Differential Sensitivity of Rat Uterine Growth and Epithelium Hypertrophy to Estrogens and Antiestrogens (43602)

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Abstract. **Triphenylethylene antiestrogens are considered weak estrogen agonists based on their limited ability to induce estrogen responses, in particular uterine growth. We compared the uterotrophic activity of naturally occurring and synthetic estrogens with that of antiestrogens by quantitating uterine wet weight and hypertrophy in the uterine luminal and glandular epithelium. Immature rats received five daily injections of either an estrogen (17β-estradiol [E_2], diethylstilbestrol [DES], or ethynyl estradiol [EE]) or an antiestrogen (tamoxifen [TAM], monohydroxytamoxifen [OH-TAM], or clomiphene citrate [CC]) (0.001–100 μg/rat/day) subcutaneously in sesame oil and were sacrificed approximately 2 hr after the last injection. Both DES and EE increased uterine weight at doses between 0.01–100 μg/rat/day; E_2 was about 10-fold less potent. The antiestrogens increased uterine weight only slightly. DES, EE, and the three antiestrogens each increased luminal epithelium hypertrophy to over 3-fold above that in controls. While the potencies of these synthetic compounds differed (DES = EE > OH-TAM > TAM = CC), each hypertrophic response occurred over two log doses, and the response curves displayed identical slopes. E_2, however, required a range of four log doses to achieve the same degree of luminal epithelium hypertrophy. The three antiestrogens elicited glandular epithelium hypertrophy up to 2-fold above controls at the same doses that induced luminal epithelium hypertrophy; the order of potency was OH-TAM > TAM = CC. However, the three estrogens increased glandular epithelium hypertrophy only marginally. Thus, under dosing conditions commonly used to assess uterotrophic activity, these "antiestrogens" are complete, albeit less potent, estrogen agonists in the luminal epithelium and, unlike estrogens, induce hypertrophy in the glandular epithelium.** [P.S.E.B.M. 1993, Vol 203]

Triphenylethylene antiestrogens are generally considered to be weak estrogen agonists because they induce only partial estrogenic responses, such as uterine weight gain (1). Antiestrogens also strongly antagonize estrogenic activity, presumably through interaction with target cell estrogen receptors (2, 3). For this reason, triphenylethylenes are commonly prescribed for treatment of breast cancer (4) and anovulatory syndrome (5, 6). Currently, several countries, including the United States, are conducting extensive trials to test the efficacy of tamoxifen as a breast cancer preventative in disease-free premenopausal women at increased risk of breast cancer (7, 8). However, in addition to the desirable estrogen antagonist activity, estrogen agonism, manifested as an increase in the vaginal karyopyknotic index, has been detected in the human reproductive tract (9, 10). Moreover, several studies have reported an increased incidence of endometrial carcinoma (an estrogen-associated malignancy) and endometrial and endocervical polyps in some breast cancer patients receiving tamoxifen therapeutically (11–14). An understanding of the effects of antiestrogens on uterine epithelia is of interest because most endometrial carcinomas are epithelial in origin (15).

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The uterine epithelium of the developing rodent is sensitive to both exogenous estrogens and antiestrogens (16–18). However, significant morphological differences exist in the uterine responses elicited by these two classes of compounds. Neonatal estrogen exposure causes hypertrophy in the luminal epithelium, which is detectable immediately after treatment and subsequently reduces the development of uterine glands (19, 20). By contrast, the appearance of luminal epithelium hypertrophy in response to neonatally administered antiestrogens is substantially delayed compared with that in response to estrogens (20) and is accompanied by the failure of uterine gland development in rats (20, 21) and mice (22, 23). Estrogen exposure in infantile rats (postnatal days 10–14) delays uterine gland appearance until estrogen-induced luminal epithelium hypertrophy subsides, whereas antiestrogen exposure during this period inhibits uterine gland appearance (24). Since antiestrogen exposure before the completion of uterine differentiation precludes examination of the glandular epithelium, the current experiments compare the uterotrophic and epithelial hypertrophic effects of estrogens and antiestrogens in the differentiated rat uterus.

Materials and Methods

Sprague-Dawley rats (bred at the National Center for Toxicological Research) were housed in a controlled environment of 12 hr of light/12 hr of dark (lights on at 0600 hr), 23°C, and 50% humidity. Purina rat chow and filtered tap water were provided *ad libitum.*

On postnatal days 20–24 (between 0800 and 0900 hr), groups of six to eight animals were given daily middorsal subcutaneous injections of the test compounds suspended in 10 μl of sesame oil (Fisher Scientific, Fair Lawn, NJ). Treatment at this age permits tissue analysis after uterine differentiation has occurred but before the occurrence of increased uterine morphological variability due to cyclical hormonal changes. The animal weights ranged between 51.2 ± 0.6 g on day 20 to 63.9 ± 2.2 on day 24. The test compounds were 17β-estradiol (E_2), diethylstilbestrol (DES), and ethynyl estradiol (EE) from Research Plus Steroid Laboratories (Denville, NJ); clomiphene citrate (CC) from Merrell Dow Pharmaceuticals, Inc. (Cincinnati, OH); and tamoxifen (TAM) and monohydroxytamoxifen (OH-TAM) from Stuart Pharmaceuticals (Wilmington, DE). Control animals were untreated, as previous experiments have shown vehicle injection to have no effect (19, 20).

Animals receiving multiple injections were sacrificed approximately 2 hr after the last injection on day 24 by cervical dislocation followed by decapitation. To assess the morphological effects of a single estrogen dose, rats were injected with E_2 (10 μg/rat, sc, in 10 μl of sesame oil) on either day 21 or 25 and sacrificed 24 hr later. In all experiments, uteri were carefully dissected free of adhering fat and mesentery, weighed, and placed in 30–40 ml of 10% neutral buffered formalin (Fisher Scientific). After 24 hr, three drops of 0.01% toluidine blue (Fisher Scientific) were added to the formalin, and 24 hr later, each uterine horn was divided into three or four pieces and processed with a 4-hr cycle in an Autotechnicon (Technicon Instruments Corp., Tarrytown, NY). Staining with toluidine blue allowed precise orientation during embedding and ensured the cutting of sections perpendicular to the long axis of each uterine horn. Sections (4 μm) were stained with hematoxylin (Gill's Formulation No. 3, Fisher Scientific) and eosin-phloxine.

Luminal and glandular epithelium heights were measured in six to eight uterine sections/animal, with six to eight animals/treatment condition, using a calibrated ocular at a magnification of ×1000. The procedure for scoring uterine glands has been described previously (19).

The uterine weight, luminal epithelium height, and glandular epithelium height data were evaluated using an analysis of variance, with fixed treatment and dose effects. Comparisons were performed using Bonferroni's multiple comparison procedure with an experimentwise significance level of $\alpha = 0.05$.

Results

DES and EE elicited log-linear increases in uterine weight that were significantly higher than control values ($P < 0.05$) between doses of 0.1 and 100 μg/rat/day (Fig. 1A). E_2 was about 10-fold less potent than the synthetic estrogens and caused significant increases compared to control values at 10 and 100 μg/rat/day. The slopes of the linear portions of the estrogen-induced uterine growth curves were parallel. The antiestrogens (Fig. 1B) induced much smaller uterine weight gains even at the highest doses administered, and the slopes of the growth curves were lower than those for the estrogens.

The dose-response curves for luminal epithelium height (Fig. 2) differed from those for uterine weight. Approximately 85% of the luminal epithelium height increase occurred between doses of 0.01 and 0.1 μg/rat/day for both DES and EE (Fig. 2A). By contrast, the slope of the E_2-induced luminal epithelium hypertrophy curve was lower than those of the synthetic estrogens, requiring four log doses to reach a maximum height response. Despite this difference in slope, all three estrogens induced significant luminal epithelium height increases ($P < 0.05$) at and above a dose of 0.1 μg/rat/day. The slopes of the luminal epithelium height-response curves for each of the antiestrogens (Fig. 2B) were virtually identical to those seen after DES and EE treatment. However, OH-TAM was 10-fold less potent ($P < 0.05$ at 1 μg/rat/day) and TAM

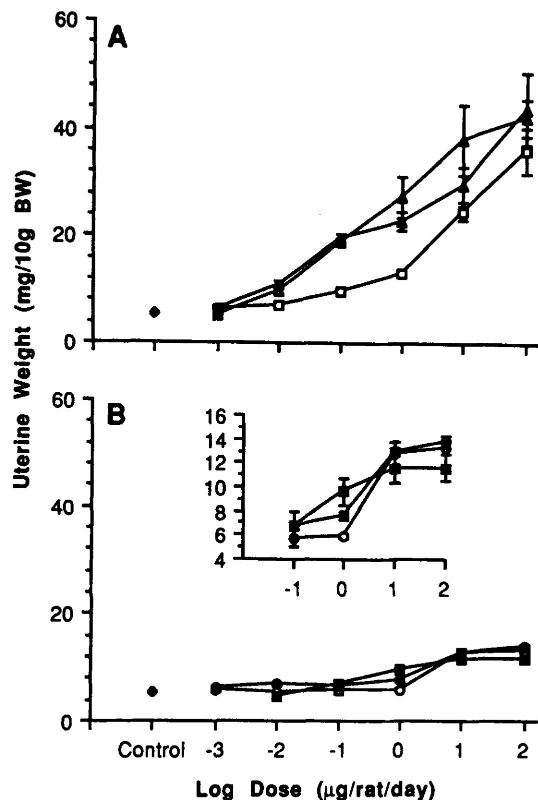

Figure 1. Effects of estrogen (A) or antiestrogen (B) on uterine weight. Groups of rats were given daily injections of DES (▲), EE (△), E_2 (□), TAM (●), OH-TAM (■), or CC (○) subcutaneously in sesame oil on postnatal days 20–24. Control rats (◆) were untreated. The inset in panel B shows the uterine weight response to antiestrogen for doses of 0.1–100 µg/rat/day on an expanded uterine weight scale. The data are mean uterine weights normalized to body weight in milligrams ± SE; n = 6–8 rats. SE bars that fall within the data points are not shown.

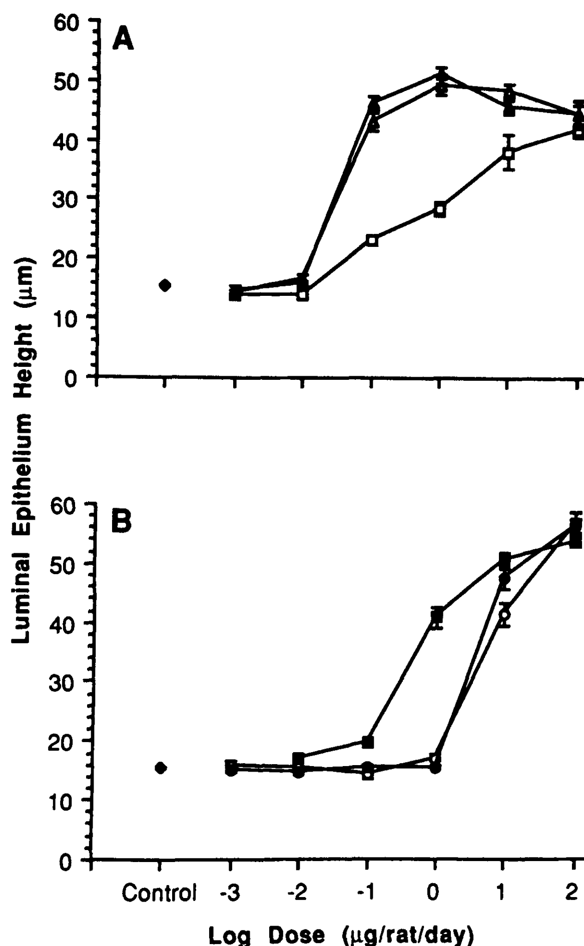

Figure 2. Effects of estrogen (A) or antiestrogen (B) on luminal epithelium hypertrophy. Groups of rats were given daily injections of DES (▲), EE (△), E_2 (□), TAM (●), OH-TAM (■), or CC (○) subcutaneously in sesame oil on postnatal days 20–24. Control rats (◆) were untreated. Luminal epithelium heights were measured using a graduated ocular at a magnification of ×1000. The data are mean luminal epithelium heights in microns ± SE of six to eight cross-sections/uterus from six to eight rats. SE bars that fall within the data points are not shown.

and CC were 100-fold less potent ($P < 0.05$ at 10 µg/rat/day) than either of the synthetic estrogens. A maximum hypertrophic response of approximately 3-fold over control values was obtained after treatment with each of the estrogens and antiestrogens, and the plateau values were not substantially different for any compound.

Neither the estrogens nor the antiestrogens altered the number of uterine glands significantly (data not shown). DES, EE, and E_2 each induced slight but insignificant ($P > 0.05$) glandular epithelium hypertrophy of about 15% over control values (Fig. 3A). By contrast, the antiestrogens each induced glandular epithelium hypertrophy of about 2-fold over control values (Fig. 3B). The dose-response curves were parallel, and OH-TAM was 10-fold more potent ($P < 0.05$ at 1 µg/rat/day) than either TAM or CC ($P < 0.05$ at 10 µg/rat/day). Increased glandular epithelium hypertrophy occurred at the same antiestrogen doses that increased luminal epithelium hypertrophy. Glandular epithelium hypertrophy induced by antiestrogens was observed both adjacent to the luminal epithelium and in the deep endometrial stroma (Fig. 4).

To determine whether a single estrogen dose could elicit significant glandular epithelium hypertrophy in immature rats, E_2 (10 µg/rat) was administered on either postnatal day 21 or 25, and the rats were sacrificed 24 hr later. E_2 increased uterine wet weight by 67% above control values on day 22 and 96% on day 26 (Fig. 5A). Luminal epithelium thickness was less responsive to a single E_2 dose than to multiple E_2 doses, but still achieved increases of 25% and 63% over control values on days 22 and 26, respectively (Fig. 5B). By contrast, E_2 increased glandular epithelium thickness by only 12% above control values on day 22 and 9% above controls on day 26 (Fig. 5C).

To make a direct comparison of the dose-responsiveness of the three parameters of uterine activity, uterine weights and luminal and glandular epithelium

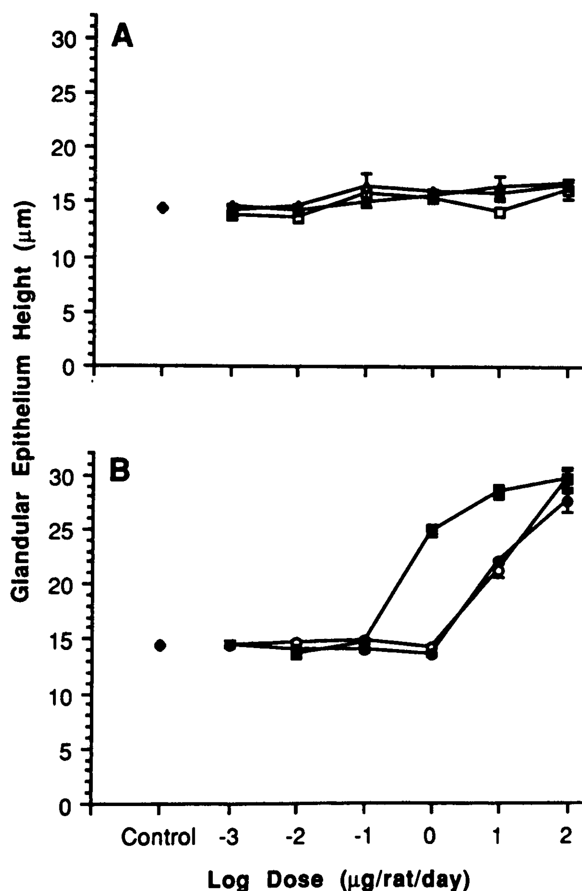

Figure 3. Effects of estrogen (A) or antiestrogen (B) on glandular epithelium hypertrophy. Groups of rats were given daily injections of DES (▲), EE (△), E_2 (□), TAM (●), OH-TAM (■), or CC (○) subcutaneously in sesame oil on postnatal days 20–24. Control rats (◆) were untreated. Glandular epithelium heights were measured using a graduated ocular at a magnification of ×1000. The data are mean glandular epithelium heights in microns ± SE of six to eight cross-sections/uterus from six to eight rats. SE bars that fall within the data points are not shown.

heights were expressed as a percent response for each compound (Fig. 6). These data indicate that at higher doses, the relative response to pure estrogens is far greater for uterine weight gain than for epithelium hypertrophy. With the antiestrogens, however, the extent of the luminal epithelium response exceeds that of uterine weight gain. Additionally, the luminal epithelium height responses were similar for all six compounds. The greater hypertrophic response of the glandular epithelium to antiestrogens compared to estrogens was also evident. These data demonstrate that whereas the magnitudes of the responses may vary with each parameter measured, any antiestrogen dose that elicits a response for one parameter also elicits a response for each of the other parameters.

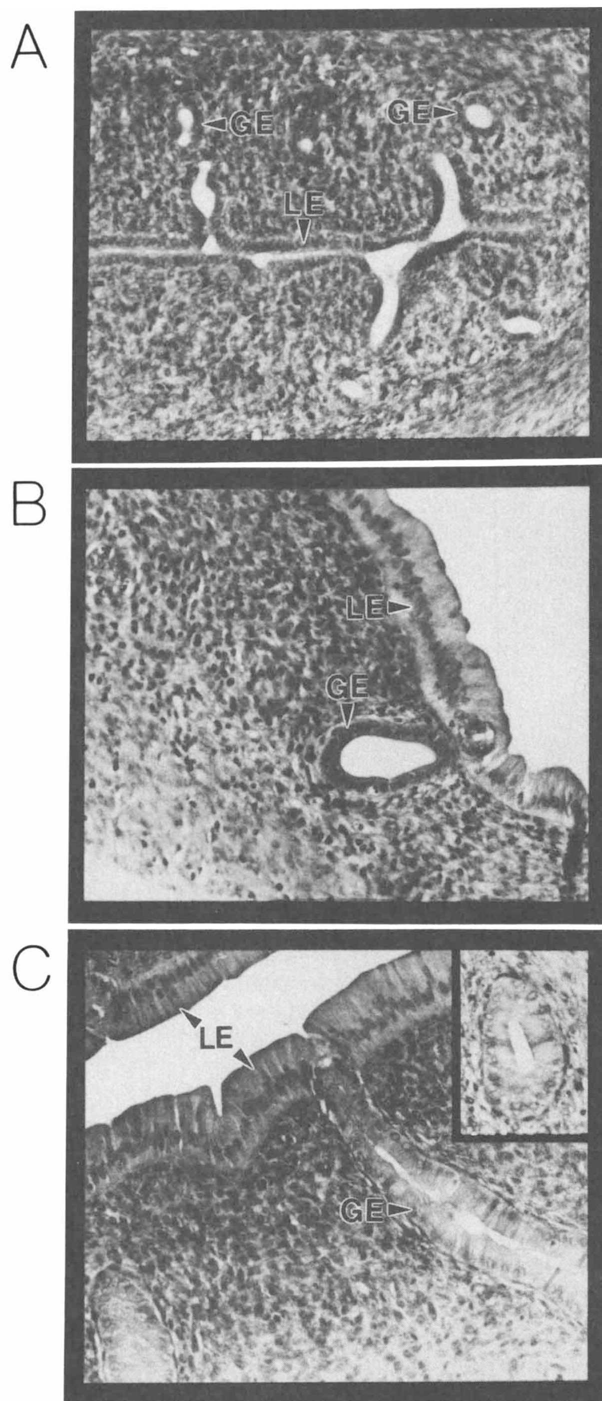

Figure 4. Effects of estrogen or antiestrogen on epithelium morphology. Both luminal epithelium (LE) and glandular epithelium (GE) heights are approximately 15 μm in untreated control animals (A). Panel B demonstrates that estrogen exposure (100 μg E_2/rat/day) induces LE hypertrophy, but little GE hypertrophy. (C) Antiestrogen exposure (10 μg CC/rat/day) causes hypertrophy in the LE and GE both adjacent to the LE and in the deep endometrial stroma (inset).

Discussion

Because the uterus of the immature rat is composed of individual tissue elements (circular and longitudinal muscle, endometrial stroma, and luminal and glandular epithelium), the complete response to estrogen, measured by uterine weight gain or biochemical parameters in whole uterine homogenates, represents the combined responses (or lack of responses) of each individual

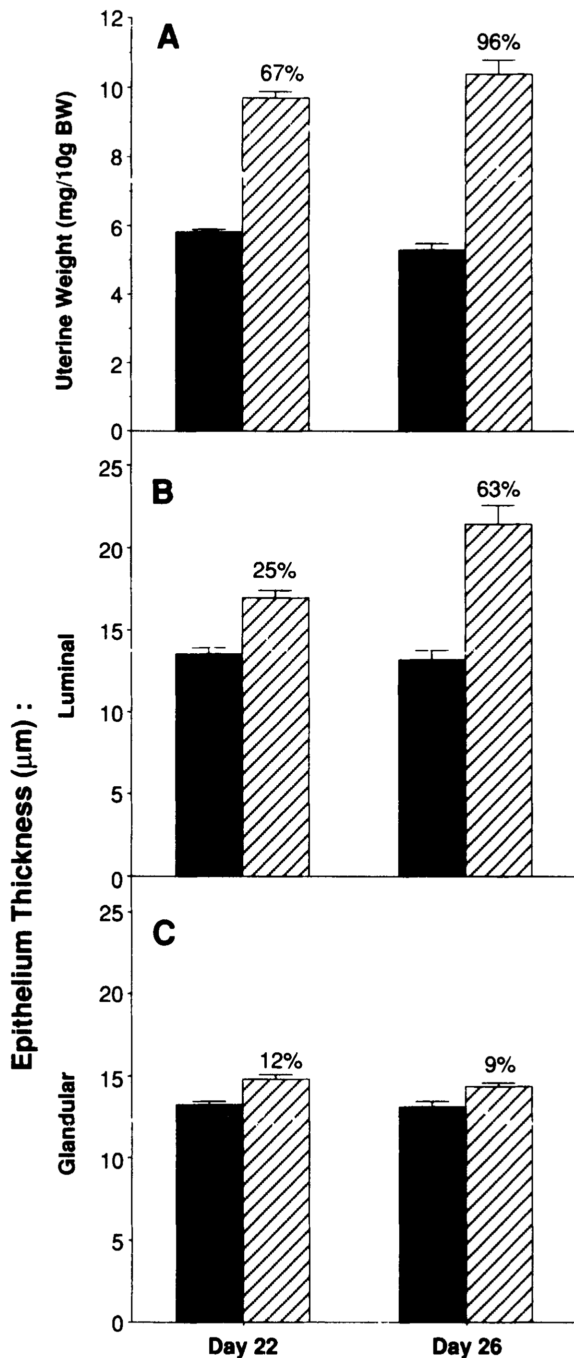

Figure 5. Uterine responses to a single estrogen dose. Groups of six or seven rats were given a single injection of E_2 (10 μg/10 μl sesame oil, sc) and killed 24 hr later. Uterine weights were recorded (A), and luminal (B) and glandular (C) epithelium heights were measured in histologically prepared uteri from both control (■) and E_2-treated (▨) rats. The data are mean uterine weights normalized to body weights in milligrams ± SE and epithelium heights in microns in six to eight uterine sections/rat from six or seven rats. The numbers above the data bars are percentages of increase over control levels.

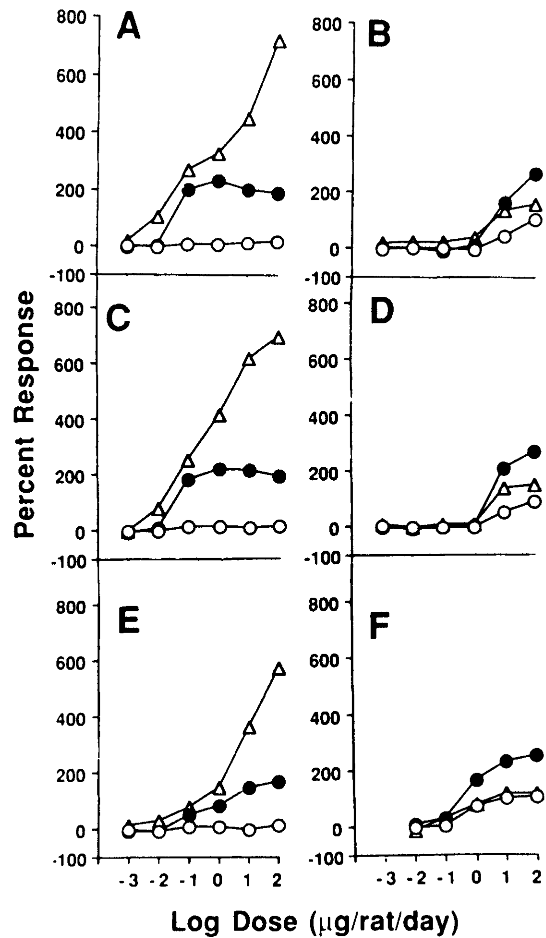

Figure 6. Uterotrophic activity of estrogens and antiestrogens as percent response. The data for uterine weight (△), luminal epithelium thickness (●), and glandular epithelium thickness (○) were calculated as a percent response by the formula: (treated − control) ÷ control. The test compounds included DES, (A), CC (B), EE (C), TAM (D), E_2 (E), and OH-TAM (F).

uterine cell type. Our interest here is with the epithelium because of its sensitivity to estrogen, its importance in uterine functions, such as secretory activity and blastocyst implantation, and its potential for transformation to uterine adenocarcinoma. While morphologically similar, significant differences exist between the uterine luminal and glandular epithelia. For example, estrogen-induced cell division is about 4- to 10-fold greater in the luminal epithelium than in the glandular epithelium in mice (25) and rats (26).

The quantitation of epithelium hypertrophy presented here clearly demonstrates a preferential sensitivity of glandular epithelium to antiestrogens versus estrogens. While the underlying mechanism controlling epithelial sensitivity remains unclear, several findings may be relevant. Ennis and Stumpf (27) have shown that significant levels of antiestrogen remain in nuclei from all uterine cell types 48 hr after dosing (the latest time sampled), whereas E_2 disappeared between 12 and 24 hr. Their data and others (28, 29) suggest that antiestrogens act in the uterus via long-term nuclear binding to provide low level, continuous stimulation of the estrogen response. In addition, Ennis and Stumpf (27) demonstrated that antiestrogen binding (labeling index) was greater in the glandular epithelium than in

the luminal epithelium at all times examined. Thus, specific long-term retention of antiestrogen in the glandular epithelium is one possible explanation for the greater hypertrophic activity of the antiestrogen in the glandular epithelium compared to that of estrogens.

The uterine weight and epithelium thickness data obtained from animals given a single estrogen dose demonstrated that substantial estrogen-induced glandular epithelium hypertrophy did not occur early in the dosing period only to decline to normal levels by the end of the multiple dosing regimen. A similarly slight increase in glandular epithelium thickness (17% over control values) in response to a single estrogen dose in 102-day-old rats has been observed (data not shown). Likewise, Arriaza *et al.* (30) reported results virtually identical to ours for luminal and glandular epithelium hypertrophy induced by a single dose of E_2. In contrast to the glandular epithelium, where either a single or multiple doses give equivalent hypertrophy, there is a cumulative increase in E_2-induced luminal epithelium hypertrophy with increasing E_2 doses.

Both DES and EE were substantially more potent than E_2 with respect to both luminal epithelium hypertrophy and uterine weight gain. At high doses, similar magnitudes of uterine responses were achieved. These results are consistent with the concept of modulation of E_2-induced responses by the serum estrogen-binding protein α-fetoprotein (31, 32), which is still present in immature rats at levels sufficient to bind lower E_2 doses (33). Since neither of the synthetic estrogens binds significantly to α-fetoprotein (32), the free hormone levels are higher than with E_2, and thus, DES and EE are more potent than E_2. This reasoning suggests that the 10-fold reduction in α-fetoprotein levels from day 22 to day 26 (33) can explain the increased potency of E_2 at the older age.

Estrogen receptor content is greatest in uterine endometrial stroma and glandular epithelium in mouse (34, 35), rat (27), and macaque (36), as demonstrated by both standard autoradiographic and immunocytochemical localization techniques. However, our data show that both estrogens and antiestrogens induced greater hypertrophy in the luminal epithelium than in the glandular epithelium. The lack of correlation between the reported epithelial cell estrogen receptor content (higher in glandular epithelium) and the epithelial cell hypertrophic response (higher in luminal epithelium) that we observed suggests the possibility of nonestrogen receptor mediation of epithelium hypertrophy. The requisite of high concentrations of either estrogens or antiestrogens to elicit nonestrogen receptor-mediated responses and, thus, nongenomic responses (37) is consistent with the high level of antiestrogen required to elicit hypertrophy in the glandular epithelium. However, it should be acknowledged that other explanations, such as stromal mediation of epithelial responses, remain tenable.

In conclusion, we have quantitated luminal and glandular epithelial cell hypertrophic responses to estrogens and antiestrogens possessing agonist activity and compared these specific responses to uterine weight gain. Tamoxifen, monohydroxytamoxifen, and clomiphene surprisingly show preferential activity in the glandular epithelium compared to estrogens. This relative specificity of these antiestrogens in the glandular epithelium may be related to the tumor inhibitory effect of tamoxifen in the breast and the uterine pathologies reported in women undergoing tamoxifen therapy. In addition, these data indicate that uterine epithelium hypertrophy may be of value in evaluating compounds with potential for use as antiestrogens, and that similar uterine epithelial responses might be elicited in women receiving tamoxifen therapeutically.

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