CC) was evaluated by assessing postnatal uterine growth and development prior to the onset of puberty in the rat. Both DES and EE, administered during the neonatal period (postnatal days 1–5), initially increased uterine weight and luminal epithelium hypertrophy. However, uterine weight declined in both DES- and EE-treated animals and fell below controls beyond day 11. Luminal epithelium stimulation generally paralleled uterine weight changes. Precocious development of uterine glands occurred after estrogenization (compared to untreated controls), but subsequently gland numbers were approximately 60% of control levels. Neonatal CC exposure induced only slight uterine weight gain but caused prolonged luminal epithelium hypertrophy and inhibited uterine gland genesis. Luminal epithelium hypertrophy appears to be a useful measure of antiestrogen activity. These data demonstrate the toxicity of DES and EE as assessed by altered prepubertal uterine gland development. Additionally, the inhibition of uterine gland genesis after neonatal CC exposure occurs in conjunction with prolonged luminal epithelium hypertrophy.

Key words: Estrogen; Antiestrogen; Uterus; Development; Neonate; Rat

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INTRODUCTION

Exposure of women to the synthetic estrogen, diethylstilbestrol (DES) during pregnancy caused hypoplastic and T-shaped uteri in offspring [1,2]. Daughters exhibiting these developmental abnormalities have subsequently experienced unsuccessful pregnancy outcome [1–3]. The similarity in developmental staging of the reproductive tract in the second trimester human and the early postnatal period in the rodent, combined with the experimental accessibility of the postnatal rodent uterus, makes it a suitable animal model to examine the developmental toxicity of estrogens and antiestrogens [4,5].

In rats, uterine glands appear rapidly and synchronously between postnatal days 10 and 14 by invagination of luminal epithelium into the differentiating mesenchyme [6]. The antiestrogen tamoxifen, which is widely used as adjuvant breast cancer therapy [7], inhibits uterine gland genesis when administered prior to uterine gland appearance [8]. This inhibition is accompanied by reduced uterine growth, prolonged luminal epithelium hypertrophy, and cellular degeneration. Recently, Iguchi et al. [9] have observed similar toxic responses in mice exposed neonatally to tamoxifen.

The naturally-occurring estrogen, 17β-estradiol (E_2), delays and reduces uterine gland development in the neonatal rat but complete inhibition is not achieved [6]. At this stage, serum contains sufficient alphafetoprotein (AFP) to bind E_2 and thus reduce the potency of E_2 to about 1% of that seen in adults [10,11]. Neither DES nor ethynylestradiol (EE), a common component of oral contraceptives [12], bind significantly to serum AFP and thus both are more potent estrogens in the neonate [13]. We therefore wished to examine the toxicity of these synthetic estrogens with respect to uterine gland genesis.

The antiestrogen clomiphene citrate (CC) is widely prescribed to induce ovulation in women experiencing anovulatory syndrome [14] and accumulates at significant levels in patients receiving chronic CC therapy [15]. While CC is not frankly teratogenic in humans [16], the reproductive tract of exposed offspring, where such effects might be predicted to occur, has not been examined. Thus, it is of interest to determine if these compounds (DES, EE, CC), all of which figure prominently in human exposure [1–3,12,14], are developmental toxicants with respect to alteration of uterine growth, disruption of postnatal uterine gland genesis, and alteration of luminal epithelium morphology.

MATERIALS AND METHODS

Sprague–Dawley rats were mated on site and the females were housed individually in a controlled environment of 12 h light/12 h darkness (lights on at 0600 h), 23°C, and 50% humidity. Purina rat chow and filtered tap water were provided ad libitum. Litters were culled according to sex within 24 h of birth (day 1), randomized, and the females reassigned at 7 or

8 pups per dam. Groups of animals were injected s.c., in the middorsal region, on postnatal days 1–5 with DES, EE (Research Plus Steroid Laboratories, Denville, NJ), or CC (Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH) suspended in 10 μl of sesame oil (Fisher Scientific, Fair Lawn, NJ). A dose-response experiment was conducted on postnatal day 5 to assess the uterotrophic potency of DES, EE, and CC in neonatal rats. The doses used (10^{-3} to 10^{2} μg/rat per day) were estimated to encompass the entire uterine weight response range based on previous experiments using neonatal administration of E_2 [6] and tamoxifen [8]. Doses of 10^{-1} μg/rat per day and 10 μg/rat per day were chosen to examine the developmental effects of the test compounds and to allow comparision with previous studies [6,8]. Normal uterine development (control animals) was examined in untreated animals since vehicle treatment was without effect in preliminary experiments [6].

Animals were killed, at intervals after dosing, by either decapitation or cervical dislocation followed by decapitation (animals 20 days and older) and weighed. Uteri were dissected free of mesometrium, weighed, and placed in 30–40 ml of 10% neutral buffered formalin. After 24 h, three drops of 0.01% toluidine blue (Fisher Scientific) were added to the formalin, and 24 h later, each uterine horn was divided into 3–4 pieces, depending on size, and processed with a 4-h cycle in an autotechnicon (Technicon Instrument Corp., Tarrytown, NY). Tissue retention of toluidine blue allowed precise orientation for cutting transverse sections (4 μm). Sections were stained with hematoxylin (Gill's Formulation no. 3, Fisher Scientific) and eosin-phloxine [17].

Glands in 6–8 uterine sections/animal were counted using 3 criteria: first, the lumen of the gland had to be visible; second, the lumen of the gland had to be separated from the uterine lumen by stromal tissue to establish it as a tubular structure and not a fold in the luminal epithelium; and third, the gland lumen had to be surrounded by epithelial cells to exclude dilated blood vessels and artifactual spaces. Questions of whether a structure represented 2 distinct glands or a plane of section through a convolution of 1 gland were resolved by examining serial sections. The number of glands per uterine section represents an average of counts from the proximal, middle, and distal portions of both uterine horns from each animal. Luminal epithelium height was measured in 6–8 uterine sections/animal and 6–8 animals/treatment condition using a calibrated graduated ocular at a magnification of 1000×. Statistical analyses were conducted using mean gland counts per uterine section, uterine weights or luminal epithelium heights as dependent variables in the model:

$$\text{dependent variable} = \text{age at death} + \text{treatment} + \text{age by treatment interaction} + \text{error}$$

Least-squares means of treatment were then compared within each age at death. This procedure results in a pooled estimate of variance across all

ages and treatments and is comparable to testing mean differences by the *t*-test. The least-squares means adjust for differences in the number of observations.

RESULTS

To assess the estrogenic potency of the test compounds in neonates, various doses of DES, EE, or CC were administered daily after birth and uterine weights and luminal epithelium heights were examined on day 5 (Fig. 1). Neonatal DES and EE exposure elicited similar uterine weight responses which plateaued at a dose of 1 μg/rat per day (Fig. 1A). Treatment with CC increased uterine weight at all doses ($P < 0.05$) except 10^{-2} μg. However, the greatest increase after CC treatment was only 25% of the estrogen-induced response. DES induced maximal luminal epithelium hypertrophy (Fig. 1B) at a dose of 10^{-1} μg ($P < 0.05$). There were no significant differences when data from animals dosed with $10^{-1}-10^{2}$ μg/rat

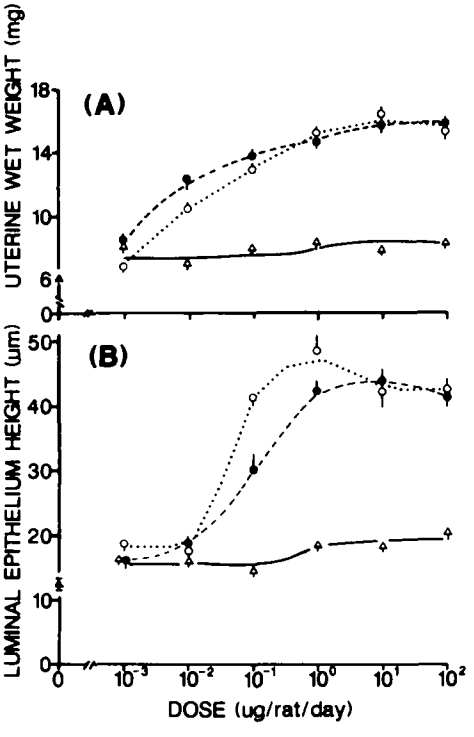

Fig. 1. Uterotrophic potency of DES, EE, and CC in neonatal rats as determined by uterine wet weight [A] and luminal epithelium height [B]. Animals were given daily doses of DES (O····O), EE (●- - - -●), or CC (Δ——Δ) and killed on day 5. Control animals (▲) were untreated. Uterine weights (mg) are means ± S.E. using a minimum of 10 animals. Luminal epithelium heights (μm) are means ± S.E. measured in 6–8 uterine cross sections/animal from 6 to 8 animals.

per day were compared. EE was slightly less potent and required a dose of 1 μg to achieve a maximal luminal epithelium response. CC stimulated slight luminal epithelium hypertrophy on day 5 but the maximal response was only 25% of that induced by either DES or EE. No uterine glands were observed on day 5 after any treatment.

To evaluate the developmental consequences of neonatal estrogen or

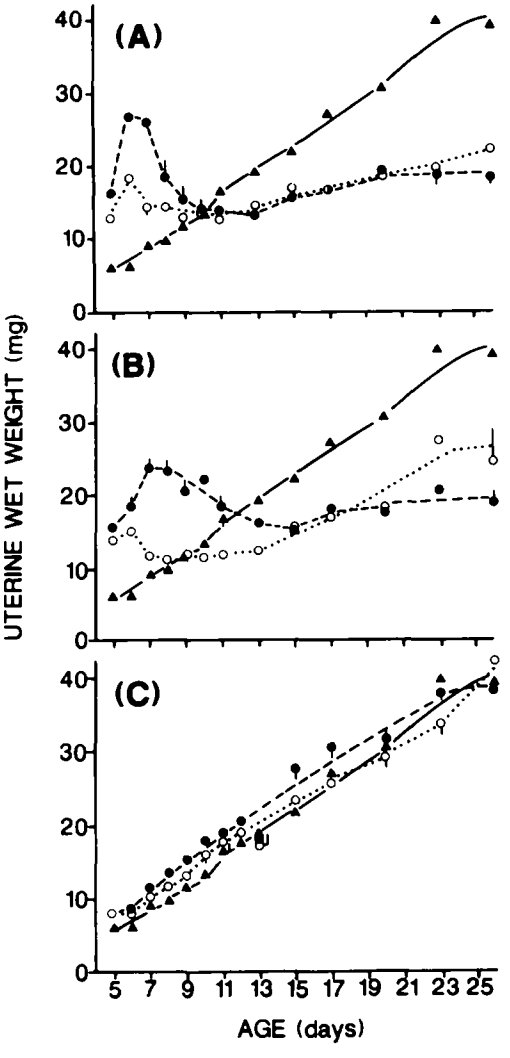

Fig. 2. Uterine wet weight after neonatal treatment with DES [A], EE [B], or CC [C]. Animals were given either 0.1 μg/rat per day (O·····O) or 10 μg/rat per day (●- - - -●) of the test compounds on postnatal days 1–5 or were left untreated (▲——▲) and killed at intervals thereafter. The data represent means ± S.E. of uterine wet weights (mg) from an average of 6 animals/examination period. Standard errors less than 1.0 mg fall within the data points and are not shown.

antiestrogen exposure, uterine responses were examined at intervals following treatment with a low dose (10^{-1} μg/rat per day) and a high dose (10 μg/rat per day) of each test compound. Uterine weight continued to increase until day 6 after both high- and low-dose DES exposure and then declined (Fig. 2A). While uterine weight declined immediately after day 6 following neonatal exposure to 10^{-1} μg of EE (Fig. 2B), uteri exposed to 10 μg of EE continued to increase in weight until day 7 and then gradually declined. Uterine weights fell below controls by day 13 ($P < 0.05$) following

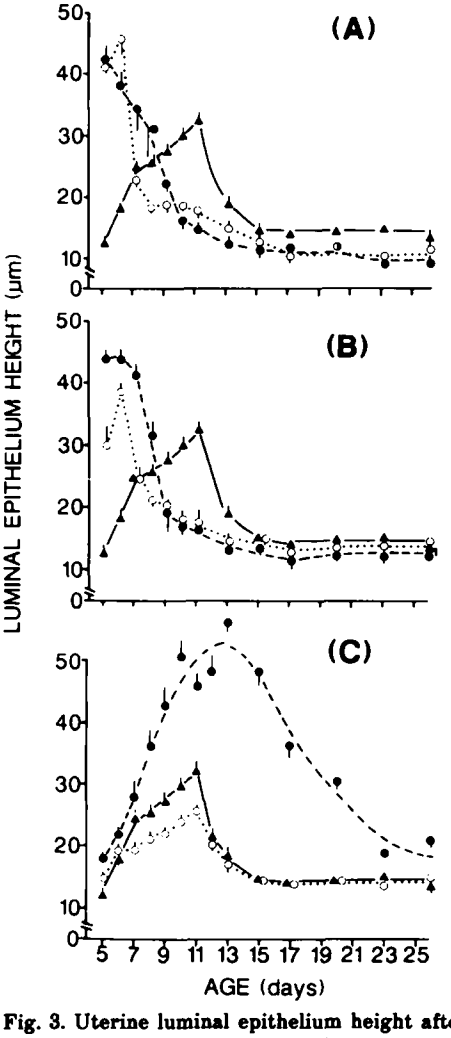

Fig. 3. Uterine luminal epithelium height after neonatal treatment with DES [A], EE [B], or CC [C]. Animals were given either 0.1 μg/rat per day (O····O) or 10 μg/rat per day (●- - - -●) of the test compounds on postnatal days 1–5 or were left untreated (▲——▲) and killed at intervals thereafter. The data represent means ± S.E. of luminal epithelium height measurements (μm) of 6–8 uterine cross sections/animal and 6–8 animals per examination period. Standard errors less than 1.0 μm fall within the data points and are not shown.

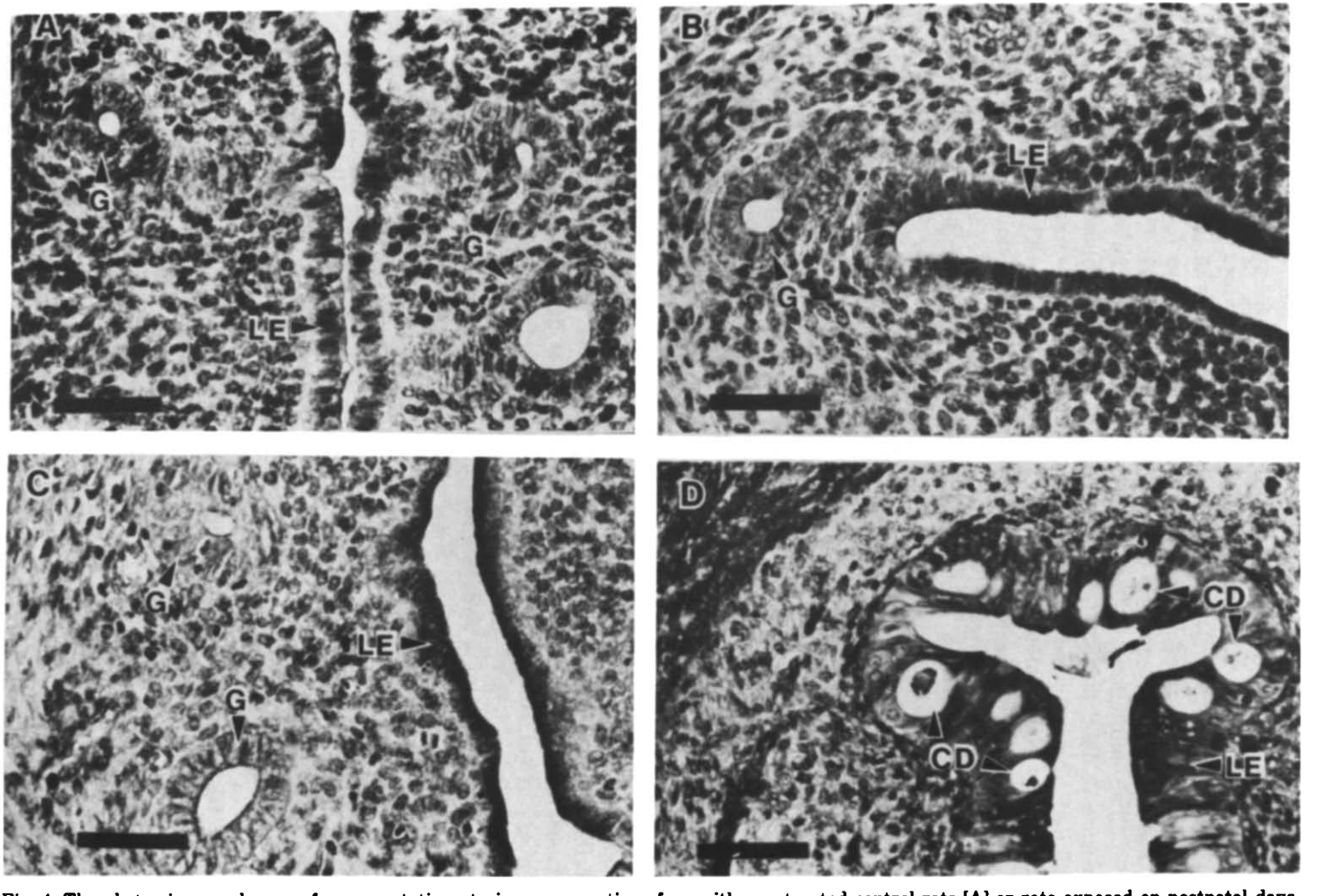

Fig. 4. The photomicrographs are of representative uterine cross sections from either untreated control rats [A] or rats exposed on postnatal days 1–5 to 10 μg of DES [B], EE [C], or CC [D]. The animals were killed on day 13 and the uteri were removed and processed for histological examination as in Materials and Methods. Cellular degeneration (CD) and luminal epithelium (LE) hypertrophy were observed on postnatal day 13 after neonatal dosing with 10 μg CC. While uterine glands (G) are visible in control and in DES- or EE-treated animals, none are present after CC treatment. The bar equals 60 μm.

exposure to each synthetic estrogen and remained below controls through day 26. The slight uterine weight increase elicited by 10^{-1} μg of CC (Fig. 2C) was maintained until day 11 ($P < 0.05$) after which uterine weights equalled those of controls ($P < 0.05$). Exposure to 10 μg of CC caused slight but significant elevated uterine weight ($P < 0.05$ for days 5, 6, 8, 9, 10, 13, 15, and 17) which returned to control levels beyond day 17.

Luminal epithelium height, examined at intervals following neonatal exposure to DES (Fig. 3A) and EE (Fig. 3B), generally followed the trends exhibited by uterine weight. Estrogen-induced luminal epithelium hypertrophy was evident until day 8 after exposure to 10^{-1} μg of DES or EE while 10 μg of the synthetic estrogens maintained luminal epithelium hypertrophy slightly longer.

The lower CC dose (Fig. 3C) caused a slight decrease in luminal epithelium height between days 7 and 11 (significant at $P < 0.05$ on days 7, 10, and 11). Beyond day 12 luminal epithelium height was indistinguishable from controls. By contrast, 10 μg of CC elicited delayed but prolonged luminal epithelium hypertrophy which was maximal on day 13. Beyond day 13 luminal epithelium height gradually declined but remained significantly elevated ($P < 0.05$) throughout the examination period. Cellular degeneration, characterized by large cytoplasmic vacuoles and nuclear karyorrhexis, accompanied the luminal epithelium hypertrophy elicited by 10 μg of CC (Fig. 4D). While the extent of cell degeneration was not quantified, similar findings were not observed in either control or estrogen-exposed rats at this age.

Both low and high estrogen doses elicited precocious appearance of uterine glands (Figs. 5A,B). However, the rates of increase in gland numbers with age were not maintained after their first appearance and the number of uterine glands reached between days 17 and 26 were approximately 60% of controls. The lower CC dose slightly delayed the appearance of uterine glands (significantly less than control on days 10, 11, 12, 13, and 15; $P < 0.05$) (Fig. 5C). Despite this delay, uterine gland genesis proceeded and gland numbers rose to control levels between days 17 and 26. By contrast, neonatal treatment with 10 μg of CC significantly depressed uterine gland numbers ($P < 0.05$) throughout the examination period.

DISCUSSION

Both DES and EE, administered neonatally, elicited greater uterine wet weight increases than were previously observed after neonatal treatment with identical doses of E_2 [6]. Binding of E_2 to serum AFP, which is present in high levels in rodents during the perinatal period, impedes entry of E_2 into target cells [11,13,18]. Since DES and EE bind serum AFP with much lower affinity than E_2 [10,13], these synthetic estrogens are more readily available for entry into target cells, and are thus more potent than E_2.

Following the initial uterine weight increases induced by DES and EE,

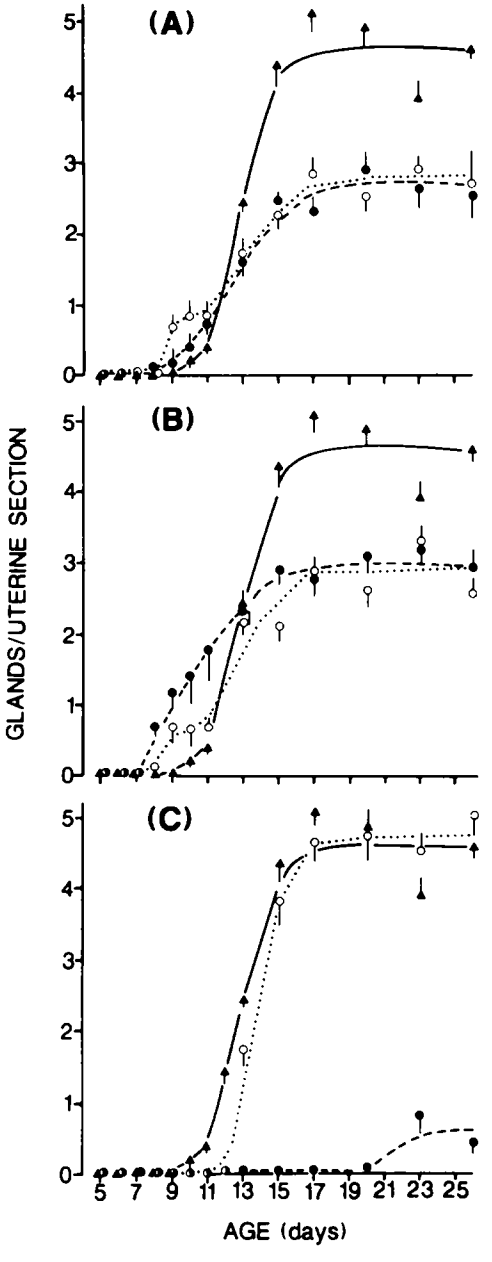

Fig. 5. Gland numbers per uterine cross section after neonatal treatment with DES [A], EE [B], or CC [C]. Animals were given either 0.1 μg/rat day (O· · · · ·O) or 10 μg/rat per day (●- - - - -●) of the test compounds on postnatal days 1–5 or were left untreated (▲———▲) and killed at intervals thereafter. The data represent means ± S.E. of uterine glands from 6 to 8 uterine cross sections/animal and 6–8 animals/examination period.

uterine growth fell below control levels. It has been demonstrated by neonatal ovariectomy [19,20] and by the use of a specific anti-estradiol serum [21] that postnatal uterine growth is mediated via ovarian estrogen secretion. It is also well documented that neonatal estrogenization in the rodent disrupts normal hypothalamic control of ovarian estrogen secretion [22]. While direct, long-term uterine toxicity cannot be ruled out, the failure of uteri to maintain normal weight after neonatal DES and EE treatment is consistent with the elimination of ovarian-dependent uterine growth. In this view, neonatal estrogenization causes functional castration with respect to ovarian estrogen secretion. Clomiphene citrate exhibited much lower neonatal uterotrophic activity than either of the synthetic estrogens and failed to depress uterine growth at later ages. The partial estrogen agonist activity of CC appeared to be insufficient to cause impairment of ovarian-mediated uterine growth.

The premature uterine gland appearance seen between postnatal days 7–9 after neonatal DES and EE exposure was also noted after neonatal treatment with high doses of E_2 [6]. Premature appearance of uterine glands may result from the estrogen-induced uterine growth described above. However, this premature gland appearance does not reflect a shift in the entire process of gland genesis. Instead, the rate of gland appearance was lowered and the number of glands ultimately reached was decreased.

The synthetic estrogens also induced premature luminal epithelium hypertrophy. As well, the peak of luminal epithelium hypertrophy (days 9–13) which normally precedes the increase in uterine gland numbers in untreated rats was eliminated and gland numbers were reduced. While our data cannot ascribe a causal link between luminal epithelium morphology and uterine gland genesis, these data reinforce our earlier suggestion [6] that premature luminal epithelium hypertrophy may affect the invagination process which is essential for normal gland genesis.

After CC treatment, prolonged luminal epithelium hypertrophy was maintained throughout the normal period of rapid uterine gland proliferation in untreated rats (days 10–15). While luminal epithelial cell hypertrophy following neonatal CC exposure has been observed [23,24], the duration and consequences were not established. The luminal epithelial cell degeneration reported here, which accompanied prolonged luminal epithelium hypertrophy, may reflect the killing of luminal epithelial cells which are present at birth and appear to be progenitors of uterine gland cells [25]. Quantitation of luminal epithelial cells in uterine cross sections from animals treated neonatally with CC and tamoxifen and killed on day 26 indicate a 55% decrease in cell number (unpublished observation). This decrease may result from either increased cell death or decreased cell division (or a combination of both). While we did not examine uterine gland numbers during or beyond puberty, only sparse uterine glands develop in adult Wistar rats treated neonatally with CC [26]. Similar reductions in uterine gland numbers have been reported in adult NMR [27] and C57BL/

Tw mice [9] and rats [8] after postnatal tamoxifen treatment. Taken together, these data suggest a permanent toxic activity of triphenylethylene antiestrogens on uterine gland genesis.

Several considerations concerning CC seem appropriate. Triphenyl ethylene antiestrogens are often referred to as "weak" estrogens because of their lower uterotrophic potency relative to "pure" estrogens. However, our data show a high degree of potency and specificity of CC in the luminal epithelium, compared to estrogens. This suggests that luminal epithelial cell effects may be useful in determining the potency of antiestrogens. Secondly, the antiestrogenic activity of the triphenylethylenes is thought to result from blockade of estrogen action. However, inhibition of uterine gland genesis by CC does not appear to occur by blocking the activity of endogenous estrogen since neonatal ovariectomy does not inhibit gland genesis [20]. Finally, estrogens are toxic to human reproductive tract development [1,2] and, as demonstrated here, CC possesses significant estrogenic potency in the luminal epithelium. Since therapeutically administered CC may remain during crucial stages of reproductive tract organogenesis in humans [15], the possibility exists that CC may induce reproductive tract lesions in humans similar to those induced by DES.

In summary, these data describe reduced uterine growth and gland development after neonatal DES and EE exposure. While CC does not reduce uterine growth it inhibits uterine gland development. Additionally, these data demonstrate toxicant-induced alterations in uterine luminal epithelium morphology which warrants further investigation.

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