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Serum Estrogen Binding Proteins in Tissues of the Immature Rat: Quantitation by Radioimmunoassay[1] (40477)

D. M. LINKIE AND A. R. LABARBERA

Departments of Obstetrics and Gynecology, and Anatomy and the Center for Reproductive Sciences, College of Physicians and Surgeons, Columbia University, New York, New York 10032

The operationally defined cellular compartments of tissue homogenates obtained by differential centrifugation contain varying amounts of extracellular material. For example, cytosol, the soluble supernatant of an homogenate, contains serum and interstitial proteins since the tissue has vascular and extravascular—extracellular components. At least two estrogen-binding moieties of vascular origin, alphafetoprotein (AFP) and albumin (RSA), have been identified in the cytosol of the immature rat uterus via tritiated estradiol-binding and analytic polyacrylamide disc gel electrophoretic studies (1). However, the selective precipitation and electrophoretic methods utilized in the identification of the two serum proteins are relatively insensitive for the quantitation of their mass in the various cellular compartments. This communication describes the development of double antibody radioimmunoassays for AFP and RSA and their utilization for the estimation of the mass of each protein in serum, crude tissue homogenates, and cytosol and nuclear extracts of several estrogen-responsive tissues of the immature rat.

Methods and materials. I. Rat serum albumin (RSA) radioimmunoassay. Rat serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO.) was utilized without further purification as immunogen, assay standard and for iodination. Anti-RSA sera for the first stage of the assay were prepared in three adult female rabbits by intradermal and multiple subcutaneous injections totalling 4.5–5.0 mg RSA emulsified in complete Freunds adjuvant (Difco Company, Detroit, MI) at intervals of two weeks (3 injections spanning 4 weeks). Blood was collected each week for 4 weeks following the final injection. Anti-rabbit gamma globulin serum prepared in sheep was obtained from Antisera for Science (New Rochelle, NY) and was used for the second stage of the assay. RSA as assay standard was solubilized in assay buffer (10 m*M* $NaPO_4$, pH 7.0; 140 m*M* NaCl; 0.01% merthiolate; 0.1% gelatin). Iodination of RSA with ^{125}I was carried out as described previously (2) and yielded specific activities of 100–150 μCi/μg protein. The ^{125}I-RSA was diluted with assay buffer and 100–200 pg (20,000 cpm) were added to each assay tube.

The assay involved the addition of the standard (1–500 μl) or sample (100–500 μl of various sera, cytoplasmic or nuclear extract preparations) to assay buffer yielding a final volume of 500 μl. The antiserum preparation used in the first stage was diluted initially to 1:400 with 50 m*M* EDTA-PBS (assay buffer without gelatin) and further diluted to 1:40,000 in 1:400 normal rabbit serum previously diluted in 50 m*M* EDTA-PBS. To each tube were added 200 μl of diluted antiserum, followed by incubation at 4° for 24 hr when 100 μl of ^{125}I-RSA were added and an additional 24-hr incubation carried out at 4°. At the end of this second 24-hr interval, 200 μl of the second stage antibody reagent (previously titrated to yield maximal precipitation of the first stage antibody preparation) were added to each assay tube and the incubation continued for an additional 72 hr at 4°. Phosphate buffered saline (PBS; assay buffer without gelatin or EDTA) was then added (3.5 ml), the tubes centrifuged (1000 g × 30 min), the supernatants decanted, and the radioactivity in the pellets counted in a Nuclear Chicago Mark II gamma spectrometer at 79% efficiency.

II. Rat serum alphafetoprotein (AFP) radioimmunoassay. AFP purified from rat amniotic fluid and used for iodination, goat anti-rat AFP as first stage antibody, and rat AFP standard (Morris hepatoma #7777 sera) were obtained from Dr. S. Sell, University of Cal-

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ifornia, San Diego. The methods of AFP isolation, purification, use as immunogen in antibody production and the employment of Morris hepatoma sera as an AFP radioimmunoassay standard have been described (3). First stage goat anti-rat AFP was diluted 1:400 with 50 m*M* EDTA-PBS and further diluted with a 1:400 normal goat serum preparation as rquired in the assay (1:1600 final dilution). Second stage antibody (rabbit anti-goat γ globulin) was obtained from Antibodies, Inc. (Davis, CA). Morris hepatoma #7777 serum (3.1 mg AFP/ml) was diluted in assay buffer. Purified rat AFP was iodinated with ^{125}I-3-(4-hydroxyphenyl) propionic acid, *N*-hydroxysuccinimide ester (1500 Ci/m*M*; New England Nuclear Company, Boston, MA) according to the procedure of Bolton and Hunter (4) to specific activities of 10–20 μCi/μg. Each assay tube received 0.5–1.0 μg of ^{125}I-AFP (20,000 cpm) diluted with assay buffer. The reagent volumes, order of additions, temperature and duration of incubation for the AFP radioimmunoassay were identical to those described for the RSA radioimmunoassay.

III. Recovery and adsorption experiments with RSA. Three sets of RSA standard curves in triplicate were prepared as above. Each tube then received an additional 100 μl of (a) assay buffer or (b) undiluted serum from the human or (c) a millionfold dilution of serum from the rat and assayed for RSA.

To estimate possible loss of RSA due to nonspecific adsorption, 9–92 μg RSA were dispensed to either glass or plastic vials and either PBS or assay buffer was added; these preparations were then assayed for RSA.

IV. Serum samples. Trunk blood was collected from rats 19 days of age and older following decapitation. Individual blood collections were allowed to clot at 4°, centrifuged (1000*g* × 20 min), and the sera quick-frozen and stored (−70°) until assayed. Sera were also obtained from the mouse, goat, monkey, and human and assayed for RSA and AFP. Amniotic fluid (human) in which the hAFP concentration had been determined in an assay specific for hAFP and partially purified preparations of ovalbumin and bovine serum albumin (Sigma Chemical Company, St. Louis, MO) were also tested for their cross reactivity with the first stage antisera of the RSA and AFP immunoassay systems.

V. Tissue extracts. Rats 21 days old were decapitated and the uterus, pituitary, hypothalamus and an equivalent section of cerebral cortex were dissected and placed in chilled Hanks balanced salt solution (Gibco, Grand Island, NY). The organ or tissue from a single animal is referred to as a "unit." The tissues were homogenized in 10 m*M* Tris, 1.5 m*M* EDTA buffer (pH 7.4 at 25°) using a ground glass homogenizer (Kontes, Vineland, NJ) at a concentration of 4 units/ml assay buffer. The homogenates were centrifuged at 105,000*g* for 60 min using a Spinco 65 Ti fixed angle rotor in a Beckman L2-65B ultracentrifuge. The 105,000*g* supernatant is referred to as the "cytosol" and the pellet as the "nuclear fraction". The cytosol was assayed without further dilution for AFP whereas a 50- to 100-fold dilution was necessary for RSA. The nuclear fractions were homogenized in 400 m*M* KCl, 10 m*M* Tris, 1.5 m*M* EDTA buffer (pH 8.5 at 25°) and extracted at 4° for 60 min to solubilize nuclear proteins. The nuclear homogenates were centrifuged at 105,000*g* × 60 min and the AFP and RSA in the undiluted nuclear extract supernatants quantitated by radioimmunoassay.

Results and discussion. The maximal percentage of precipitable first antibody associated iodinated-protein for both radioimmunoassays was 85–88%. The use of first stage anti RSA and anti AFP at final dilutions of 1:200,000 and 1:8000, respectively, resulted in the precipitation of 30–40% of the added radioactivity. This amount of binding was assigned as 100% of binding in control tubes. Data regarding the characteristics of both radioimmunoassays were analyzed by the method of Rodbard *et al.* (5) and were characterized by within assay sample variations of 4% and 2% and between assay variations of 15% and 12% for RSA (n = 6 assays) and AFP (n = 5 assays) respectively. The assay sensitivities were defined as the amount of standard required to inhibit binding to 90% of that occurring in the absence of nonradioactive antigen and were 0.81 ± 0.10 ng RSA and 4.24 ± 0.61 ng AFP.

Dose response curves depicting the decrease in antibody bound ^{125}I-RSA in the presence of increasing amounts of nonradio-

active antigen are shown in Fig. 1. Displacement of ^{125}I-RSA by dilutions of sera from the rat and other species is also shown where it is noted that all sera from the rat (adult or immature) required a millionfold dilution. The radioimmunoassay estimates of albumin in serum reported in Table I agree with those obtained by either paper electrophoresis or spectrophotometric analysis (6). In addition, the quantitation of RSA by the bromocresyl green method (7) for ten serum samples yielded results similar to those obtained by radioimmunoassay (29 ± 1.8 mg/ml, bcg vs 31 ± 2.4 mg/ml, RIA; $\bar{x}$ ± SEM).[2]

The rabbit anti-RSA sera developed for these studies possessed a high degree of species specificity. The amino acid composition of rat serum albumin (8) is similar to that of serum albumins from the primate (9) and bovidae (10). However, there is a six log unit disparity between inhibition curves generated by the serum albumins of these different species which is to be contrasted to a single log unit difference cited using the less discriminatory agar gel electrophoretic method (11). These results suggest that the tertiary structure of albumins differs significantly between species and that the conformational differences can be amplified by this more sensitive radioimmunoassay technique. Such a species difference is further inferred by the experiment in which RSA was quantitatively recovered following addition to undiluted serum from the human (Table II). The overestimation of added RSA in the presence of serum from the human reflects a measure of cross reaction given that approximately 3 × 10^6 ng of human serum albumin was present in each sample. Correction for the endogenous RSA in the added serum from the rat also revealed quantitative recovery.

The content of RSA in the immature rat uterus approximated 44 pmol (2.93 μg/unit; 67,000 mol wt); 1–2% of this amount was in the nuclear extract. This finding is supported qualitatively by a double antibody immunoperoxidase localization procedure (not shown) we have employed utilizing the same antibody for RSA in which RSA was demonstrated at both cellular and extracellular loci of uteri via deposition of the oxidation product of 3-3′-diaminobenzidine (6 nm sections). In contrast, reagent controls consisting of RSA adsorption of the antibody prior to its application or normal rabbit serum did not yield deposition product which suggests that endogenous RSA cannot be totally removed from tissues. Indeed, the immunohistochemical method evidenced albumin in all tissues studied (uterus, brain cortex, hypothalamus, pituitary and liver) which is in accord with both the known tissue albumin content (Table I) and, in the case of the liver, an hepatic site of synthesis. While the content of RSA in the extravascular-extracellular

[2] Clinical Chemistry Laboratory, Columbia-Presbyterian Medical Center.

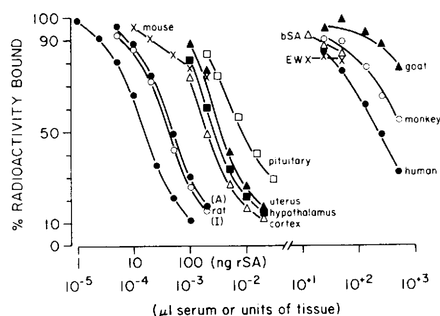

FIG. 1. Dose-response curves for albumin in the RSA-RIA: Fraction V RSA Standard (●—●), serum from the adult –A (●—●) and immature –I (○—○) rat, and the adult mouse (×—×), all at 0.1–1 × 10^{-3} μl/tube; serum from the adult human (●—●), monkey (○—○), and goat (△—△), all at 20–500 μl/tube; partially purified bovine serum (△—△) and avian egg (×—×) albumins, both at 1–500 mg/tube; and immature rat tissue extracts (cytosol) at 1–50 × 10^{-3} units/tube, uterus (▲—▲), brain cortex (△—△), pituitary (□—□) and hypothalamus (■—■).

TABLE I. CONCENTRATIONS OF ALBUMIN AND α-FETOPROTEIN IN TISSUES OF THE 21-DAY-OLD FEMALE RAT.

Tissue	Albumin	AFP
	mg/ml	μg/ml
Serum	32.12±4.08 (16)[a]	62.97±4.79 (22)
	μg/unit	*ng/unit*
Uterus	2.93±0.56 (7)	4.05±0.88 (5)
Brain cortex[b]	4.40±2.33 (3)	9.22±2.87 (3)
Hypothalamus	3.26±1.44 (3)	6.16±1.92 (3)
Pituitary	0.75±0.23 (3)	0.93±0.38 (3)

[a] Mean ± SEM (number of samples)

[b] Section = 3.5% total brain weight inclusive of hypothalamic region.

space was not measured directly in this study, it can be estimated as 60% of the serum RSA content based on the lymphatic (thoracic)/serum protein ratio (6) so that even if the vascular space of an organ (as per uterus) is cleared, interstitial serum proteins would remain entrapped. Less than 5% of the predicted immunologically identifiable amount of albumin was measured in the uterine cytosol assuming that the uterus of the 21-day-old rat weighs 27 mg (blood volume ~7.9% organ wt). It seems likely that this low recovery may represent (a) limited vascular perfusion of the uterus of the 21-day-old animal and subsequent minimal transfer of serum proteins to the interstitial space, (b) a limited interstitial volume, and/or (c) disruption of the tissues in a nonprotein containing buffer results in the nonspecific loss of tissue proteins. A possible role for the protective effect of protein in the buffer to retard tissue protein loss *in vitro* was supported by the adsorption experiment: regardless of the mass of RSA added, assay buffer as diluent correlated with recoveries of 79–93% whereas the PBS only diluent yielded recoveries of 16–21%. Such a nonspecific loss of tissue protein is especially important when studying subcellular fractions of tissues of limited size such as the pituitary or hypothalamus where the protein/volume ratio is low. An analogous reduction in the recovery of protein subsequent to iodination has been reported wherein glass adsorption of ^{125}I-protein increased as the protein mass decreased (12).

TABLE II. RECOVERY OF ALBUMIN IN THE PRESENCE OF SERUM FROM THE RAT OR HUMAN.

Albumin (ng)		
	Recovered	
Added[a]	+ rat serum[b]	+ human serum[c]
1.6	3.65	2.40
3.2	4.75	5.15
6.0	6.55	8.10
12.0	12.82	19.25
24.0	20.82	37.00
50.0	46.15	89.00
	$y = 1.14x - 1.85$	$y = 0.56x + 1.14$
	$r = 0.998$	$r = 0.998$

[a] Antigen carried in assay buffer.
[b] Corrected for endogenous rat serum albumin (2.85 ± 0.45 ng/0.1 ml; $n = 9$).
[c] Endogenous human serum albumin. (3,100,000 ± 240,000 ng/0.1 ml; $n = 10$).

The rat AFP radioimmunoassay showed some cross reactivity with a serum component from the pregnant mouse which is presumed to be mouse AFP (Fig. 2). This component, however, did not yield a parallel inhibition curve upon further dilution. Dilutions of rat serum up to ten thousandfold were required for AFP measurement for the immature rat whereas serum from the adult could be assayed directly (≤25 ng AFP/ml). The rat AFP radioimmunoassay system also did not cross react with any components in sera or amniotic fluid from the human where copious amounts of hAFP (≥10 mg/ml, third trimester) have been measured in a specific human AFP double antibody radioimmunoassay (Linkie, unpublished observations).

The relationship of serum AFP content with age (Fig. 3) indicated a $T_{1/2}$ of 31.2 hr which is 30% greater than that reported by Sell, *et al.* (13) but in agreement with their observation of a trend towards decreased clearance beginning with the third week of the postnatal period. This may represent altered or selective catabolism of one or more of the isoforms of AFP (14) based on their variable content of carbohydrate during this period of reduced synthesis. In comparison, a direct relationship for the increased clearance of hormonal glycoproteins following their desialation is known to occur (15). This age-related change in serum titer of AFP in

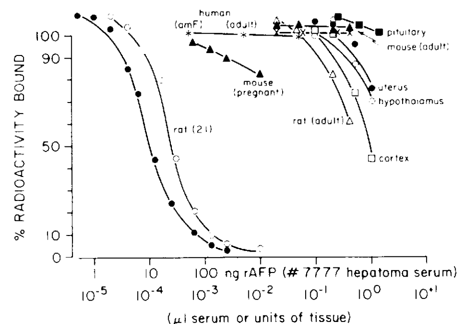

FIG. 2. Dose-response curves for serum α-fetoprotein in the AFP-RIA: Morris hepatoma #7777 serum standard (●—●); sera from the immature (21 days of age, ○—○) and adult (△—△) rat; sera from the adult nonpregnant (▲—▲) and pregnant (diluted, ▲—▲) mouse; sera (×—×) and amniotic fluid (amF, ∗—∗) from the adult human; and immature rat tissue extracts (cytosol) at 1–10 × 10^1 units/tube uterus (●—●), hypothalamus (○—○), brain cortex (□—□), and pituitary (■—■).

the immature rat, when coupled with the high affinity of AFP for estradiol (Ka $\sim 10^8\ M^{-1}$), could influence the analysis of "estrogen receptors" in estrogen target tissues in the following way. One uterus or hypothalamus from a 21 day old rat contains 4–6 pmol of AFP (Fig. 2, Table I) but since the amount of uterine estrogen receptor (Ka $\sim 10^9\ M^{-1}$) in the supernatant (cytosol) of a homogenate approximates 1–2 pmol (16), this mass of AFP should not compete with the higher affinity receptor for estrogen when low estrogen concentrations obtain. However, in the hypothalamus, where the estrogen receptor concentration is 1% that of the uterus (17), quantitation of such a small amount of estrogen receptor could be influenced by contaminating AFP and RSA (Ka $\sim 10^6\ M^{-1}$) i.e. 86 fmol AFP + 48,700 fmol RSA vs ~10–20 fmol estrogen binding sites. Most analyses of radioactive estrogen which is "specifically" bound to receptor are made by the dextran-charcoal adsorption, gel filtration or hydroxylapatite procedures. Excess nonradioactive diethylstilbestrol, which does not readily associate with AFP, is often employed to assess "nonspecific" binding of estradiol. However, the partitioning of the added steroids amongst the specific receptor, AFP, RSA and their unbound states at equilibrium is not known i.e. displacement of receptor bound radioactive estrogen by nonradioactive steroid differentially shifts the radioactive ligand to AFP, RSA and its unbound state. None of the indicated nonequilibrium assay methods simultaneously excludes both contributions of AFP and RSA to the apparent total binding of steroid by specific receptor. Furthermore, the necessary identical cell fractions which receive only the radioactive steroid also remain undefined in their distribution of steroid with the endogenous AFP and RSA moieties. Failure to prepare the estrogen responsive tissues by optimally reducing serum contamination, especially in the immature rat, complicates the above analytic methods by increasing the amounts of serum proteins carried through the receptor assays (unpublished). A neglect of this precaution most likely has contributed to a hypothesis that AFP is the major estrogen binding component in the immature rat uterus (18), a finding which we (1) and others (19–21) have been unable to support.

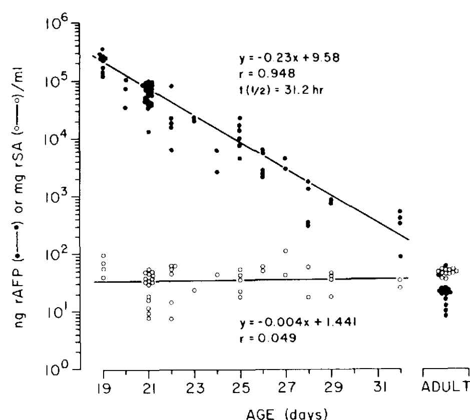

FIG. 3. Serum albumin (mg/ml, ○—○) and α-fetoprotein (ng/ml, ●—●) levels as a function of age.

The role of circulating serum estrogen-binding proteins such as AFP as modulators of estrogen availability for the estrogen-responsive centers in the brain of the developing rat is the subject of numerous investigations and remains an unresolved issue. Since the target tissues of the immature rat are commonly selected for studies of the estrogen receptor and since the present results indicate that such tissues contain sufficient nonreceptor estrogen binding proteins which can influence receptor quantitation and qualification, attention must be paid to the methods of preparation of the tissue receptor to minimize such artifacts of serum origin.

Summary. Radioimmunoassays specific for rat serum albumin and rat α-fetoprotein have been described. These systems are capable of measuring the serum proteins in as little as one nanoliter of rat serum. Negligible cross reaction with other albumins and globulins was observed in sera from a variety of animal species. These radioimmunoassay systems have proved most useful in the quantitation of RSA and AFP in extracts from tissues where their mass and their affinities for estradiol could interfere with the estrogen receptor and hence compromise analyses of the binding events in estrogen action studies. Such interference would be amplified in the less mature rat, be influenced by the method of tissue preparation, and most likely occur in tissues characterized by femto and subfemtomolar levels of estrogen receptor.

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