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The Relative Binding Affinity of Diethylstilbestrol to Uterine Nuclear Estrogen Receptor: Effect of Serum and Serum Albumin (42573)

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Abstract. The relative binding affinity (RBA) of diethylstilbestrol (DES) was determined in nuclear fractions of the rat uterus. DES displayed a two- to threefold greater affinity (RBA = 245 ± 36) than estradiol (RBA = 100) for nuclear E receptor. The RBA of DES to nuclear E receptor was lowered significantly in the presence of rat serum (43 ± 1) or human serum (52 ± 7). Dilution of human serum resulted in a progressive increase in the RBA of DES which approached that observed in the absence of serum. Addition of purified human serum albumin mimicked the decrease in RBA of DES that was observed with serum. The IC_{50} of estradiol was not changed in the presence of either rat serum or albumin. These data show that DES possesses a greater affinity for nuclear E receptor than estradiol and that serum albumin can modulate DES binding to uterine E receptor.

The synthetic nonsteroidal estrogen diethylstilbestrol (DES) has seen widespread use clinically (1) and argiculturally (2). Because estrogens have been implicated in cancers of the reproductive tract (3, 4), the known teratogenic and possible carcinogenic potential of DES may be related to its estrogenic potency (5, 6).

The potential estrogenic activity of a compound has been estimated by its relative binding affinity (RBA) to uterine cytosolic E receptor preparations (7). The RBA of DES for uterine cytosolic E receptor in various species has been shown to be greater than (7), less than (8), or equal to (9) that of estradiol (E_2). Because plasma proteins can also bind estrogens, these discrepancies may be attributable to serum contamination of cytosolic E receptor preparations during tissue homogenization. In addition, the interaction of hormones with serum proteins has been shown to affect biological response (10, 11). The present study was undertaken to determine the RBA of DES to nuclear estrogen receptor and examine the influence of serum and serum albumin on the RBA of DES.

Materials and Methods. Labeled steroid [2,4,6,7,-$^3H_4$] estradiol 17β (^{3}H-E_2; 110 Ci/mmole) was obtained from New England Nuclear Corp. (Boston, MA) and stored in ethanol (100 μCi/ml) at −20°C. Radioinert steroids and DES were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from standard commercial sources and were reagent grade or better.

Saline was buffered with 10 m*M* Tris–HCl (pH 7.4). Buffer A_{10} contained 50 m*M* Tris–HCl, 1 m*M* EDTA, 12 m*M* monothioglycerol, and 10% glycerol (v/v) (pH 7.5); buffer B contained 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, and 12 m*M* monothioglycerol (pH 7.5). Dextran-coated charcoal suspension (DCC) contained 0.5 g Norite A (Sigma) and 50 mg Dextran-T70 (Pharmacia Fine Chemicals, Piscataway, NJ) in a 100 ml of 10 m*M* Tris–HCl and 1 m*M* EDTA (pH 7.5). Scintillation counting solution was toluene–Triton X-100 (2:1, v/v) with 5 g diphenyloxazole (PPO) and 50 mg 1,4-*bis*(3-(5-phenyloxazolyl))benzene (POPOP) per liter.

Serum. Human female serum was obtained from women who were within their reproductive years and who were not taking oral contraceptives. Rat serum was obtained from adult female Sprague–Dawley rats. Endogenous steroids were removed from serum samples by incubation of serum with an equivalent volume of DCC for 30 min at 30°C and centrifugation as described below.

Nuclear receptor preparation. Randomly cycling adult female rats (Sprague–Dawley) housed under controlled conditions (14-hr light, 10-hr dark photoperiod) were killed by cervical dislocation. Uteri were removed rapidly, stripped of fat, and mesentery, slit lon-

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gitudinally, blotted, weighed, and placed in ice-cold buffered saline. All subsequent procedures were performed at 0–4°C unless otherwise noted.

Tissues were rinsed and homogenized (1/10, w/v) in buffer A_{10} with a Tekmar homogenizer (Tekmar Co., Cincinnati, OH) and the homogenate was centrifuged at 800*g* for 15 min. The nuclear fraction was washed twice by resuspension in the homogenization buffer with intervening centrifugations at 800*g* for 15 min. Nuclear E receptor was extracted from the washed pellet by resuspension in buffer A_{10} containing 0.5 *M* KCl. The nuclear suspension was incubated for 1 hr with mixing at 15-min intervals, and centrifuged at 170,000*g* for 1 hr to remove nuclear debris. The resultant supernatant was the nuclear E receptor extract used for competition assay.

All nuclear preparations were used fresh. The number of preparations used in this study is equivalent to the n values indicated in the text for each experiment. Each nuclear preparation consisted of pooled uterine nuclear extract from three to six animals.

Competition assay. The competitive binding activity of DES and of steroids for rat uterine nuclear E receptor was determined according to the method of Korenman (12). Nuclear E receptor (300 μl), prepared as described above, was incubated with ^{3}H-E_2 (0.75 n*M*, 100 μl) and different concentrations (0.04 n*M*–4 μ*M*, 100 μl) of unlabeled competitor with or without charcoal-stripped serum. Incubations were conducted at 0–4°C for various time periods or at 30°C for 1 hr (see Results). After incubation, samples were chilled where appropriate and unbound ligand was removed from each sample by incubation (10 min) with 500 μl DCC followed by centrifugation at 1500*g* for 4 min. Radioactivity was counted in the supernatant fractions at 33% efficiency. Total specific binding was determined by the difference between binding in the absence or presence of excess estradiol (4 μ*M*). The IC_{50} was the concentration of competitor required to decrease ^{3}H-E_2 binding by 50%. RBA was calculated as: IC_{50} (E_2)/IC_{50} (competitor) × 100.

Results. Competition assays, using nuclear E receptor, showed that DES is a more potent competitor than E_2, i.e., 2.5 times (RBA = 252 ± 8) that of E_2 (Fig. 1). The data shown in Fig. 1 are from competition assays conducted at 0–4°C for 20 hr. When the competition assays were conducted at 30°C for 1 hr or at 24, 36, or 48 hr at 0–4°C, no significant alteration in RBA of DES was observed (data not shown). Because uterine cytosolic preparations are susceptible to serum contamination, we next determined whether a serum component could account for the discrepancies in RBA of DES cited above (see introduction).

The RBA of DES for uterine E receptor de-

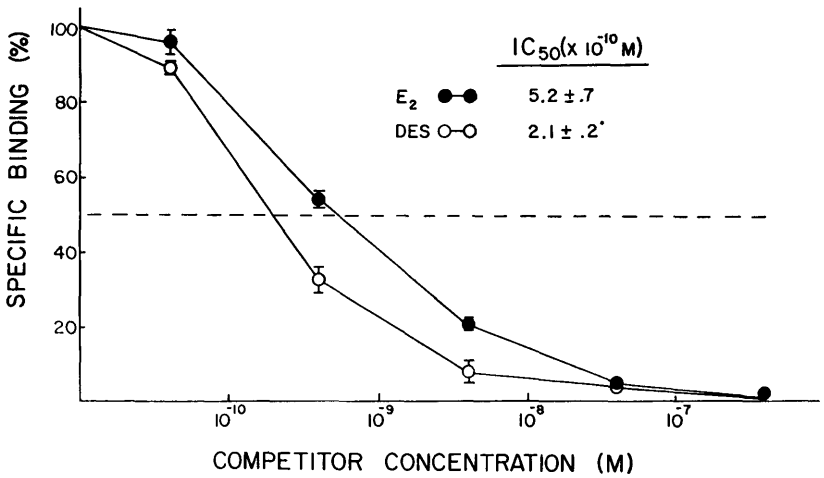

FIG. 1. Competition of ^{3}H-E_2 binding to rat uterine nuclear E receptor. Horizontal dashed line represents the 50% inhibition level of ^{3}H-E_2 binding. Each point represents the mean ± SEM (N = 4). The IC_{50} of DES was significantly different ($P < 0.05$, Students' t test) from E_2.

creased significantly in the presence of adult female rat serum (1/20, v/v, final) from 252 ± 8 in the absence of rat serum (Fig. 1) to 45 ± 7 in the presence of serum (Fig. 2A). In contrast to DES, the IC_{50} of E_2 was unaffected by the presence of rat serum (Fig. 1, Fig. 2A).

Human female serum contains sex-steroid-binding globulin (SHBG) which possesses a high binding affinity for testosterone and estradiol (13). In order to assess the effect of human female serum on nuclear E receptor binding, it was necessary to differentiate ^{3}H-E_2 bound to receptor from that bound to SHBG. The order of potency for displacement of ^{3}H-E_2 bound to human female serum is T > E_2 ⋙ DES. Thus, we used excess testosterone to block SHBG binding sites. We determined that ^{3}H-E_2 binding to SHBG was completely inhibited in the presence of excess testosterone (4×10^{-6} *M*). Also, we showed that this concentration of testosterone did not displace ^{3}H-E_2 bound to rat uterine nuclear E receptor, nor did it alter the RBA of DES in the absence of serum (data not shown). Testosterone-supplemented human serum was used in all subsequent experiments.

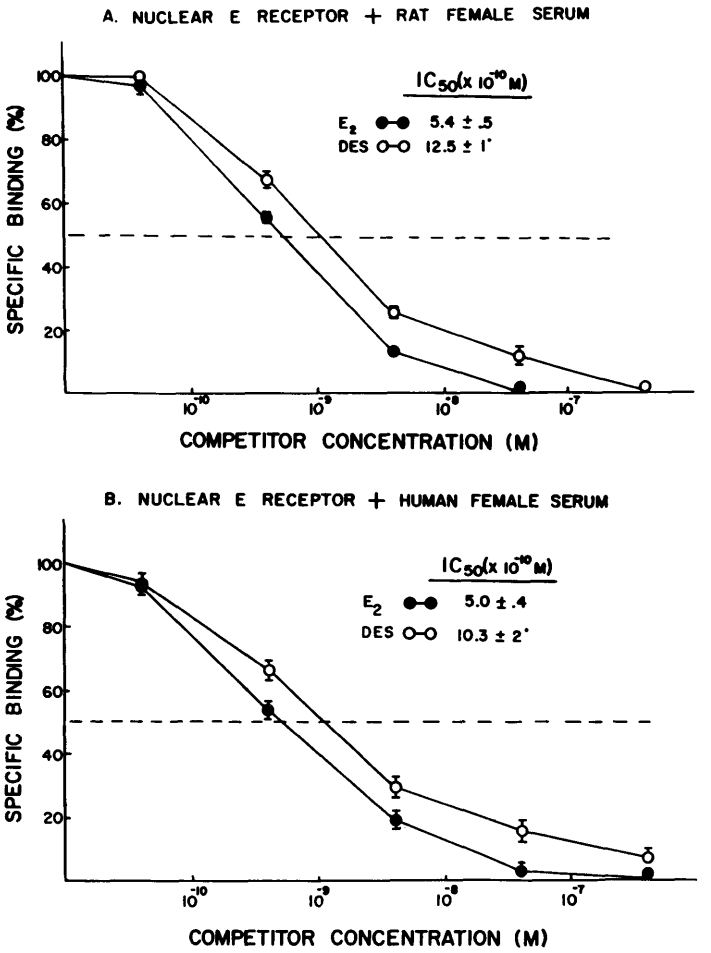

FIG. 2. Competition profile of ^{3}H-E_2 binding to nuclear E receptor by E_2 and DES in the presence of rat female serum (A) or human female serum (B). Horizontal dashed line represents the 50% inhibition level of ^{3}H-E_2 binding. Each point represents the mean ± SEM ($N = 4$). The IC_{50} of DES was significantly different ($P < 0.05$, Students' *t* test) from E_2 in (A) and (B). SHBG binding in human serum was blocked with testosterone (4×10^{-6} *M*).

The RBA of DES for uterine nuclear E receptor decreased from 245 ± 36 in the absence of human serum to 52 ± 7 in the presence of serum (1/20, v/v, final) (Fig. 2B). Furthermore dilution of human female serum resulted in a progressive reduction in the IC_{50} of DES for uterine nuclear E receptor (Fig. 3). At a serum dilution of 1/40 to 1/80, the IC_{50} of DES was almost indistinguishable from that observed in the absence of serum. Human serum did not alter the IC_{50} of E_2.

We next determined if purified human serum albumin could mimic the effect of human serum on the RBA of DES. Equivalent protein concentrations of purified human serum albumin and human serum were examined for their effect on the RBA of DES. Although the addition of either purified serum albumin or human serum did not affect the IC_{50} of estradiol (Fig. 4a), the RBA of DES was decreased markedly in the presence of purified serum albumin to a level similar to that observed in the presence of human female serum (Fig. 4b).

Discussion. The relative strength of binding of a compound to the E receptor has been a useful means by which to estimate its estrogenic potency. This study has shown that the RBA of DES for nuclear E receptor is two to three times greater than that of E_2.

It has been generally appreciated that a variety of factors, including plasma protein binding, can modulate the dynamics of interaction between hormone and receptor which ultimately can affect the biological response. This study has shown that the RBA of DES to nuclear E receptor was altered significantly in the presence of rat or human female serum. Furthermore, dilution of human serum was shown to result in a progressive increase in RBA of DES for nuclear E receptor which approached that observed in the absence of serum, whereas the RBA of E_2 for nuclear receptor was unaffected by serum.

Serum albumin appears to be responsible for the alteration in the RBA of DES for nuclear E receptor. In the presence of purified human serum albumin, the RBA of DES to uterine nuclear E receptor paralleled that observed in the presence of human serum. The addition of albumin markedly lowered the IC_{50} of DES but did not alter the IC_{50} of E_2. Sheehan and Young (14) showed a 10- to 20-fold greater binding affinity of DES to bovine serum albumin compared to E_2. Thus, the effect of albumin on the IC_{50} of DES, but not E_2, is most likely explained by a greater binding affinity of albumin for DES which results in a decrease in the free concentration of DES available for binding to uterine E receptor.

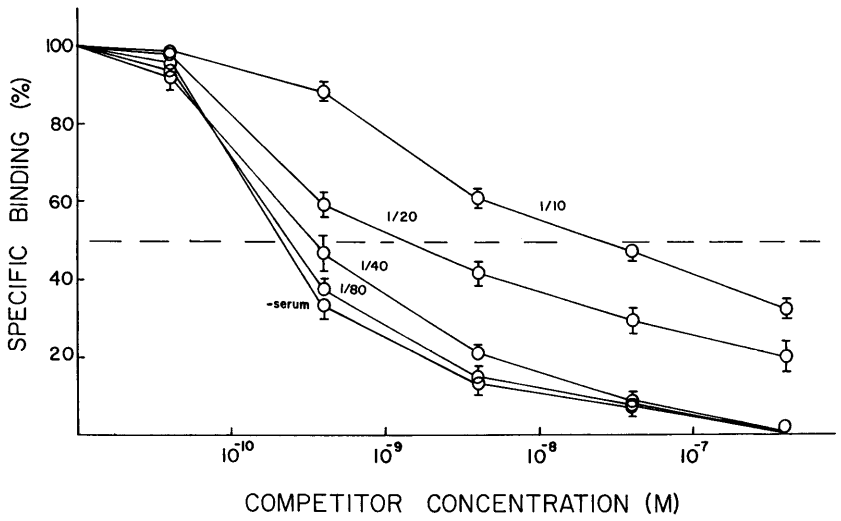

FIG. 3. Effect of dilution of human serum on the IC_{50} of DES for nuclear E receptor. Competition of ^3H-E_2 binding to nuclear E receptor by DES was determined in the presence of the indicated dilutions of human female serum (v/v, final). Horizontal dashed line represents the 50% inhibition of binding. Each point is the mean ± SEM (N = 4). SHBG binding in human serum was blocked with testosterone (4×10^{-6} M).

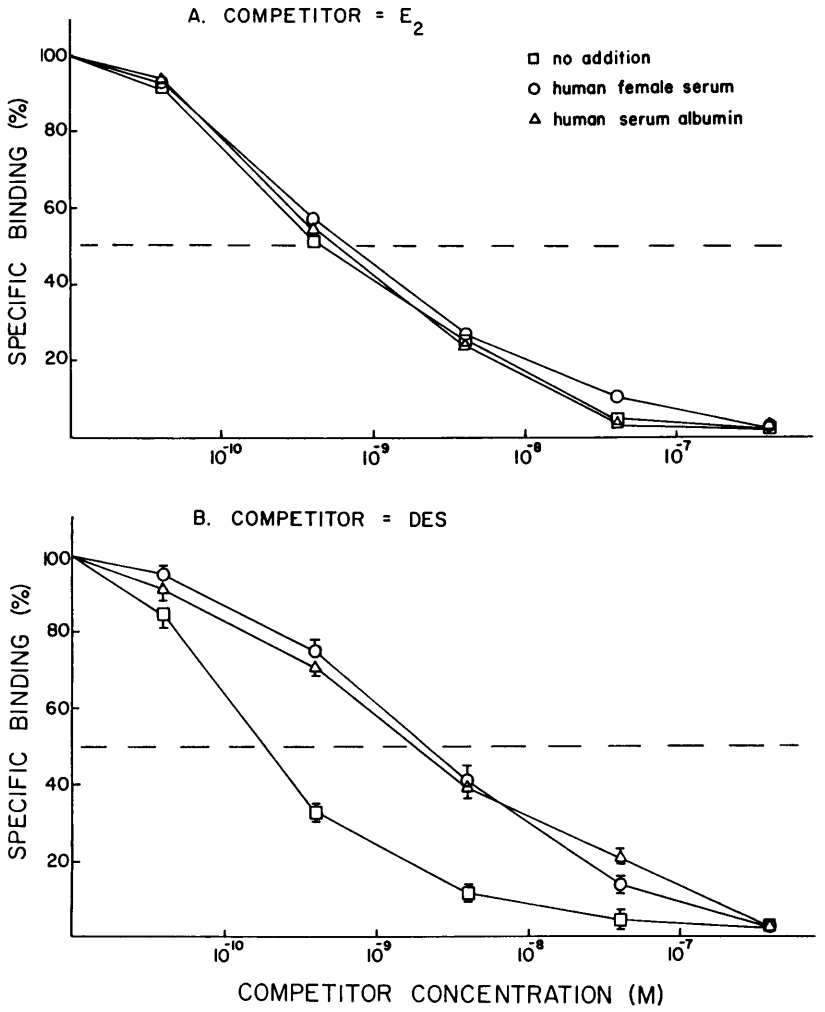

FIG. 4. Competition of ^{3}H-E_2 binding to nuclear E receptor by E_2 (A) and DES (B) in the presence of purified human serum albumin or human serum. Horizontal dashed line represents the 50% inhibition of ^{3}H-E_2 binding. Each point is the mean ± SEM (N = 4). The SEM in (A) is not shown for clarity of the figure. SHBG binding in human serum was blocked with testosterone (4×10^{-6} M).

A recent report by Steingold *et al.* (15) has shown that extraction of DES by the rat uterus or liver was unaffected by the presence of serum or bovine serum albumin (BSA), whereas the extraction of DES by the brain was reduced significantly in the presence of serum or BSA. These results suggest that differences in the local microcirculation between organs may affect the delivery or dissociation of DES from albumin. These experiments were performed in ovariectomized rats following a 15- or 18-sec perfusion. Estrogens are known to cause uterine hyperemia, increased capillary permeability, and local albumin accumulation. These changes in the uterine microcirculation in concert with DES–albumin interactions could conceivably alter the duration of exposure of proximal target cells to DES. The present study has focused only on *in vitro* interactions of DES, albumin, and the nuclear E receptor. Further studies are necessary to determine the biological significance of these interactions.

Varying levels of serum contamination may explain why the RBA of DES for uterine cytosolic E receptor has been reported to range

from less than to greater than that of E_2 (7–9). This conclusion is supported by a previous report that has shown uterine cytosol from immature rats to contain a 50-fold higher albumin concentration than nuclear preparations (16). The potential serum contamination of cytosolic preparations suggests that RBA estimates may be best determined with nuclear E receptor, as described in the present study. Alternatively, the nuclear exchange assay of Medlock *et al.* (17) may also be used to avoid serum contamination.

Plama proteins have been shown to affect the distribution, availability, and metabolism of steroid hormones (18, 19). Thus, plasma proteins can serve as important modulators of target cell response to the endogenous secretion or exogenous administration of a hormone. The results of this study, together with previous reports (14, 20), support a role for serum albumin as a modulator of DES action. This study has shown that DES–albumin interactions, coupled with the high binding affinity of DES for the nuclear E receptor, could be important contributing factors in the etiology of DES-induced reproductive tract abnormalities. Further study will be required to determine the biological significance of the high affinity of DES for uterine nuclear E receptor and the role of serum and serum albumin in the modulation of DES action.

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