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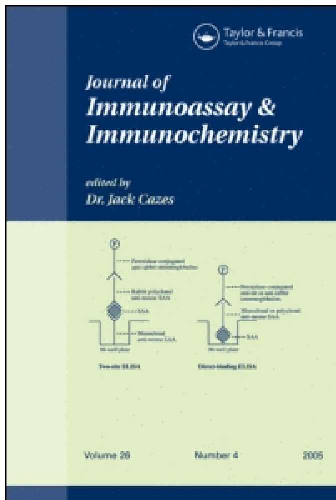

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Linkie, Daniel M. and Labarbera, Andrew R.(1980) 'Immunoassay of Serum Alphafetoprotein and Albumin Reflects the Mode of Tissue Preparation: Implications for Steroid Hormone Receptor Assays', Journal of Immunoassay and Immunochemistry, 1: 2, 271 — 284
To link to this Article: DOI: 10.1080/01971528008055788
URL: http://dx.doi.org/10.1080/01971528008055788

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JOURNAL OF IMMUNOASSAY, 1(2), 271-284 (1980)

IMMUNOASSAY OF SERUM ALPHAFETOPROTEIN AND ALBUMIN REFLECTS THE MODE OF TISSUE PREPARATION: IMPLICATIONS FOR STEROID HORMONE RECEPTOR ASSAYS

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(KEY WORDS: SERUM PROTEINS IN TISSUES; SERUM PROTEINS IN RECEPTOR ASSAYS).

ABSTRACT

The effect of various modes of tissue preparation on the retention of distinct serum proteins was determined. Specific and sensitive radioimmunologic methods were used to measure serum alphafetoprotein(AFP) and albumin(SA) in 105,000 x g hypotonic soluble (cytoplasm) and hypertonic (400mM KCl) solubilized pellets (nuclear extracts) from homogenates of immature rat estrogen target tissues. The tissue level of both serum antigens was influenced by the preparative method: cervical dislocation followed by organ isolation and a single rinse yielded the highest levels of apparent tissue AFP and SA while decapitation followed by exsanguination, organ isolation and multiple rinses resulted in the lowest levels. For the uterus, exposure of the uterine lumen enhanced reduction of serum antigen detection. The recommended procedure minimizes the inclusion of serum proteins which can interfere in the subsequent *in vitro* measurement of specific receptors in hormone responsive tissues.

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INTRODUCTION

The reduction of serum hormone binding proteins in tissue homogenates and subcellular preparations has great importance in the subsequent analysis of hormone binding to receptors. The presence of receptors at extremely low levels is reflective of the overall physiologic status of the tissue and the concentration of circulating hormone. Rodents (rats, mice, guinea pigs) in various physiologic states are commonly used for these steroid hormone receptor studies; circulating sex steroid binding proteins therefore assume particular importance since copious and variable quantities of these high-affinity binding moieties obtain in such species (1-3). As an example, immature rats and mice are characterized by an α_1-serum globulin (alphafetoprotein, AFP) derived from amniotic and hepatic tissues during gestation and from subsequent hepatic synthesis as late as the third postnatal week (4). AFP has a high average affinity for estradiol-17β ($K_d \sim 10^{-8}M^{-1}$), mass abundance ($3\text{-}30M^{-9}ml^{-1}$), and a prolonged clearance rate ($t(\frac{1}{2}) \sim 30$ hr) which collectively allows competition *in vivo* with the intracellular estrogen receptors (ER). In addition, the use of tissues from these animals for *in vitro* cell-free steroid receptor analyses is likely compromised through the inclusion of variable and unknown amounts of this and other serum proteins. While specific synthetic steroids may compete for receptor binding sites, such steroids are characterized by elevated non-specific binding to serum proteins (5) and hence are of diminished value when low levels of specific

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receptor obtain. Methods of tissue preparation are therefore sought which reduce serum interference. We demonstrate in this article that 1) the apparent tissue content of serum proteins, using AFP and albumin as markers, can be markedly influenced by the method of tissue isolation and preparation and 2) that the use of excess radioinert synthetic steroids for the measure of receptors in cell-free binding assays may prove artifactual given the steroid binding functions of circulating serum proteins.

MATERIALS AND METHODS

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Immature (19-21-day-old) female rats of the Sprague Dawley strain (Blue Spruce Farms, Altamont, N.Y.) were decapitated and exsanguinated (DEX) and the tissues rapidly isolated and either homogenized directly or rinsed from one to seven times in chilled 10mM Tris [Tris (hydroxymethyl) aminomethane],1.5mM EDTA (ethylene diamine tetraacetic acetate disodium), pH 7.4 at 25°, containing 0.1% gelatin (w/v; TEG). Uteri were either slit along the antimesometrial border or left intact. Additional rats were killed by cervical dislocation (CD) without exsanguination and the tissues processed as above. Uterine, pituitary, left lateral cerebral cortex and hypothalamic tissues were all placed in respective 10 ml rinse volumes of homogenization media. The hypothalamic area was defined and limited as follows: anterior-optic chiasm; lateral-hypothalamic fissures; posterior-mammillary bodies. All tissues were homogenized with a Polytron (PT_{10}) tissue disintegrator (Brinkmann, Westbury, N.Y.) at a setting of

6 for 30 sec using chilled homogenization buffer. The homogenates were centrifuged (105,000 x g) in a Sorvall fixed angle rotor (type 65) using a Beckman L2-65B ultracentrifuge for 60 minutes to yield operationally defined cytosol (supernatant) and nuclear (pellet) fractions. The latter were rinsed and further homogenized in buffer containing KCl at 400 mM and incubated at 2° for 60 minutes to solubilize nuclear proteins. Centrifugation as per above (105,000 x g) for 60 minutes then yielded a "nuclear extract" (supernatant) and pelleted debris. Radioimmunoassays for rat serum AFP and albumin were carried out as described previously (6). AFP and SA are reported as pmol/unit or pmol/ml wherein a unit is defined as the equivalent of one organ, e.g. homogenization of 5 uteri/ml TEG yields 1 uterus/0.2 ml. The molecular weights of AFP and SA were taken as 72,000 and 67,000 daltons, respectively.

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RESULTS

Effect of In Vitro Uterine Rinse Sequence and Exposure of the Uterine Lumen on Subsequent AFP and SA Quantitation

Uteri collected from 21-day-old rats following decapitation were either slit longitudinally or left intact. They were rinsed zero, one or seven times prior to homogenization. The amount of immunologically detectable cytoplasmic AFP and SA approximated 1 and 55 pmol/unit respectively when uteri were neither slit nor rinsed; a single rinse reduced both levels 30% to 0.7 and 37.0 pmol/unit (Fig. 1). A more significant reduction (90%) of uterine-associated AFP could be effected, however, if the organ was

first slit and subjected to seven rinses (with blotting in between) prior to homogenization, i.e. to 0.1 pmol/uterus. SA concentrations were reduced by this procedure only 45% to 30 pmol/unit. The nuclear content of both antigens, regardless of the various tissue manipulations, was ∿0.05 pmol AFP/unit and 2.0 pmol SA/unit.

Uterine AFP and SA Content as a Function of the Mode of Animal Death

A comparison of cervical dislocation (CD), isolation of the uterus and one rinse before homogenization was made with decapitation-exsanguination (DEX) and seven uterine rinses in the 20-day-old rat. The results are shown in Fig. 2 in which the left panel records the AFP and SA in the tissue rinses; the right panel summarizes the cytoplasmic, nuclear pellet rinses, and nuclear content of both serum antigens. Approximately 0.8 pmol AFP was measured in the single rinse from uteri obtained from CD animals, whereas successive rinses (n=3) of ∿0.8 pmol/rinse were evident in the DEX group, thereby suggesting that additional AFP can be removed from uteri. This observation is supported by a parallel rinse pattern for SA (CD = 24 pmol; DEX = 16 + 19 + 20 pmol). The right hand panel on Fig. 2 also indicates that tissue (cytosol) levels of AFP were greater in uteri from CD than DEX animals, i.e. 1.8 pmol vs. 0.6 pmol/unit. Similarly, SA levels in uterine cytosol were 8 pmol/unit for CD animals and 3 pmol/unit in DEX animals. More AFP and SA were evident in the

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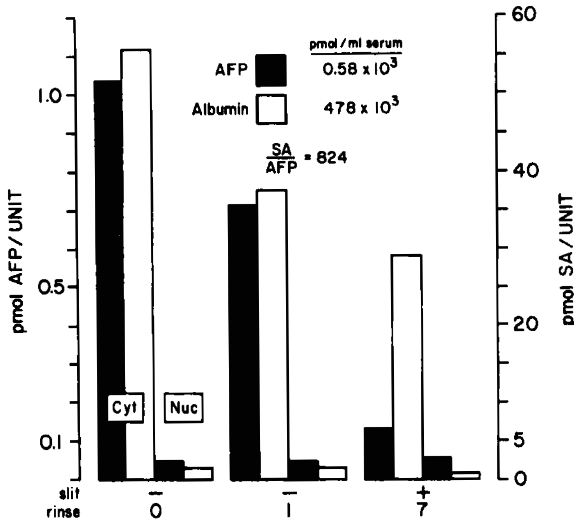

FIGURE 1. Uterine cytoplasmic and nuclear associated AFP and SA (pmol/unit) determined by radioimmunoassay (RIA) for the 21-day-old immature rat killed by decapitation. Bars indicate the concentrations as a function of the tissue dissection and rinsing procedures. The ratio SA/AFP (pmol/ml serum) indexes animal age; SA is relatively constant, AFP decreases with age. A dramatic increase in SA/AFP results during the critical third-fifth week of postnatal life in this species.

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"nuclear rinse" (1 ml TEG applied to high-speed pellet) and nuclear extract of CD than DEX animals.

AFP and SA Levels in Various Tissues of the Immature Rat Following Cervical Dislocation and One Tissue Rinse

The content of AFP and SA in the cytosol and nuclear extracts of the hypothalamus, pituitary, and brain cortex as well as uterus were determined for the 19-day-old rat (Fig. 3). These tissues were obtained in the least fastidious manner, i.e. CD with one rinse. This procedure yielded the highest level of AFP in uterine

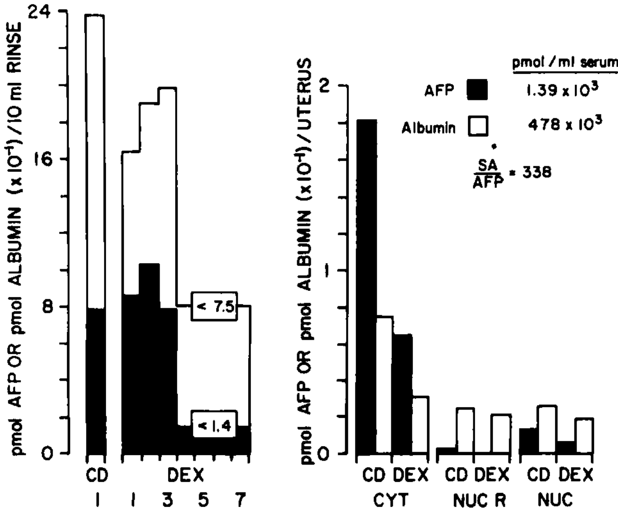

FIGURE 2. Left panel: AFP and SA levels (per 10 ml Tris - EDTA - 0.1% gelatin buffer) in the rinse(s) of uteri obtained from 20-day-old rats killed by cervical dislocation (CD) or decapitation followed by exsanguination (DEX). The levels of AFP and SA in the fourth - seventh rinse of DEX uteri were below the level of detectability (given the volume assayed) for their respective RIA. Right panel: Comparison of AFP and SA content of uterine cytosol pellet rinse, and pellet extract (pmol/unit) for animals killed by either CD or DEX. SA/AFP day 20 < day 21.

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cytosol (5.0 pmol/unit) as well as nuclear-associated AFP (0.17 pmol/unit). SA in the cytosol approximated 22 pmol/unit while a range of 5-10 pmol/unit was obtained for nuclear extracts. Cytosol of hypothalamus, pituitary, and cerebral cortex had AFP levels of 0.17, 0.10 and 0.30 pmol/unit, respectively; nuclear-associated AFP was 0.08, 0.07 and 0.11 pmol/unit for these same tissues. SA in the cytosol and nuclear extracts of the hypothalamus, pituitary, and cerebral cortex ranged from 5-11 pmol/unit.

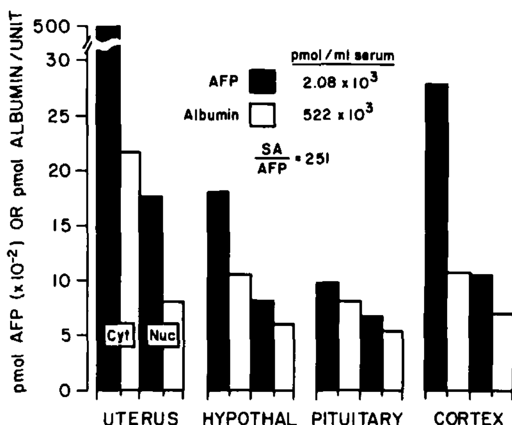

FIGURE 3. Cytoplasmic and nuclear associated AFP and SA for the uterus, hypothalamus, pituitary, and brain cortex of the 19-day-old rat (pmol/unit). Animals were killed by cervical dislocation and the tissues rinsed but once. SA/AFP day 19 < day 20 < day 21.

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DISCUSSION

The present studies were designed to investigate the extent to which the method of preparation of a tissue can influence the inclusion of serum in cellular extracts. The results may be extrapolated to steroid hormone analyses where the desired measure of specific cellular receptor is affected by serum proteins. An optimal method for obtaining rodent uteri, in which estrogen receptor analyses are sought, is decapitation and exsanguination followed by rapid organ removal, exposure of the lumen, and multiple rinses with chilled buffer (Fig. 1). This method significantly reduces the tissue content of potentially compounding serum estrogen binding proteins in subsequent estrogen receptor analyses. This approach can be extended to other estrogen-responsive tissues whose intracellular receptor density is much less

than that of the uterus, e.g. pituitary, hypothalamus, pineal. Uterine, hypophyseal, brain and hypothalamic cytosols from the immature rat have also been analyzed by immunoassay for AFP and SA following sucrose density gradient fractionation; both serum proteins were present and migrated at 4.2-4.3S (3). An additional preliminary procedure of _in situ_ tissue or whole animal perfusion should enhance removal of serum from all organs, including the non-tubular, and thereby augment the outlined tissue preparation method.

Figure 2 indicates that multiple rinses effectively reduced serum protein levels in the tissues; however, there appeared to be no additional removal after the third rinse which suggests their entrapment in an extravascular compartment. Indeed, serum albumin is found in the extravascular-extracellular and cellular compartments of such uteri when studied by a specific peroxidase labelled antibody procedure (6). It is also noteworthy that sex hormone binding globulin (SHBG), a serum estrogen and androgen binding protein in the human and monkey, has also been localized in the cytoplasm of uterine endometrial cells and cells of the estrogen responsive MCF-7 tumor cell line (7) thereby supporting biochemical data that serum proteins cannot be totally eliminated from tissue fractions and hence may pose potential competitive interference in receptor assays. Figure 2 clearly indicates the limitations of killing by cervical dislocation and a limited (one) rinse procedure.

The immunologically detected AFP in uterine cytosol from the

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19-day-old rat (Fig. 3) approximated 5 pmol/unit. This experiment utilized a tissue preparation method commonly employed in estrogen receptor studies (8). Since the average K_d of AFP for estradiol-17β is $\sim 10^{-8}M^{-1}$ (1,9), at least 10-20 pmol are estimated to compete equally with receptor (10) assuming that 1) both AFP and receptor have a single steroid binding site and 2) all immunologically detected AFP competes equally with receptor. The immunologic assessment of AFP may of course not bear directly on a mechanism of estrogen action since antigenic determinants need not correlate with an estrogen binding function (11). The evidence for heterogeneity of rat AFP (12,13) and differences in the affinity of AFP isoforms for estradiol further compound this relationship. On the other hand, cytoplasmic receptor from mature rat uteri that has bound estradiol, when studied _in vitro_ in dialysis against various dilutions of sera from the immature rat, binds significantly less steroid as the concentration of AFP increases (data not shown). A similar observation was reported following _in vitro_ incubation of immature rat uteri at saturation levels of estradiol with sera from animals of different ages (14). Albumin can be expected to compete with receptor for steroid but at a concentration far in excess (1×10^6 pmol/unit) of that measured in these studies.

The masses of serum estrogen-binding moieties in the uterus of the 19 - 21-day-old rat would not appear to compromise receptor assessment but the potential AFP competition for steroid would increase with the less mature animal since much more AFP is pre-

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sent (3-30 nmol/ml). Pituitary associated AFP (0.10 pmol), at least in the 19-day-old rat, could influence estrogen action given an equivalent glandular receptor density. Since measured AFP (0.18 pmol; Fig. 3) approximates endogenous receptor (10-20 fmol) in the hypothalamic region, estrogen binding may be compounded *in vivo*, or when subsequently assessed *in vitro*. The relationship could be important during the intrauterine and immediate postnatal development and differentiation periods, assuming that the dramatically elevated levels of AFP would withhold steroid from the estrogen-sensitive intracellular environment.

Finally, many studies of steroid receptors in a variety of tissues and in cells in culture have been conducted with cell-free preparations utilizing saturation analysis with natural or synthetic hormones. An improper choice of discriminating method for receptor analysis (adsorption, filtration, charge, etc.) when coupled with an initial inadequate mode of tissue preparation would include variable amounts and types of serum proteins which bind steroids, e.g. AFP, SHBG, CBG, PBG, AAG, albumin. The inclusion of such steroid binding entities theoretically complicates estrogen, androgen, progestin and corticosteroid receptor analyses even if excess radioinert steroids are utilized because the amount of the variable endogenous binders and their affinities are unknown as is the partition of the endogenous and added steroid between all lower affinity binding moieties and the specific hormone receptor. The choice of excess competing

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steroid is particularly critical for while synthetic steroids (e.g. R5020, R2858, diethylstilbestrol) have proved useful in the characterization of receptors, an associated nonspecific binding in excess of the natural hormone in some cases limits their application when low levels of receptor obtain (5). Hence the present study indicates that 1) the levels of such immunoassayable serum proteins as AFP and albumin, can be used to monitor the method of tissue preparation, 2) the outlined procedure may serve as a model for other serum protein-tissue receptor systems and 3) since many serum proteins bind steroids both specifically and non-specifically, any assessment of tissue steroid receptor content must discriminate between the endogenous cellular binding site and included serum proteins to effectively minimize artifacts in receptor quantitation.

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ACKNOWLEDGEMENTS

This work was supported in part by NIH grant 10090 and Population Centers Award 06132.

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