SPECIFIC ESTROGEN-RECEPTORS IN THE NEOPLASTIC AND LACTATING MAMMARY GLAND OF THE RAT*

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SUMMARY

Specific receptors of ^{3}H-estradiol-17β were demonstrated by a sucrose gradient assay of cytosols from the lactating mammary gland and the R3230AC mammary adenocarcinoma of the rat. These estrogen receptors, each of which sedimented at approximately 8S, exhibited similar dissociation constants ($K_d \sim 1 \times 10^{-9}$M) and steroid binding specificities. Ovariectomy of the host had no apparent effect on the number of binding sites/mg protein in the R3230AC tumor.

Several laboratories, including our own, have shown that certain experimental mammary tumors in rodents (1-8) and primary carcinomas of the human breast (9-12) possess specific estrogen-binding capacity. The ability of an estrogen-responsive tissue (e.g., uterus, vagina or anterior pituitary) to take up and retain ^{3}H-estradiol-17β can be attributed to the presence of specific macromolecules termed receptors (11, 13, 14).

The R3230AC tumor, a transplantable rodent mammary adenocarcinoma, has been classified as an estrogen-responsive neoplasm by Hilf (15). This paper describes several of the biochemical characteristics of the specific estrogen receptors identified in this breast neoplasm as well as those of the receptors found in the normal mammary gland.

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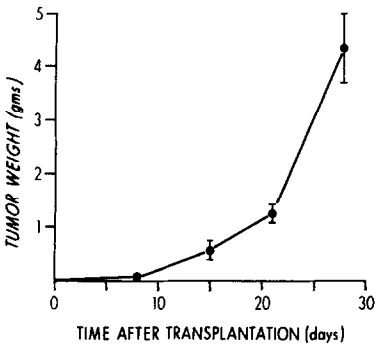

Fig. 1.

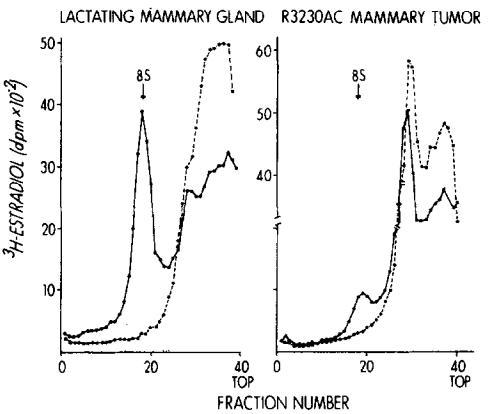

Fig. 2.

Figure 1. Growth of the R3230AC mammary tumor in the female Fischer rat. Points represent Mean ± S.E.M.

Figure 2. Isotopic profiles following sucrose gradient centrifugation of cytosols from the lactating mammary gland and the R3230AC tumor incubated in vitro with 2×10^{-9}M ^{3}H-estradiol-17β (solid line). The dotted line represents reactions preincubated 10 minutes with 20 μM CN-55,945-27. The 8S refers to the location of the estrogen receptor from uterine cytosol separated under comparable conditions.

MATERIALS AND METHODS

2,4,6,7-^{3}H-Estradiol-17β (95-110 Ci/mmol) and scintillation fluor (Omnifluor^{R}) were purchased from New England Nuclear Corporation. Radiochemical purity was checked by thin-layer chromatography. The antiuterotropic agent, CN-55,945-27, was provided through the courtesy of Dr. J. Reel of Parke-Davis and Co., Ann Arbor, Michigan.

Female Fischer 344 rats, 80-90 gm, were implanted with R3230AC tumor using a sterile trocar technique and then sacrificed by cervical dislocation at various times after transplantation. Experiments were performed on tumors removed during the period of exponential growth, usually at 20-25 days (Figure 1). After removal of the tumor, the tissue was weighed and homogenized in Duall homogenizers (Kontes Glass Co.), using an appropriate volume of cold 10 mM Tris·HCl, pH 7.4, contain-

ing 1.5 mM EDTA as diluent. The homogenate was centrifuged at 105,000 x g (0° C) for 30 minutes and the supernatant (cytosol) was removed without disturbing the upper lipid layer. Cytosols of the lactating mammary gland, 14 days post-partum, were prepared in a similar fashion.

A sucrose gradient procedure similar to that previously described (11,12) was used for measurement of specific estrogen-binding capacity of the receptor in the cytoplasm. Aliquots (200 µl) of cytosol were incubated with 50 µl of the Tris·EDTA buffer alone, or with 50 µl of buffer containing the antiuterotropic substance, CN-55,945-27, at 2×10^{-5}M for 10 minutes at 0°C. After incubation, ^{3}H estradiol-17β was added to a final concentration of 2-3 nM. A 200 µl portion of each reaction mixture was layered immediately onto a cold, linear gradient of sucrose (5-40%) also containing Tris·EDTA buffer, pH 7.4. The gradients were centrifuged for 12-15 hours (0°C) at 308,000 x g using the Spinco SW-56 rotor in a Beckman L2-65B ultracentrifuge. Specific binding was estimated as the difference in radioactivity bound in the 8 Svedberg (S) region of the gradient in the presence or absence of the inhibitor, CN-55,945-27. Specific estrogen-binding capacity is expressed as fmol (10^{-15} mol) estradiol-17β bound per mg cytosol protein. Protein was determined using the method of Lowry, et al. (16).

RESULTS AND DISCUSSION

Although other investigators (17-20) have shown that normal breast can bind ^{3}H-estradiol following injection *in vivo* or incubation *in vitro*, the presence of specific estrogen-receptor complexes has not been conclusively demonstrated. One reason for the apparent difficulty is that the quiescent breast is composed primarily of adipose cells and connective tissue. We have circumvented this problem by studying the mammary gland during lactation, a stage at which the gland is epithelial in nature and is under profound hormonal stimulation.

The presence of estradiol-binding macromolecules was demonstrated in the cytosols of the lactating mammary gland and the R3230AC tumor using a cell-free system (Figure 2). Specific estrogen-binding substances, sedimenting at approxi-

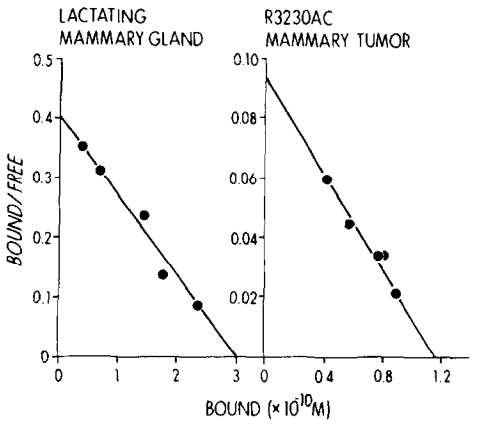

Figure 3. Scatchard analysis of titration results using the sucrose gradient procedure to separate the 8S estradiol-receptor complex.

mately 8S, did not bind ^{3}H-estradiol-17β when preincubated with CN-55,945-27 (Figure 2). However, non-specific binding, as indicated by a lack of inhibition by CN-55,945-27, was observed in the 4-5S region of the gradient. Binding in thi region has been reported by others (5,8,11,12,14) and may be attributed to contamination by plasma protein, e.g., albumin.

Titration of a constant volume of cytosol from either the tumor or mammary gland with increasing concentrations of ^{3}H-estradiol-17β (usually 0.5-3.0 nM) indicated that each of the 8S receptors was saturated between 1-2 nM. The dissociation constant (K_d) of the steroid-receptor complex was approximately 8×10^{-10} M for the lactating mammary gland and 12×10^{-10} M for that of the R3230AC tumor as estimated (Figure 3) according to the method of Scatchard (21). The number of binding sites/mg cytosol protein was calculated as 20 fmol for the mammary gland and 6 fmol for the tumor (Figure 3). The binding capacity of the receptor in the lactating gland ranged from 15-50 fmol/mg protein while that of the tumor ranged from 2-16 fmol/mg.

Preliminary measurements of the binding specificities of the receptors were made from competition studies employing several steroid hormones. Each of the unlabeled competitive substances, at the concentration designated in Table I,

TABLE I: INHIBITION OF ^{3}H-ESTRADIOL-BINDING TO CYTOPLASMIC RECEPTORS

COMPETITIVE SUBSTANCE	CONCENTRATION ($\times 10^{-6}$ M)	^{3}H-ESTRADIOL-17β BOUND (%)*	
		R3230AC TUMOR	LACTATING MAMMARY GLAND
NONE	0	100	100
CN-55,945-27(Parke-Davis)	10	31	10
ESTRADIOL-17β	1	17	9
HYDROCORTISONE	1	100	91
PROGESTERONE	1	100	100
TESTOSTERONE	1	-	100
DIHYDROTESTOSTERONE	1	88	-

*Conditions of incubation in text. Binding of ^{3}H-estradiol-17β (2-3 nM) to receptor preincubated with competitive substance expressed as percent of control value.

TABLE II: ^{3}H-ESTRADIOL-BINDING CAPACITY OF CYTOPLASMIC RECEPTORS FROM THE R3230AC TUMOR*

HOST (No.)	TUMOR WT. (gm)	ESTROGEN-BINDING CAPACITY		
		fmol/mg PROTEIN	fmol/mg TUMOR	TOTAL fmol/TUMOR
INTACT (11)	$1.7 \pm 0.4^{+}$	5.4 ± 1.3	0.21 ± 0.03	431 ± 142
OVEX (9)	1.2 ± 0.2	5.6 ± 1.4	0.20 ± 0.02	260 ± 75

* All tumors were assayed 20-22 days after transplantation.

+ Mean ± standard error.

was preincubated with the cytosol preparation for 10 minutes at 0°-3° C. ^{3}H-Estradiol-17β was added at a concentration of 2-3 nM and the binding capacity measured using the sucrose gradient assay. Binding of ^{3}H-estradiol-17β to specific receptor sites was significantly inhibited by CN-55,945-27 at a concentration of 10 μM or by 1 μM unlabeled estradiol-17β (Table I). Progesterone, hydrocortisone, testosterone or dihydrotestosterone, at a concentration of 1 μM, did not significantly inhibit binding of estradiol-17β by cytosols from tumor or mammary gland (Table I). These data indicate a high degree of hormonal specificity of the ligand binding.

To determine if endogenous estrogen production has an influence on the specific estrogen-binding capacity of the neoplasm, tumors were implanted in intact or ovariectomized rats (5-7 days after ablation). Twenty-two days after implantation, tumors were removed and the estrogen-binding capacity was measured. The data in Table II indicate that there was no significant effect of the endogenous estrogen levels in the host on the estrogen-binding capacity of the receptor in the tumor. Tumor growth was similar in intact or ovariectomized hosts (Student t-test). It is known that the response of the R3230AC tumor to estrogen results in alterations in the levels of certain enzymes; however, the tumor is not dependent upon estrogen for growth (15).

Cytoplasmic macromolecules, which bind ^{3}H-estradiol-17β specifically and with high affinity, have been reported in a number of normal and neoplastic tissues (1-14, 17-20). Although the binding characteristics of these receptors are similar, the binding capacity, i.e., number of binding sites/unit weight, varies over a wide range. It has been suggested recently (22) that the relative hormonal autonomy of the R3230AC tumor may be due to a paucity of specific estradiol-receptor proteins in the cytosol fraction. However, this conclusion appears unlikely in view of the data reported here, which show that the R3230AC tumor contains significant quantities of these receptors. In several tumors we have observed binding capacities which approach those of the lactating mammary gland. Perhaps the lower level of estrogen-binding capacity in the R3230AC

tumor, an estrogen responsive neoplasm, is a reflection of a heterogeneous cell population in which a variable number of cells demonstrates estrogen sensitivity or a more homogeneous cell population in which individual cells exhibit variable levels of estrogen-binding capacity. Experiments to determine the cellular distribution of ^{3}H-estradiol binding are in progress.

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