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DIFFERENCES IN THE MOLECULAR HETEROGENEITY OF ALPHA-FETOPROTEIN FROM UTERUS AND SERUM OF IMMATURE RATS

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SUMMARY.- AFP is found in uterine cytosol extract of 20 day old rats although a part of this AFP (8.5 %) comes from extracellular contamination. Two uterine AFP populations were isolated by chromatography on anti-AFP-immunosorbent : an antigenic free form obtained at normal salt conditions (Fraction I) and a second population which is retained on anti-AFP column at higher salt concentration (Fraction II). PAGE and affinity experiments with lectins were used to reveal that the uterine AFP exhibits the same molecular heterogeneity as serum AFP. However differences were observed in the relative amounts of each molecular variants between AFP's from sera or uterus.

INTRODUCTION

Although little is known about the biological role of AFP, it has been demonstrated that rat AFP possesses a high estrogen binding capacity (1-3). This property can be connected to the observation of Uriel *et al.* (4) showing that AFP is present in rat uterine cytoplasmic extracts. Nevertheless recent data (5) suggest that in uterine cytosol of immature rat two macromolecules (i.e. AFP and uterine estradiol receptor) bind estradiol with high but different binding specificity and it was concluded that AFP is not a subunit of the estradiol receptor.

Several molecular variants of rat AFP have been characterized by polyacrylamide gel electrophoresis (6-9) and lectin affinity chromatography (10-15). Age-dependent variations have been observed in the relative amounts of these iso-AFP isolated from the sera of newborn rat from birth until 20 day old suggesting differential metabolism of preferential uptake by some target tissues (16

Abbreviations : AFP, alpha-fetoprotein ; AFP_A, slow moving and AFP_B, fast moving electrophoretic variants. RCA_I, *Ricinus communis* agglutinin of molecular weight 120 000. Con A, concanavalin A ; LCA, *Lens culinaris* agglutinin. CIAE, crossed-immuno-affino-electrophoresis.

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In this work evidence is presented that AFP from uterine cytosol exhibits qualitatively the same iso-AFPs as encountered in serum but quantitatively some differences are noticeable in their distribution.

MATERIALS AND METHODS

Antisera.- Monospecific antisera against rat AFP and rat albumin were prepared as previously described (6).

AFP and albumin estimation.- AFP and albumin were estimated by fused rocket immunoelectrophoresis (17). Total protein concentration was determined by the method of Lowry *et al.* (18) using serum albumin as standard.

Isolation of AFP from sera and uterine cytosol.- AFP was isolated from sera and uteri of 20-24 day old female Wistar rats by immunoaffinity chromatography on an anti-AFP Sepharose column (15). 75 rat uteri were rapidly excised and seven time washed for 2 hours at 4°C in 20 ml Tris-2 mM Na_2 EDTA-2 mM mercaptoethanol pH 7.4 (TE buffer) (4). Uteri were then crushed with 20 ml TE buffer for 1 minute with the ultraturrax and homogenized at 4°C. The mixture was then centrifuged at 12 000 rpm (10 000 g) for 1 hour at 4°C. The supernatant was estimated for its antigenic AFP and albumin content by the direct electroimmunoassay. AFP from uterine cytosol was immunoadsorbed on an anti-AFP Sepharose column (3 x 13 cm) equilibrated with the TE buffer and eluted with pH 2.8 glycin buffer (Fraction I). The unbound cytosol material i.e. devoided of free AFP as controlled by electroimmunoassay, was made 0.4 M KCl (TEK buffer) with solid KCl and kept under stirring for 4 hours at 4°C in order to dissociate macromolecular complex (4). This solution was then immunoadsorbed again on fresh anti-AFP Sepharose column equilibrated with TEK buffer. Immunoadsorbed AFP (Fraction II) was eluted as above.

Polyacrylamide slab gel electrophoresis.- Analytical electrophoresis was carried out on 1 mm thick gel slab, using 12 % acrylamide gel concentration in the gel system of Davis (19). For detection of the proteins, the slab gels were stained for 2 hours with 0.5 % Coomassie Brillant Blue dissolved in ethanol, acetic acid, water (9:2:9 V/V) and destained in ethanol, acetic acid, water (5:2:9 V/V). The gels were scanned at 650 nm with a Joyce Loebl densitometer.

Crossed immuno-affino-electrophoresis.- The CIAE method described by Bøg-Hansen *et al.* (20) was performed using free lectins (Con A or LCA) in the first dimension gel.

Lectins and antibodies were included at 50°C in 1 % Agarose A (Pharmacia) melted in 37 mM Tris, 24.5 mM Veronal, 0.36 mM calcium lactate and 0.2 M sodium azide solution pH 8.6. In the first dimension electrophoresis 5 µl samples containing 100 µg/ml AFP were run at 10 V/cm for 2 hours in a gel containing a fixed concentration of lectin. The second dimension was performed at 2 V/cm for 18 hours in an anti-AFP impregnated agarose gel.

Chromatography on Ricinus Sepharose column.- The *Ricinus* agglutinin RCA_I (molecular weight 120 000) specific for D-galactose residues, was coupled to the Sepharose 4B previously described (16).

AFP isolated from uteri or sera was applied on RCA_I column, washed with 20 mM phosphate buffer pH 7.2-0.15 M sodium chloride. The bound material was eluted with the same buffer containing 0.1 M D-galactose.

TABLE I.- AFP and albumin estimation in the serum and uterine cytosol extract of 20 day old rats.

	serum	uterine cytosol**	$\frac{\text{cytosol prot.}}{\text{serum prot.}}$
AFP*	257	63.75	0.248
Albumin*	34000	2890	0.085

*Concentrations are expressed in µg/ml and determined by immunoelectrophoresis.

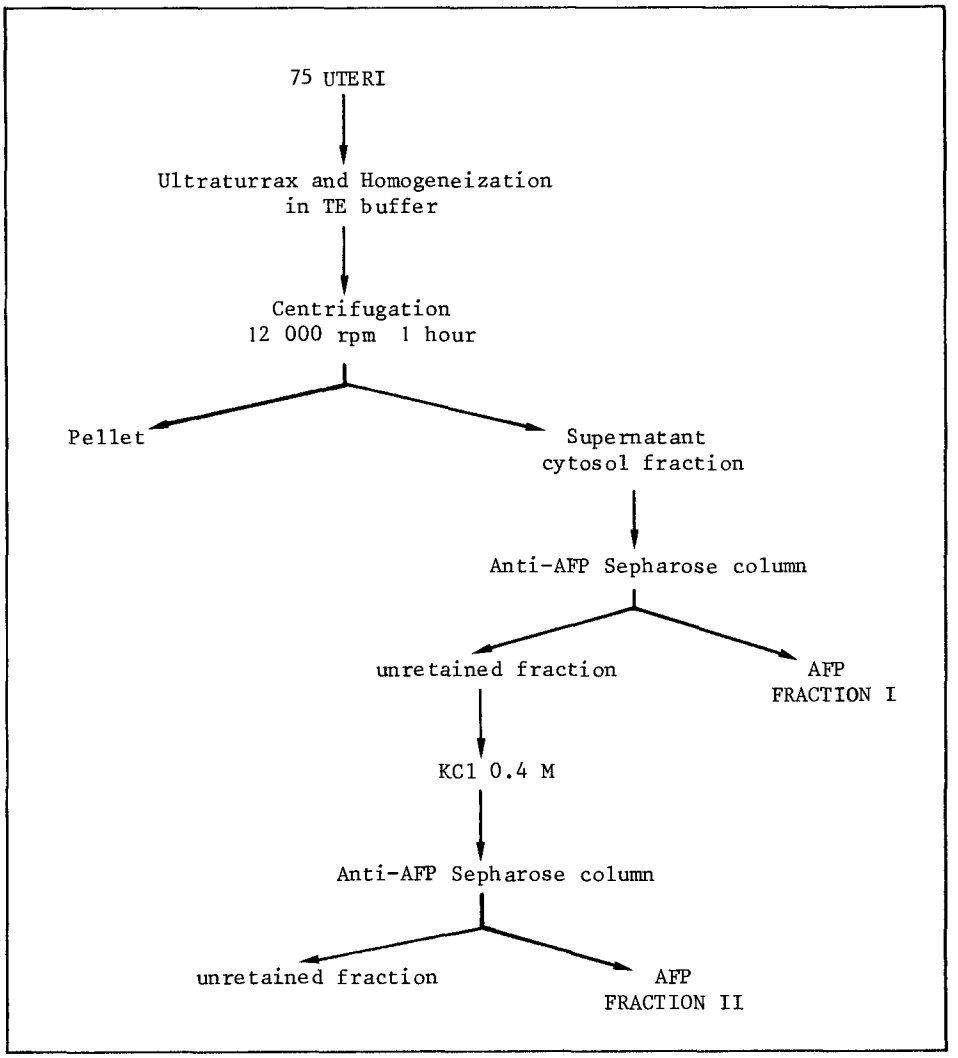
**The values correspond to 75 uteri that occupied 1 ml volume.

RESULTS AND DISCUSSION

The cytosol extract yields 0.85 µg AFP per uterus. However this value must be lowered owing to extracellular contamination (21). Indeed, even after seven washing cycles 8.5 % of the albumin serum content are still recovered in the uterine cytosol extracts (Table I). The higher AFP values previously reported (4) (1.6 µg per uterus) probably result from more gentle washing conditions.

This AFP can be retained on anti-AFP Sepharose column equilibrated in TE buffer (AFP Fraction I ; Table II). No detectable antigenic AFP remains in the unadsorbed material as controlled by immunoelectroassay in TE buffer medium. On the other hand, when this material is brought to 0.4 M KCl (TEK buffer) some AFP (0.08 µg per uterus) becomes immunoprecipitable. This new AFP fraction (Fraction II ; Table II) can be adsorbed on the anti-AFP column in TEK buffer conditions. Consequently, in agreement with the previous findings of Uriel *et al.* (4) the uterine cytosol extract contains two kinds of AFP (i) a free and antigenic form in TE buffer and (ii) a TE buffer-non antigenic form which turns antigenic in TEK buffer.

TABLE II.- Purification scheme of AFP from uterine cytosol



Analysis of these two AFP fractions by polyacrylamide gel electrophoresis reveals the presence of two electrophoretic variants as in the sera of the same 20 day old animals (Fig. 1). However densitometric measurements of the stained proteins bands indicate distribution differences between serum and uterine cytosol iso-AFPs. The slow moving electrophoretic variant (AFP_A) which is predominant in the serum decreases in the uterine AFP Fraction I and appears only at the same amount as the fast moving variant (AFP_B) in the uterine AFP Fraction II (Table III).

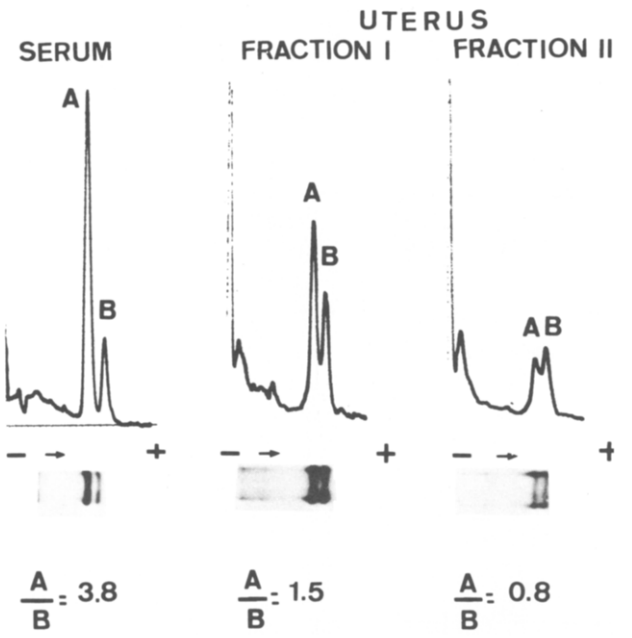


Fig. 1.- Polyacrylamide gel electrophoresis patterns of immunoadsorbed AFP from a) serum of 20 day old female, b) uterine cytosol extract : Fraction I, c) uterine cytosol : Fraction II. The obtained gel was scanned with a Joyce-Loebl microdensitometer and the ratio of each peak (A : AFP_A and B : AFP_B) was obtained by integrating the surfaces.

Using affinity chromatography on RCA_I-column it was shown that 10 % of AFP isolated from fetal rat serum (14-18 days gestation) is RCA_I-reactive (20). In newborn serum, the RCA - reactive AFP declines progressively with the age of the animals. While only traces (1.4 %) of RCA - reactive AFP still remain in the sera of 20 day old animals, significant higher values can be recovered in the uterine cytosol Fraction I (7.6 %) and Fraction II (12.8 %) (Table III).

In crossed-immuno-affino-electrophoresis, interaction with lectins included in the first dimension gel, results in the retardation of some AFP components so as to reveal carbohydrate variations of the AFP molecules (Kerckaert *et al.*, in preparation). This technique was used to compare the lectin affinity of serum AFP and uterine AFP Fraction I. Three precipitation

TABLE III.- Serum and uterus distribution of the three AFP variants : AFP_A, AFP_B and RCA reactive AFP.

	Serum								Ut. I	Ut. II
Age[(a)]	-8	-4	0	4	8	12	16	20	20	20
% AFP_A	61.5	60.0	58.7	59.9	61.8	62.9	66.8	78.0	60.0	48.7
% AFP_B	38.5	40.0	41.3	40.1	38.2	37.1	33.2	22.0	40.0	51.3
$A/_B$	1.59	1.60	1.42	1.49	1.61	1.69	2.01	3.54	1.50	0.95
% RCA reactive	10.0	8.8	3.8	3.2	3.0	2.6	2.1	1.4	7.6	12.3

(a) Age of animals are expressed in days. Negative numbers indicate days before birth. Results are expressed in percentage. Ut. I and Ut. II are abbreviations for uterine fraction I and uterine fraction II.

peaks are obtained with LCA and Con A (Fig. 2). The fast moving peak (peak (a)) corresponds to lectin-unreactive material, the other retarded peaks (b) and (c) represent lectin-weakly reactive and lectin-reactive AFP respectively. Although the serum and uterine AFP patterns look very similar it should be note only that the LCA-unreactive AFP is less important in uterus than in serum.

Beyond the recent controversial question whether or not AFP is part of the estrogen receptor (4, 5), it remains that AFP is effectively present in uterine cytosol extracts. However, direct demonstration of the true intracellular localization of the AFP molecules has not yet been established. In this work, we present evidence that even if serum contamination occurs in uterine extracts, uterine AFP and serum AFP displays distinct molecular

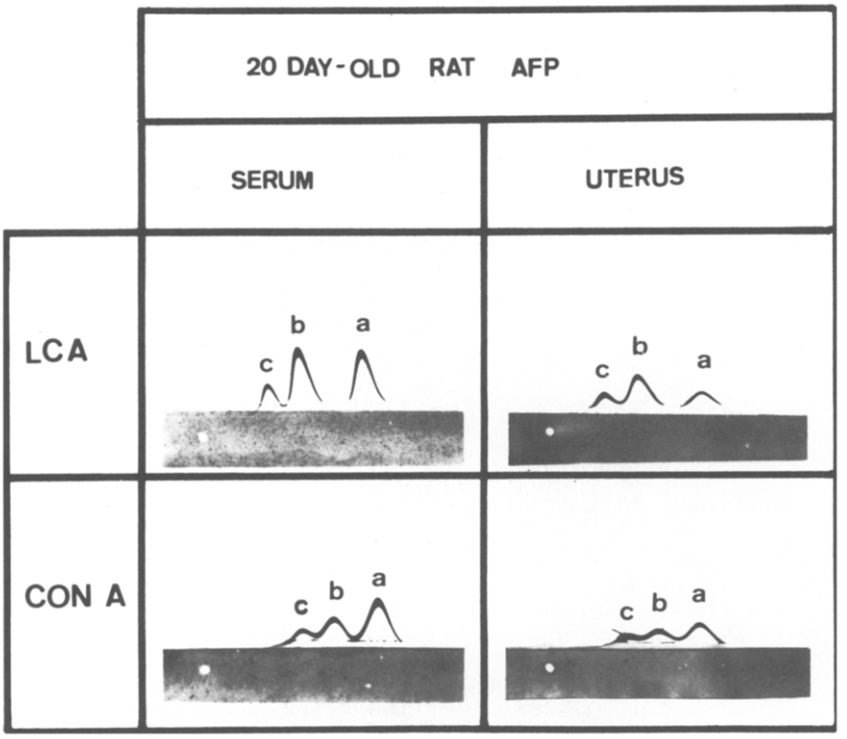


Fig. 2.- Comparison between the CIAE patterns of AFP purified from the serum and from the uterine cytosol (Fraction I) of 20 day old rats. The first dimension gel contains 100 µg/cm^2 of free lectin (LCA or Con A)
Peak a : LCA or Con A non reactive AFP
Peak b : LCA or Con A weakly reactive AFP
Peak c : LCA or Con A reactive AFP

polymorphism. Two possibilities could be put forward to explain these differences in the iso-AFP distribution : (i) existence of a preferential uptake by uterus cells of some variants like the fast moving electrophoretic form and the RCA_I-reactive AFP ; (ii) possibility that the uterus cell assumes its own AFP synthesis or processing.

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