RAT ALPHA-FETOPROTEIN HETEROGENEITY

Affinity chromatography on *Ricinus communis* Sepharose column

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1. Introduction

Rat AFP heterogeneity has been demonstrated by polyacrylamide gel electrophoresis [1–4]. The two electrophoretic variants exhibit similar immunological and physico-chemical properties [1]. However some slight differences were observed in their molecular weight, CNBr cleavage fragments [5] and estrogen binding capacity [4].

Furthermore, a second kind of heterogeneity has been described by affinity chromatography on immobilized lectin. Because of their high specificity, lectins or plant agglutinins are useful tools in demonstrating the microheterogeneity of the carbohydrate moiety of many glycoproteins. Particularly, the ability of the lectin Concanavalin A to react with some carbohydrate chains of the α_1-fetoprotein has permitted to isolate a new molecular population of AFP [6,7]. The specificity of this immobilized lectin is now well defined [8,9] but the interaction between Concanavalin A and glycoproteins has frequently been misinterpreted.

Recently, Harboe et al. [10] and Surolia et al. [11] have demonstrated the affinity of the *Ricinus communis* agglutinin RCA_I for some adult serum glycoproteins which contain terminal non reducing D-galactose residues. But it has not been established if the fetal proteins and particularly AFP variants participate in the precipitin reaction.

The aim of this work is to examine the interaction between *Ricinus* agglutinin with whole AFP, and with its electrophoretic variants, by affinity chromatography using Sepharose coupled lectin. The composition of the conjugated carbohydrate chains and specially the external end groups which might be expected to be important in any putative biological function were investigated.

Abbreviations used: AFP, alpha-fetoprotein; RCA_I and RCA_{II}, *Ricinus communis* agglutinin of mol. wt 120 000 and 60 000 according to the nomenclature of Nicolson et al. [20]; 3′-MDAB, 3′-methyl-4-dimethylaminoazobenzene; NANA, *N*-acetyl neuraminic acid

2. Materials and methods

2.1. *Source of AFP*

AFP was isolated from amniotic fluid, fetal newborn sera and hepatoma bearing rat serum. Fetal serum was collected after decapitation of fetuses 13 to 18 days old. Newborn serum was also obtained by decapitation of 4, 8, 12, 16, 20 and 24-day-old Wistar Rats. Hepatoma bearing rat serum was collected from rats which have been fed with 3′-MDAB for 3 weeks [12].

2.2. *AFP purification and antisera production*

AFP was purified by affinity chromatography on an anti AFP-Sepharose column. The two electrophoretic variants (AFP_A, AFP_B) were prepared as described previously [1].

2.3. *AFP quantitative analysis*

The concentration of AFP in whole sera or in

chromatographic fractions was measured using unidirectional electroimmunoassay [13]. Total protein concentration was assayed by the Lowry method [14].

2.4. *Polyacrylamide gel electrophoresis*

Analytical electrophoresis was carried out on 1 mm thick gel slab, using 12% acrylamide gel concentration in the gel system of Davis [15].

2.5. *Carbohydrate composition*

Total hexoses were determined by the orcinol-sulfuric method [16], hexosamines by the Elson-Morgan reaction [17] and N-acetylneuraminic acid by the diphenylamine procedure [18]. Molar ratios of monosaccharides were evaluated by gas liquid chromatography [19].

2.6. *Isolation of* Ricinus communis *agglutinin*

Ricinus agglutinin RCA_I (mol. wt 120 000) [20], was prepared using a combination of affinity chromatography and gel filtration. First, the *Ricinus* seed extract was subjected to affinity chromatography on a Sepharose-*N*-ε-amino-caproyl-β-D-galactopyranosylamine column prepared according to Gordon [21]. Two lectins RCA_I and RCA_{II} were eluted with 0.1 M galactose and then separated on a G-150 Sephadex column equilibrated with a pH 7.2 phosphate buffered saline.

The RCA_I agglutinin was coupled to the Sepharose 4B activated according to the procedure of March et al. [22].

2.7. *Affinity chromatography on RCA_I-Sepharose column*

The different AFP containing media were applied on the RCA_I-column, washed with 20 mM phosphate buffer pH 7.2–0.15 M sodium chloride until the $A_{280\ nm}$ returned to the baseline. The bound material was eluted with the same buffer containing 0.1 M D-galactose. The commercial D-galactose obtained from The Nutritional Biochemicals Corporation was purified on a charcoal column [23].

3. Results

3.1. *Affinity chromatography on RCA_I-column*

RCA_I agglutinin reacts specifically with glycoproteins containing terminal non-reducing D-galactose residues. Among the whole normal human plasma proteins only IgA, IgG, IgM, α_2-macroglobulin and haptoglobin are retained on RCA_I-column [10]. When freshly prepared rat fetal serum is applied on this column another component seems to be retained. Electrophoretic analysis of the unbound and bound fractions shows (fig.1) that the unbound material contains most of the typical proteins of fetal rat serum i.e., γ-globulins, haptoglobin, transferrins, two electrophoretic forms of the α_1-fetoprotein, albumin and α_1-acid glycoprotein. The bound fraction is composed of only a few components, whose electrophoretic mobilities correspond to IgM, haptoglobin and the slow moving AFP (AFP_A). As illustrated in fig.2, this last component was confirmed to be AFP by immunological determination.

The same experiment was carried out with purified rat AFP isolated from the serum of fetuses 13–18 days old. The unbound AFP, (fig.2A, fractions 4–13) was eluted with phosphate saline buffer and represented 90–92% of the total AFP. The recycling of this unbound fraction on fresh Sepharose-lectin showed that there was no further binding on the lectin. Electrophoresis on 12% acrylamide gel revealed that this fraction contains two distinct bands (fig.2B) called AFP_B and AFP_{A1}. The bound AFP (fractions 22–23) was eluted with 0.1 M galactose in phosphate saline buffer and accounted for about 8–10% of the total AFP. Polyacrylamide gel electrophoresis of bound AFP displays a single band termed AFP_{A2}. From these results, rat AFP contains three distinct molecular populations: two slow moving forms AFP_{A1} (RCA_I non-reactive) and AFP_{A2} (RCA_I reactive) and one fast moving, RCA_I non-reactive form, AFP_B.

3.2. *Carbohydrate analysis of the different AFP variants*

Due to the fact that the *ricinus* lectin is specific for non reducing galactose terminal residues, we presumed that the unbound AFP_{A1} and bound AFP_{A2} variants differ in their carbohydrate composition. To verify this, we analyzed the carbohydrate content of these two molecules by quantitative gas liquid chromatography and colorimetric estimation of the different monosaccharides. Gas liquid chromatog-

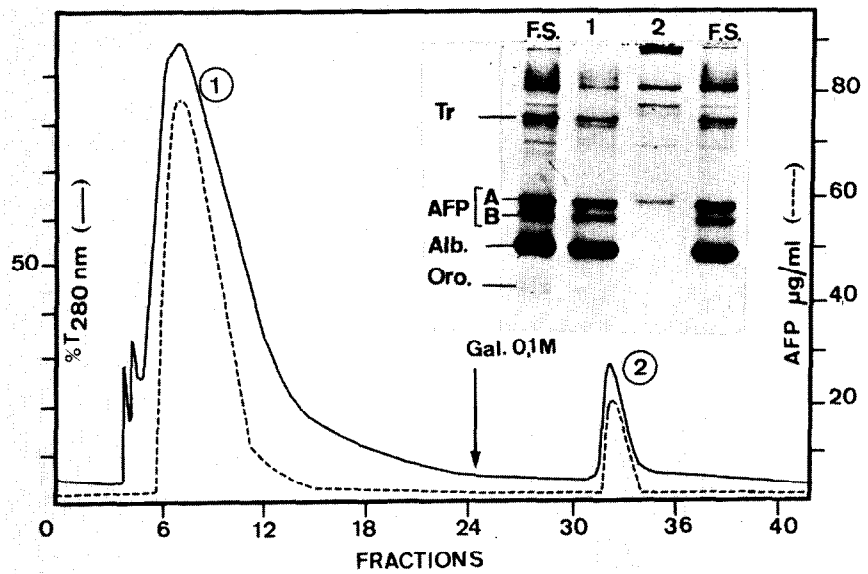

Fig.1. Elution profile of fetal rat serum (2 ml) through a column containing the *Ricinus* agglutinin RCA_I coupled to Sepharose 4B. The first peak (fractions 4–15) contains unbound proteins, the second peak (fractions 31–34) contains proteins bound to the insolubilized *Ricinus* agglutinin and subsequently eluted with 0.1 M D-galactose (Gal). Transmittance of the effluents was monitored at 280 nm (——). Fractions of 2 ml were collected and assayed for AFP content (- - - -) by electroimmunoassay. The proteins in each fraction were characterized by polyacrylamide gel electrophoresis. Samples: FS, original fetal serum; 1, pooled fractions 4–15; 2, pooled fractions 31–34. Tr: transferrin; Alb, albumin; Oro, orosomucoid.

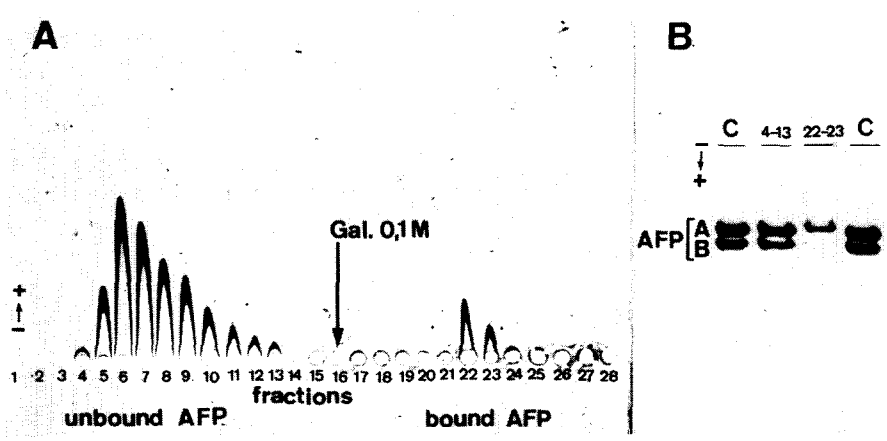

Fig.2. Fractionation of AFP variants on a column containing *Ricinus* agglutinin coupled to Sepharose. (A) Fused rocket immunoelectrophoresis of fractions eluted from the *Ricinus* agglutinin column. The agarose gel contains 1.5 µg/cm² of purified anti AFP and the electrophoresis is performed at 20 V/cm for 2 h. The immunoplate is stained with Commassie brillant blue R. Fractions 4–13 contain the unbound AFP (about 90% of the total AFP) whereas fractions 22–23 contain AFP (about 8% of the total AFP) which is bound to the insolubilized *Ricinus* agglutinin and eluted with 0.1 M D-galactose (Gal). The arrow indicates the start of the elution with D-galactose. (B) Polyacrylamide gel electrophoresis of bound and unbound AFP obtained by affinity chromatography on *Ricinus* Sepharose column. Samples: C, Original AFP; fractions 4–13 correspond to the unbound proteins and contain AFP_{A1} and AFP_B; fractions 22–23 correspond to the bound proteins and contain a single band corresponding to the variant AFP_{A2}.

raphy of previously liberated monosaccharides (0.5 M methanol/HCl, 80°C 20 h) shows that bound and unbound AFP_A exhibit the same content in neutral monosaccharides (mannose and galactose) and in *N*-acetylglucosamine. Nevertheless a difference exists in *N*-acetylneuraminic acid content: the bound AFP_{A2} variant contains only 4 residues while the unbound AFP_{A1} variant contains 6. This result was confirmed when total *N*-acetylneuraminic acid was estimated by the colorimetric diphenylamine procedure.

As shown in fig.3A, AFP_A variants treated with neuraminidase were significantly retained on the *Ricinus* column. It was then confirmed by gas-liquid chromatography that neuraminidase removes quantitatively all the *N*-acetylneuraminic acid residues and unmasks D-galactose terminal residues. However, contrarily to AFP_A, treatment of AFP_B with neuraminidase did not increase its binding; only 4.6% became RCA_I-reactive. This may be partly explained by the fact that the neuraminidase treatment was shown to remove only 50% of the total *N*-acetyl neuraminic acid residues of AFP_B.

3.3. *Estimation of the three variants in the rat serum*

The relative amounts of the three forms AFP_{A1}, AFP_{A2} and AFP_B seemed to vary according to the origin of the α_1-fetoprotein and the age of the animal. Thus, the concentration of the different AFP variants was estimated by immunoelectrophoresis in the sera from 13 and 18 day embryos and 4, 8, 12, 16, 20, 24-day old newborn rats.

The unbound AFP_{A1} variant was always predominant in the sera and its relative amount increases with the age of the animal. This form represents about 52% of the total α_1-fetoprotein in the sera of 13–18 day old embryos and reaches 75% in 24-day old newborn rat sera. With respect to the percentages of the AFP_B and AFP_{A2} variants, the former decreased slightly (38–24%) while the latter declined sharply (10–1%).

AFP reappears frequently in adults with primary

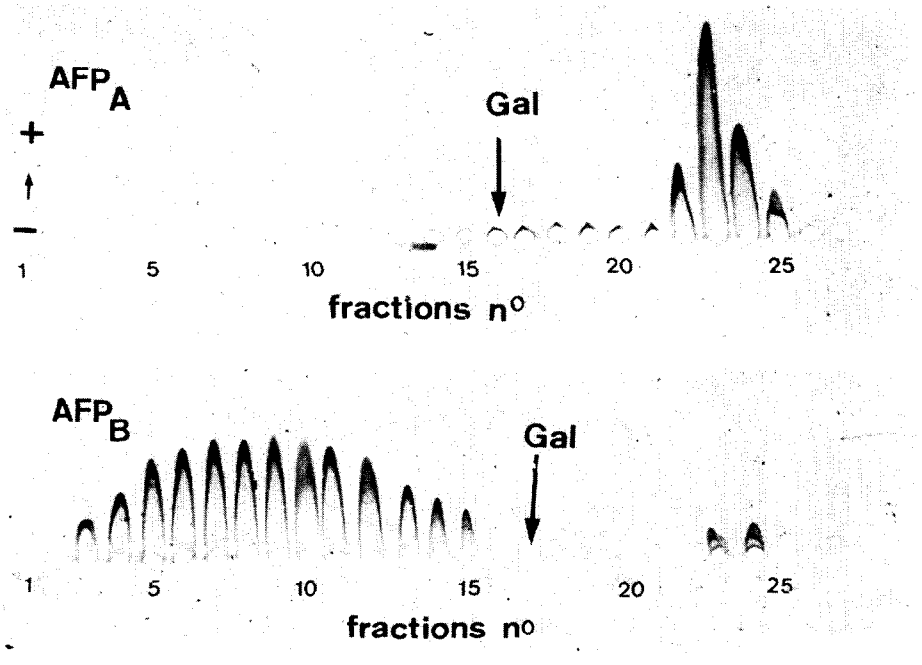

Fig.3. Elution profile of neuraminidase treated AFP_A and AFP_B variants on *Ricinus* agglutinin column. Enzymic desialation was carried out with neuraminidase from *Vibrio cholerae* (100 units/mg of protein). Estimation of AFP was carried out with the immunoelectrophoresis procedure. Sample of 0.5 mg of sialic acid-free AFP_A was passed through the column. 98% of the protein was retained on the column and eluted on a single peak (fractions 22–25) with 0.1 M D-galactose (Gal). The same experiment was performed on neuraminidase treated AFP_B (0.5 mg) but only traces were detected on the bound fraction.

liver cancer [24]. We investigated the 3 forms of AFP described in fetal and newborn rats, in hepatoma bearing adult rats, to see if, in fact, any variations from this pattern became evident. Indeed serum from hepatoma bearing rats contains the three variants AFP_{A1}, AFP_{A2} and AFP_B in the relative amounts 55.6%, 1.7% and 42.7% respectively.

4. Discussion

Although slight carbohydrate variations cannot be detected by classical colorimetric or gas-liquid chromatography procedures, however the use of lectin-Sepharose affinity chromatography reveals some carbohydrate heterogeneity. It was demonstrated that the RCA_I-reactive AFP_{A2} had a lower content of sialic acid (4 residues per mole) as compared to the RCA_I non-reactive AFP_{A1} (6 residues per mole). The most common external sequence of the carbohydrate chain of different serum glycoproteins being *N*-acetylneuraminic acid → galactose → *N*-acetylglucosamine [25], it can be postulated that the lack of two *N*-acetylneuraminic acid residues exposes two D-galactose terminal sites in the reactive form allowing it to react with the *Ricinus* lectin. This observation was confirmed by enzymic desialation of AFP_A which unmasks all D-galactopyranosyl sites and allows the quantitative binding of the desialated variant. In contrast AFP_B glycans appeared to be less accessible as shown by their uncomplete in vitro enzymic desialation and their non-reactivity with the *Ricinus* lectin.

α_1-Fetoprotein is not the unique serum glycoprotein which exhibits such heterogeneity, i.e., variable degrees or sialation of the carbohydrate chains. Indeed previous reports describe the occurence of partially desialated ceruloplasmin [26] and fetuin [27]. The differences in *N*-acetylneuraminic content result either from a partial enzymic desialation by neuraminidase or an uncomplete transfer of this monosaccharide by the glycosyltransferase system to these glycoproteins. The carbohydrate moiety of this partially sialated variant (AFP_{A2}) might play a role in estrogen binding. For example, it could correspond to the minor class of rat AFP described by Benassayag et al. [4] shown to be present in the slow electrophoretic fraction and possessing a very high affinity for 17β estradiol.

This partially sialated form is invariably found in small but significant quantities in fresh sera or adult hepatoma sera and is not an artefact due to the method of isolation. Dosage of these proteins in the serum of rats from birth until the 24th day reveals that AFP_B and AFP_{A2} disappear faster than the AFP_{A1}. This may signify either that they become preferentially localized in certain organs or that they are more rapidly catabolized in the sera.

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