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Estrogen Binding by Neoplastic Human Thymus Cytosol*

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Abstract—*A binding activity specific for estrogens has been identified in the cytosol of human thymoma. The binder is resolved into 4–5S and 8–9S components by density gradient centrifugation. The analysis of the binding data according to Scatchard revealed the presence of two binding components, one with high affinity and the other with low affinity. The receptor is distinct from the cytoplasmic glucocorticoid receptor on the basis of binding specificity. Specific estrogen binding could be demonstrated in two out of four thymoma cytosols.*

INTRODUCTION

THE ANTAGONISTIC endocrine relationship between the thymus and the gonad was discovered early by Hammar [1], who observed hypertrophy of the thymus following gonadectomy. The studies of Evan and Simpson [2], Korenchevsky *et al.* [3] and Golding and Ramirez [4] are of historical as well as actual interest with regard to the relation of sex steroids to thymus. More recently, Sobhon and Jirasattham [5] reported that testosterone, estrogen and a combined dose of progesterone–estrogen caused a marked decrease in relative weight of the thymus in ovariectomized rats. Progesterone by itself showed little thymic suppressive effect only when given at high dose levels. The relative and absolute number of circulating lymphocytes were also decreased by treatment with testosterone and estrogen, while the number of circulating neutrophils increased. Histologically, testosterone and estradiol caused a preferential depletion and destruction of lymphocytes in the thymic cortex. Since these suppressive effects were reduced but still significantly present after removal of adrenal glands, the authors concluded that estrogen may have a dual action i.e., directly on the thymus and indirectly through its influence on the adrenal gland.

It is well known that cytoplasm of estrogen-responsive tissues contains a specific estrogen receptor protein [6]. The interaction of the hormone with the receptor is thought to be essential for the development of tissue response to hormonal stimulation. By analogy, it was postulated that some of the observed estrogen effects on the thymus could be mediated by similar estrogen-binding proteins. This paper presents preliminary characterization of a receptor macromolecule(s) for 17β-estradiol found in two out of four cases of human thymoma. The receptor is distinct from that which binds glucocorticoids also present in the cytosol of tymoma cells.

MATERIALS AND METHODS

Reagents and chemicals

17β-(^{3}H)estradiol (56 Ci/mmole) and (^{3}H)triamcinolone acetonide ¶ (27 Ci/mmole)

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¶The abbreviations and trivial names used were: triamcinolone acetonide, pregna-1,4-diene-9α-fluoro-11β, 16α, 17α,21-tetranydroxy-3,20-dione-16,17-acetonide; dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; ethinylestradiol, 1,3,5(10)-estratrien-17α-ethinyl-3,17β-diol; TETS buffer, 0.25M sucrose 0.01M thioglycerol: 0.01M Tris-HCl: 0.0015M EDTA, pH 7.4: DES,diethylstilboestrol; DCC dextran coated charcoal.

were obtained from the Radiochemical Centre, Amersham, U.K.; a radiopurity of at least 95% was confirmed by thin-layer chromatography in benzene: ethyl acetate (3 : 2) (for 17β-estradiol) and in methylene chloride: acetone (70 : 30) (for triamcinolone acetonide). Non-radioactive dexamethasone was a gift of Merck Chemical Co., Darmstadt, Germany. All other steroids, charcoal (Norit A) and Trizma base, were obtained from Sigma Chemical Co., St. Louis, Mo. U.S.A.; Silica gel F-254 plates, 20 × 20 cm, were purchased from Merck Chemical Co., Darmstadt, Germany; Dextran T-70 was purchased from Pharmacia, Uppsala, Sweden; scintillation fluor (Instagel) was purchased from Packard Instruments International S.A., Zurich, Switzerland. All other chemicals were reagent grade.

Tumour specimen

Four histolically confirmed thymoma specimens were obtained from Catholic University Hospital at the time of operation and promptly stored at −20°C until used. The stability of the receptor was not affected by freezing up to a period of 4 weeks.

Preparation of cytosol

Cytosol fraction from the tumour was prepared in TETS buffer (4 ml buffer/g tissue) as previously described [7]. Protein was determined by the method of Lowry *et al.* [8].

Sucrose density gradients

Cytosol was incubated for 5 hr at 2°C with 17β-(^{3}H)-estradiol alone (6×10^{-9}M) or in combination with a 100-fold molar excess of various unlabeled steroids. Aliquots of 0.25 ml were applied on the top of linear 10–30% sucrose gradient in TETS buffer. Gradients were centrifuged at 48,000 rev/min for 16 hr at 2°C in a Beckman L5-65 ultracentrifuge with a Spinco SW 50.1 rotor. Fractions (6 drops) were collected from the top of the tubes by injection of concentrate sucrose at the bottom of the gradients. Radioactivity was measured after addition of 8 ml Instagel. The counting efficiency for tritium was 40%. ^{14}C-labeled proteins ovalbumin and human γ-globulin were run simultaneously to calculate sedimentation coefficients.

DCC assay

Aliquots (0.1 ml) of cytosol were incubated with 17β-(^{3}H)estradiol (2×10^{-10}–2×10^{-8}M) alone or in the presence of a 100-fold molar excess of DES in a final volume of 0.25 ml. At the end of the incubation, 0.25 ml of TETS buffer containing 1% Norit A and 0.1% dextran was added and incubated for an additional 10 min at 2°C. Following centrifugation at 3000 rev/min for 5 min, 0.25 ml supernatant was removed and counted in 6 ml Instagel. For determination of the concentration and apparent equilibrium dissociation constant (K_D) of receptor sites, specific binding data were analyzed by the Scatchard equation [9] using an Olivetti P 6060 desk top computer. The curvelinear plot was resolved into linear components according to Rosenthal [10]. The intra-assay reproducibility was approximately 5% and the interassay reproductibility in experiments done on the same specimen never exceeded 10%.

Thin-layer chromatography

Following ether extraction of the tissue cytosol previously incubated with 17β-(^{3}H)estradiol and treated with DCC to adsorb free steroid, the solvent was evaporated under nitrogen and the residue was dissolved in benzene: ethanol (9 : 1) for application on silica gel plates. Authentic 17β-estradiol was added to the dissolved residue. The chromatogram was developed with benzene: ethyl acetate (3 : 2), and the derivatives were visualized by spraying the plates with sulfuric acid: potassium bichromate mixture.

RESULTS

The sucrose density gradient centrifugation pattern of human thymoma cytosol incubated with 17β-(^{3}H)estradiol indicated the presence of a peak of bound radioactivity in the region 8–9S of the gradient with a reproducible 4–5S shoulder (Fig. 1). In the presence of 100-fold excess of unlabelled ethinylestradiol or DES, there was a complete loss of the 8–9S peak and some reduction in the 4–5S shoulder. The addition of either testosterone or progesterone was without effect on both the 8–9S and the 4–5S components (Fig. 1).

In order to evaluate further the nature of these estrogen-binding components, the binding affinity was evaluated by equilibrium binding. An example of the Scatchard relationship observed between the ratio of bound to free steroid and the concentration of bound steroid is illustrated in Fig. 2. The plot revealed a curvilinear relationship indicating,

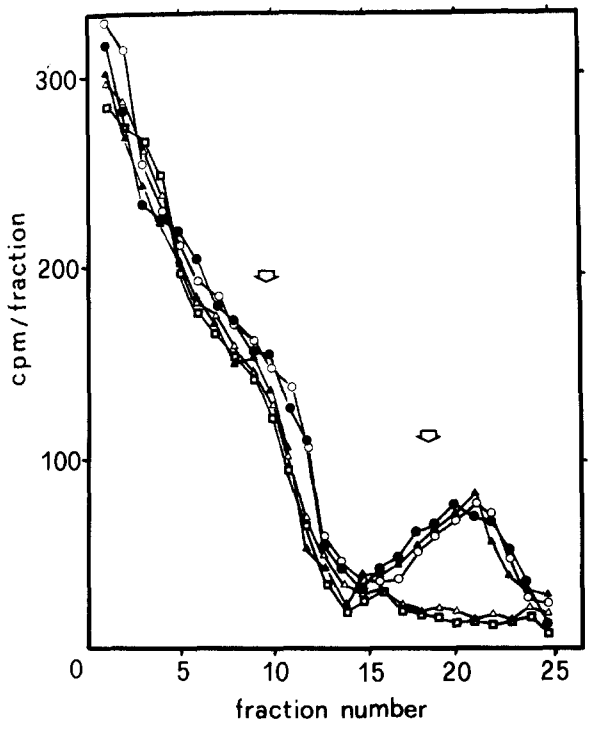

Fig. 1. Sucrose density gradient centrifugation of 17β-(^{3}H)estradiol-labeled cytosol from human thymoma. Cytosol was incubated for 5 hr *at* 2°C *with* 6×10^{-9} M 17β-(^{3}H) *estradiol alone* (○) *or in presence of a* 100*-fold molar excess of nonradioactive DES* (□), *ethinylestradiol* (△), *testosterone* (●) *or progesterone* (▲). *At the end of the incubation the samples were treated with DCC and* 0.2 ml *aliquots layered on* 10–30% *linear sucrose gradient in TETS buffer and centrifuged for* 16 hr *at* 48,000 rev/min *in a Beckman SW* 50.1 *rotor.* ^{14}C*-labeled ovalbumin* (3.6S) *and* ^{14}C*-labeled human γ-globulin* (7.1S) *were included as marker proteins* (*arrows*).

as did the results of the density gradient study, that cytosol contained more than one estrogen binding component. The curve was resolved into linear components by the method of Rosenthal [10]. The first component (K_1) had an apparent dissociation constant (K_D) of 0.79×10^{-9}M and a binding capacity of 8.7 fmole/mg of cytosol protein; the second component (K_2) was characterized by a K_D of 41.5×10^{-9}M and had a binding capacity of 99.6 fmole/mg of cytosol protein. The number of low and high affinity binding sites and the relative K_D measured in four thymomas are given in Table 1.

The identity of the receptor-bound steroid was investigated by thin-layer chromatography as described in the Materials and Methods section. Following development, the chromatograms were cut into 1.0 cm/segments and counted in 10 ml of Instagel. The distribution of radioactivity on the chromatograms indicated that more than 80% of the total bound hormone co-migrated with 17β-estradiol. These data suggest that 17β-estradiol, and not some thymic metabolite, was the ligand observed in the binding reactions.

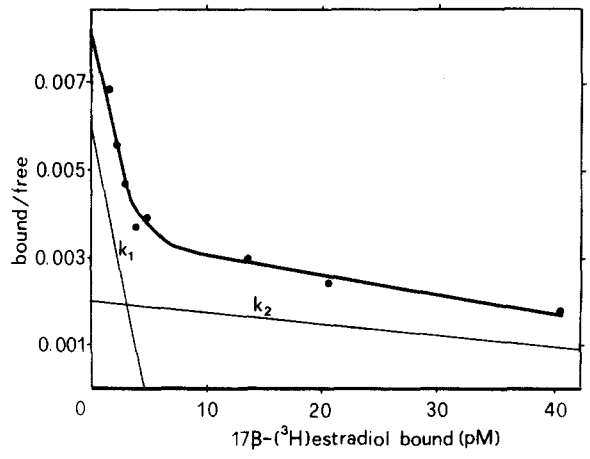

Fig. 2. Scatchard plot of binding data for 17β-(^{3}H) *estradiol by human thymoma cytosol. Aliquots* (0.1 ml) *of the cytosol were incubated for* 18 hr *at* 2°C *with increasing concentrations* (2×10^{-10}–2×10^{-8}M) *of radioactive* 17β*-estradiol alone or in the presence of a* 100*-fold molar excess of non-radioactive DES. Specific binding was determined after exposure to DCC followed by centrifugation and counting of the supernatant as described in Materials and Methods.*

Table 1. Binding capacity and binding affinity of estrogen receptor from cytosol of human thymomas

Tumour	Binding sites (fmole/mg protein) high affinity (K_1)	low affinity (K_2)	K_D ($M \times 10^{-9}$) high affinity (K_1)	low affinity (K_2)	Histology
T_1	8.7	99.6	0.79	41.5	Lymphoepithelial thymoma
T_2	5.0	52.0	0.74	35.0	Epithelial thymoma (spindle cells)
T_3	—*	—	—	—	Epithelial thymoma (round–oval cells)
T_4	—	—	—	—	Lymphoepithelial thymoma

*Indicates the absence of specific binding.

It has previously been demonstrated that cytosol from hyperplastic human thymus contains specific glucocorticoid receptors with sedimentation coefficients similar to those described here for 17β-estradiol [7]. Glucocorticoid receptors have also been found in five cases of human thymoma.* Consequently, it was investigated whether the two binding sites could belong to the same macromolecule. We reasoned that, if this is the case, the binding of radioactive glucocorticoid to glucocorticoid receptors would be displaced by the addition of non-radioactive 17β-estradiol. In the experiment reported in Fig. 3, cytosol was incubated with (^{3}H)triamcinolone acetonide in the presence or absence of various unlabeled steroids including 17β-estradiol. The binding capacity of control incubation was 217 fmole/mg protein. In the presence of a 10-fold molar excess of dexamethasone or cortisol the binding of (^{3}H)triamcinolone acetonide was reduced to 45 and 80% of the control value, respectively. In the presence of a 1000-fold molar excess of 17β-estradiol or testosterone, the binding was almost the same as in the control. These results indicate that the estrogen and glucocorticoid binding sites are associated with two distinct (and non-interacting) binding sites.

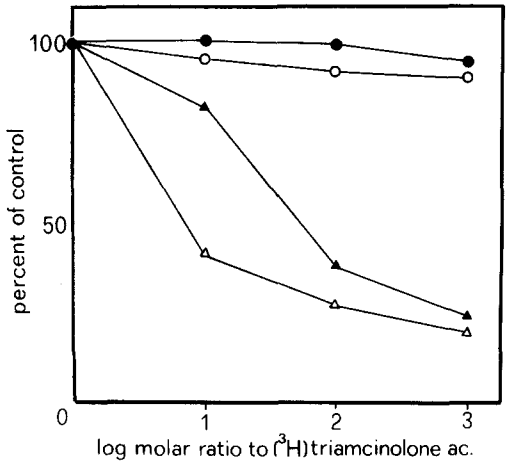

Fig. 3. Competition for glucocorticoid receptor in human thymoma cytosol. Aliquots (0.1 ml) *of the cytosol were incubated for* 18 hr *at* 2°C *with* (^{3}H) *triamcinolone acetonide* (2×10^{-8}M) *alone or in the presence of unlabeled competitors. Total specific binding in the absence of unradioactive competitor was taken as* 100% *and averaged* 217 ± 15 fmole/mg *protein.* (●) *testosterone;* (○) *17β-estradiol;* (▲) *cortisol;* (△) *dexamethasone.*

DISCUSSION

The data presented demonstrate, for the first time, that cytoplasm of two out of four human thymomas contains receptors that bind 17β-(^{3}H)estradiol specifically and with high affinity. The affinity constant and sedimentation coefficients of these binding components are similar to those described for estrogen receptors in other tissues. Moreover the receptor is distinct from that which binds glucocorticoids, as already observed for glucocorticoid and estrogen receptors in rabbit uterus [11] and in mouse mammary tumours [12]. The presence of an estrogen receptor in the thymus of the rat has recently been reported [13]. The physicochemical properties of the binder, including binding capacity, steroid specificity and sedimentation characteristics are quite similar to those reported here.

Histologically, human thymoma consists of a mixture of lymphoid and epithelial cells, often with marked variations of these components form one tissue area to another. As mentioned in the introduction, it appears that gonadal steroids exert their suppressive action mainly on the lymphocytes of the thymic cortex and that the population of epithelial cells is less affected. However, studies by Friedman *et al.* [14] have demonstrated that implantation of intrathymic pellets of 17β-estradiol in rats led to lymphatic involution accompanied by marked epithelial squamous or glandular hyperplasia. Thus it is possible that both lymphoid and epithelial cell populations are modulated in some manner by estrogens in their physiological activity.

In this respect, it should be noted that of the two receptor-positive tumours, one consisted of an admixture of lymphoid and epithelial cells, while the other was composed predominantly of epithelial cells (Table 1). This lack of correlation between estrogen receptor activity and cell type composition of the tumour suggests that the binding protein may be present in lymphoid and/or epithelial cell populations.

We do not know at present if the presence of estrogen receptors in thymoma is related to the neoplastic state of thymic cell such as rate of growth or biochemical differentiation, as there is not yet information about the occurrence of these receptors in normal human gland. In the case of steroid-sensitive diseases, such as breast cancer, it has been shown that tumours with higher mitotic activity and thymidine labeling indices tend to be estrogen receptor negative [15]. Moreover, the estimation of estrogen receptor content in the tumour has been found useful not only in predicting clinical response to hormonal therapy, but also in predicting the natural his-

*Manuscript in preparation.

tory of the disease regardless of treatment [16, 17]. It is certainly possible that the presence of estrogen receptors in thymoma cells may simply be a biochemical marker of cellular differentiation regardless of histology of the tumour. More extensive investigation on the presence of estrogen receptor in a larger number of histologically different thymomas as well as in normal thymus in correlation with the stage of glandular differentiation is requested to confirm this possibility.

The mechanism by which the action of estrogens on normal thymus takes place is unknown, but the participation of an estradiol-receptor complex in a direct action of the hormone on the gland is an intriguing possibility. Since recent data [18] already suggested that physiological levels of estrogens induce enzyme systems in cultured human lymphocytes, it would be of interest to investigate the role of this estrogen binding component in normal as well as in neoplastic thymic cell populations.

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