A HIGH-AFFINITY, LOW-CAPACITY RECEPTOR FOR ESTRADIOL IN NORMAL AND ANEMIC MOUSE SPLEEN CYTOSOLS[1]

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SUMMARY: A high-affinity, low-capacity receptor activity for 17 β-estradiol has been identified in cytosols of mouse spleen, normal or erythropoietic. It appears to be a protein which sediments at 5S in high-salt gradients and with a peak at 4S and a shoulder at 8-10S in low-salt gradients. In addition to 17 β-estradiol, only 17 α-estradiol and 5(10)-estren-3 α, 17 β-diol competed successfully with the steroid ligand. A K_d of 1.5 x 10^{-9} M and 4.1 fmoles/mg protein for the number of binding sites were calculated for the receptor activity of early erythropoietic spleen cytosols.

Estrogens have been reported to display a paradoxical effect on hematopoiesis in mice. Pharmacologic doses led to a decrease of the hematopoietic stem cell populations of bone marrow and spleen, with a consequent leukopenia and thrombocytopenia (1, 2). However, anemia did not occur: bone marrow erythropoiesis was markedly depressed, but splenic erythropoiesis increased sufficiently to maintain normal circulating erythrocyte levels. Although plasma erythropoietin levels of the estrogen-treated animals were elevated, the authors concluded that this change was not responsible for the rise in splenic erythropoiesis, which also occurred at decreased erythropoietin levels, i.e., polycythemia.

While these and many other reports describe steroid modulation of erythropoiesis, information is sparse on the interaction of steroids with erythroid precursor cells. Minguell and Grant studied in vivo uptake of testosterone by rat bone marrow cells and found little, if any, metabolic alteration of the hormone following cell accumulation (3). Subsequently a

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nuclear protein was extracted from rat marrow cells which specifically bound testosterone and, by inference from competition experiments, 5 α- and 5 β-dihydrotestosterone (4). To date, however, a comparable receptor in the cytoplasm of rat marrow cells has not been identified, possibly due to the paucity of early erythroid precursors in this tissue.

In order to obtain a large population of early erythroid precursor cells, we have assayed the cytosol binding of steroid hormones in the mouse spleen following phenylhydrazine-induced hemolytic anemia. In the adult mouse the spleen is normally a minor organ of erythropoiesis, but after bleeding or hemolysis it becomes a major site of red blood cell production. Following administration of phenylhydrazine, the conversion from a predominantly lymphoid spleen to an erythropoietic one is an orderly sequence of events of reproducible magnitude (5). At the peak of splenomegaly, at least 70% of the nucleated spleen cells are erythroblasts.

The present study reports the presence of a high-affinity, low-capacity receptor for 17 β-estradiol in the cytosol of early erythropoietic spleen following phenylhydrazine-induced hemolytic anemia. No comparable receptor activity was observed for testosterone or its derivatives. The 17 β-estradiol receptor was also present in preanemic and erythropoietic spleens induced by bleeding.

MATERIALS AND METHODS

C57Bl/6J mice, 20-25 g, were made anemic by a series of doses of phenylhydrazine (6). On the fourth day after the onset the mice were killed by cervical dislocation. The spleens were removed and placed on ice, and the subsequent steps were done at 4°C. After weighing, the spleens were minced and suspended in 5 volumes of Buffer A (0.25 M sucrose - 0.02 M Tris-HCl, pH 7.5 - 0.005 M β-mercaptoethanol - 0.001 M EDTA - 10% [v/v] glycerol). The minces were washed by low-speed centrifugation and resuspended in 4 volumes of Buffer A containing chloroquine (10^{-4} M), a stabilizer of lysosome structure. The suspension was homogenized with a motor-driven Teflon pestle, and the homogenate was filtered through four layers of gauze and then subjected to three sequential centrifugations: 500 x g for 10 min, 14,000 x g for 10 min, and 105,000 x g for 60 min. The final supernatant was the cytosol used in the binding experiments. The protein concentration of the cytosols was determined by the method of Lowry et al. (7) using bovine serum albumin as the standard.

Samples of the cytosol were incubated with [^{3}H]17 β-estradiol at 4°C for 90 min. The capacity of the receptor activity was tested by add-

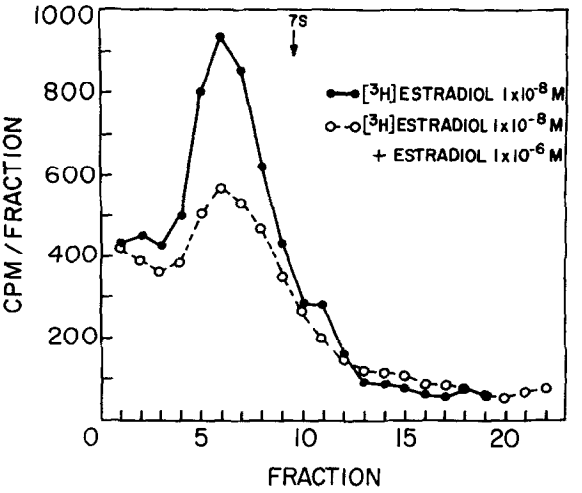

Figure 1: Sucrose density gradient centrifugation of macromolecular-bound [^{3}H]17 ß-estradiol. Samples of spleen cytosol, 17 mg protein, were incubated in a final volume of 1.12 ml with [^{3}H]17 ß-estradiol, 1 x 10^{-8} M, in the absence and presence of unlabeled estradiol, 1 x 10^{-6} M, for 90 min at 4°C. Following the incubation, free steroid was removed by adsorbtion to Dextran-charcoal, and 0.3-ml aliquots of the supernatants were transferred to tubes containing 5-20% sucrose gradients in 0.01 M Tris-HCl, pH 7.5 - 0.001 M EDTA - 0.005 M mercaptoethanol - 10% glycerol. [^{14}C]-Formaldehyde-labeled human gamma globulin, prepared by the method of Rice and Means (11), was added as an internal marker. The gradients were centrifuged in an SW56 Ti rotor at 300,000 x g for 17 h. The gradient fractions were collected and assayed for radioactivity.

ing a 200-fold excess of unlabeled 17 ß-estradiol 10 min prior to the isotope. Other steroids were tested for their ability to compete for the receptor in the same manner. The separation of free and macromolecular-bound steroid was performed using Dextran-coated charcoal (8).

Thin-layer chromatography of [^{3}H]17 ß-estradiol was performed on silica gel F-254 plates, 20 x 20 cm (Brinkmann Co.). Following ether extractions of the tissue samples, the solvent was evaporated under nitrogen and the residue dissolved in methanol for application. The chromatogram was developed in chloroform:ether (190:10), and the derivatives were visualized with ultraviolet light or by exposure to iodine fumes.

C57Bl/6J mice were purchased from Jackson Laboratories, Bar Harbor, Maine. [^{3}H]17 ß-Estradiol (91 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Massachusetts, and was used following isolation by thin-layer chromatography. The nonradioactive steroids were purchased from Steraloids, Inc., Pawling, New York.

RESULTS AND DISCUSSION

Preliminary experiments indicated preferential binding of testosterone, compared to its enantiomer epitestosterone, to early erythropoietic

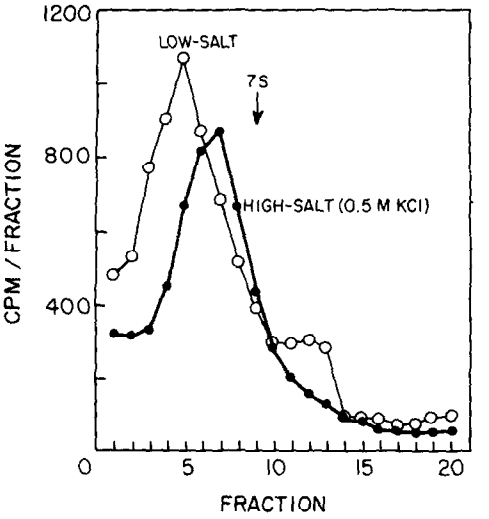

Figure 2: Sucrose density gradient centrifugation of macromolecular-bound [^{3}H]17 ß-estradiol in low- and high-salt gradients. The experimental details were as described for Fig. 1, except that the addition of unlabeled estradiol was omitted and 0.5 M KCl was present in the high-salt gradient.

spleen cytosol. However, suppression of the radioactive steroid binding with an excess of unlabeled testosterone was not demonstrated either with density gradient centrifugation, Dextran-charcoal separation, or gel filtration. In contrast, a distinct [^{3}H]17 ß-estradiol receptor complex was observed after incubation of the steroid with early erythropoietic spleen cytosol at 4°C. After density gradient centrifugation, a major peak of the [^{3}H]17 ß-estradiol:receptor complex was present at 4S, with a reproducible shoulder of radioactivity at 8-10S (Fig. 1). In the presence of a 100-fold excess of unlabeled hormone, there was approximately a 40% reduction of the radioactivity in the 4S peak and a complete loss of the shoulder at 8-10S. The sedimentation of macromolecular-bound [^{3}H]17 ß-estradiol differed in gradients containing high-or low-salt concentrations (Fig. 2). In gradients containing 0.5 M KCl a single peak of radioactivity was present at 5S; while in the low-salt gradients a 4S peak as well as a shoulder at 8-10S was present.

The steroid specificity of the receptor was assayed by comparing

Table 1. Effect of Steroids on [^{3}H]17 β-Estradiol Binding to Erythropoietic Spleen Cytosol

	Competing Steroid	% Binding
I.	None	100
	17 β-Estradiol	46
	17 α-Estradiol	73
	Androsterone	93
	Cortisol	96
	Etiocholanolone	100
	Testosterone	101
II.	None	100
	17 β-Estradiol	51
	17 α-Estradiol	71
	5(10)-Estren-3 α, 17 β-diol	72
	5(10)-Estren-17 β-ol-3-one	92
	5(10)-Estren-3, 17-dione	98
	4-Estren-17-one	100
	Testosterone	103
	5 α-H Dihydrotestosterone	103
	19 Nortestosterone Proprionate	108

the inhibition of [^{3}H]17 β-estradiol binding by 200-fold excesses of various unlabeled steroids. In this experiment and subsequent ones, [^{3}H]17 β-estradiol was present at 2 nM, and the bound steroid was separated from the free by Dextran-charcoal sedimentation. Excess 17 β-estradiol reduced binding by about 50% (Table 1). Of the other steroids tested, only the enantiomer 17 α-estradiol and 5(10)-estren-3 α, 17 β-diol showed activity as inhibitors. None of the other compounds interfered with [^{3}H]estradiol binding. The suppression of [^{3}H]estradiol binding by unlabeled 17 β-estradiol or 5(10)-estren-3 α, 17 β-diol was 76% and 58% respectively, while testosterone and 19 nortestosterone had no effect even at a 2,000-fold excess of the labeled steroid (Fig. 3).

The excess of 17 β-estradiol concentration on total, specific, and nonspecific binding was determined over a 50-fold range and the results are shown in Fig. 4A. Specific binding is the difference in

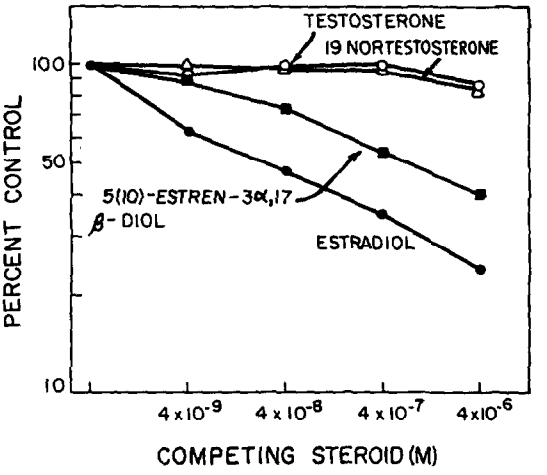

Figure 3: Effect of concentration of competing steroids on macromolecular [^{3}H]estradiol binding. The control was the [^{3}H]estradiol bound in the absence of unlabeled steroids.

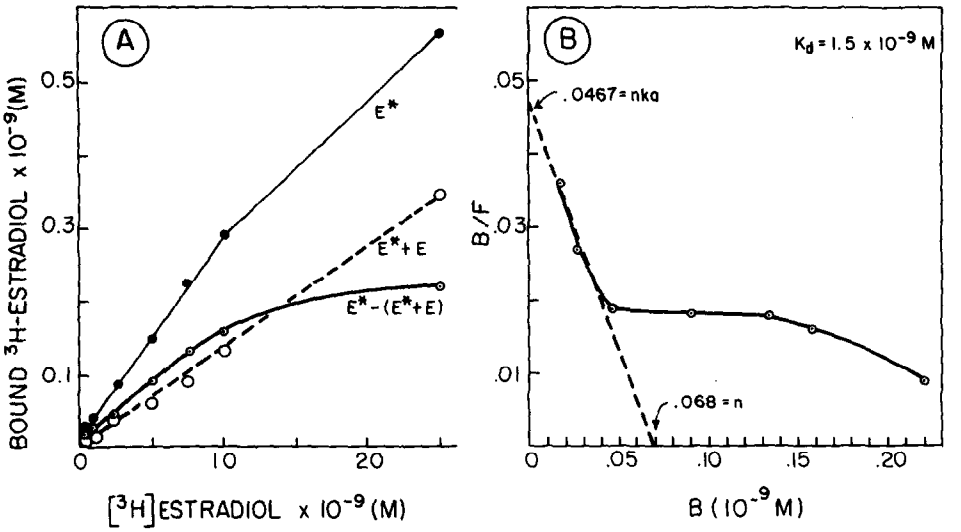

Figure 4: Specific binding of [^{3}H]estradiol in early erythropoietic mouse spleen cytosol. A: Variation in total, specific, and nonspecific binding of [^{3}H]estradiol to cytosol receptors as a function of estradiol concentration. (Total, specific, and nonspecific binding are defined in the text.) B: Scatchard plot of specific [^{3}H]estradiol binding shown in A. The abbreviations are: E^*, [^{3}H]17 β-estradiol; E^* + E, [^{3}H]17 β-estradiol + 200-fold excess of unlabeled estradiol; E^* - (E^* + E), difference between [^{3}H]-17 β-estradiol alone and [^{3}H]17 β-estradiol in the presence of excess unlabeled estradiol; B, bound [^{3}H]17 β-estradiol; F, free [^{3}H]17 β-estradiol. The cytosol protein concentration was 17 mg/ml. The abscissa and ordinate intercepts are designated n and nka, respectively. The number of high-affinity binding sites = 1/n and the dissociation constant, K_d, was determined from the equation nka = n/K_d.

macromolecular-bound radioactivity after incubation in the absence (total binding) and in the presence (nonspecific binding) of a 200-fold excess of unlabeled hormone. The curve of specific binding demonstrated near saturation at concentrations of 10 nM or greater (Fig. 4A). When the values were plotted according to Scatchard (9), a biphasic curve was obtained (Fig. 4B). The points at the lower steroid concentrations gave values of 1.5×10^{-9} M for the K_d and 4.1 fmoles/mg protein for the number of binding sites. Bullock and Bardin have reported a K_d of 1.4×10^{-9} M and the number of binding sites as 44 fmoles/mg for the mouse kidney cytosol binding of 17 β-estradiol (10). In our study specific estradiol binding for normal mouse kidney, spleen, and lung cytosols was 13.4 ± 0.14, 6.6 ± 2.1, and 2.2 ± 0.21 (fmoles/mg protein ± S.D.) respectively. There were no significant differences between these extracts as regards the effects of 5(10)-estren-3 α, 17 β-diol and testosterone on binding.

The splenic cytosol receptor is probably a protein, as about 60% of specific binding was destroyed by pronase. Heating of the cytosol for 10 min at 50°C led to a 66% loss of specific binding. Preincubation with 17 β-estradiol had no effect on the proteolytic or thermal inactivation. Addition of DNase or RNase had no effect on specific binding.

The identity of the receptor-bound steroid was investigated by thin-layer chromatography as described in the section on methods. Following development, the chromatograms were cut into 1.0-cm sections and eluted with methanol. The distribution of radioactivity on the chromatograms indicated that 81% of the total bound in the absence of excess hormone and 78% of the total bound in its presence comigrated with 17 β-estradiol. Between 9 and 11% of the total radioactivity was in a peak which migrated to a position 2 cm ahead of 17 β-estradiol. However, this was not a metabolite, as it was also present in unreacted 17 β-estradiol stock solutions. These data suggest that 17 β-estradiol, and not some splenic metabolite, was the ligand observed in the binding reactions.

Although a 17 β-estradiol receptor activity was identified in cytosols of early erythropoietic spleen cytosols formed in response to a phenylhydrazine-induced hemolytic anemia, its role in erythropoiesis is as yet uncertain. A biological effect of estrogen on splenic erythropoiesis has been demonstrated by Fried and his co-workers (1, 2). At pharmacologic levels of the hormone, a compensatory increase occurred in splenic erythropoiesis secondary to an increase in the erythropoietin-responsive cell population of the mouse spleen (2). In our studies, estradiol binding activity was present in cytosols of preanemic mice as well as in the early erythropoietic phase after bleeding or phenylhydrazine-induced hemolysis. However, one association of the receptor activity with erythropoiesis was apparent from the extent of estradiol binding of 105,000 x g supernatants of hemolysates and reticulocyte-rich lysates. Hemolysates of preanemic mice specifically bound 0.26 ± 0.12 fmoles/mg protein, while the lysates of phenylhydrazine-treated animals bound 2.06 ± 0.22 fmoles. In the latter group, the peripheral reticulocytes were 30-40% of the total erythrocyte count (6). In order to delineate the role of the receptor in erythropoiesis, studies are in progress on the in vivo retention of 17 β-estradiol in the splenic cytoplasm and nuclei of normal and anemic C57Bl/6J mice as well as in mutants with known defects in erythropoiesis.

REFERENCES

1. Fried, W., Tichler, T., Dennenberg, I., Barone, J. and Wang, F. (1974) J. Lab. Clin. Med. 83, 807-815.
2. Anagnostou, A., Zander, A., Barone, J. and Fried, W. J. (1976) J. Lab. Clin. Med. 88, 700-706.
3. Minguell, J. and Grant, J. K. (1972) J. Steroid Biochem. 3, 803-805.
4. Valladares, L. and Minguell, J. (1975) Steroids 25, 13-21.
5. Dickerman, H. W., Cheng, T-c., Kazazian, H. H., Jr. and Spivak, J. L. (1976) Arch. Biochem. Biophys. 117, 1-9.
6. Spivak, J. L., Marmor, J. and Dickerman, H. W. (1972) J. Lab. Clin. Med. 79, 526-540.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
8. Korenman, S. G., Perrin, L. E. and McCallum, T. P. (1969) J. Clin. Endocrin. Metab. 29, 879-883.
9. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672.
10. Bullock, L. P. and Bardin, C. W. (1975) Endocrinology 97, 1106-1111.
11. Rice, R. H. and Means, G. E. (1971) J. Biol. Chem. 246, 831-832.