CHARACTERIZATION AND ISOLATION OF NINE RAT ALPHA-FETOPROTEIN VARIANTS BY GEL ELECTROPHORESIS AND LECTIN AFFINITY CHROMATOGRAPHY

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SUMMARY.- AFP purified from Rat amniotic fluid by immunosorbent chromatography, can be divided into nine different microheterogeneous forms. Preparative gel electrophoresis is first used for obtaining the two AFP_A and AFP_B populations. Upon affinity chromatography on RCA_I-Sepharose, the slow electrophoretic form, AFP_A, consists for 90 % of an unbound fraction (A_1) and 10 % of a bound fraction (A_2). Every one of the three AFP fractions (A_1, A_2 and B) are then resolved on Con A-Sepharose in three new AFP components : Con A-non reactive (A_{1a}, A_{2a} and B_a) ; Con A-weakly reactive (A_{1b}, A_{2b}, B_b) ; Con A-reactive (A_{1c}, A_{2c}, B_c). The molecular nature of this heterogeneity is discussed.

INTRODUCTION

The biological role of alpha-fetoprotein (AFP) is still unknown, although informations involving an estrogen-binding ability (1, 2) and an immunosuppressive activity (3, 4) can be considered. However, recent discrepancy in both the estrogen-binding capacity (5, 6) and immunosuppressive potency (7, 8), raise the problem of the purity and of the homogeneity of the AFP preparations. In this regard, the molecular heterogeneity observed in AFP purified from human (9-12) or rat (10, 11, 13-18) might be an important feature. Previous reports on rat AFP have indicated the existence of several molecular species by polyacrylamide electrophoresis (13-17), Concanavalin A (Con A) (11, 18-20) and *Ricinus communis* agglutinin (RCA_I) (21) affinity chromatography. Using a combination of these three separation procedures at a preparative scale, we describe here the characterization and isolation of nine rat AFP variants.

MATERIALS AND METHODS

AFP purification.- The purification of AFP from Wistar rat amniotic fluid (14-18 days gestation) was performed according to the procedure de-

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tailed elsewhere (21). Briefly, it consists in an initial step in obtaining purified anti AFP antibodies by immunoabsorption on an AFP-Sepharose column of Rabbit Anti AFP antisera. The pooled anti AFP antibodies were then coupled to Sepharose 4B (Pharmacia Fine Chemicals) to give a very specific high capacity immunosorbent. This anti AFP-Sepharose was routinely used for the isolation of hundreds milligrams of AFP.

Preparative polyacrylamide gel electrophoresis.- The two AFP electrophoretic populations -AFP_A (slow moving form) and AFP_B (fast moving form)- were obtained by electrophoresis on 20x20x0.5 cm polyacrylamide gel slabs as previously described (16).

RCA_I-Sepharose affinity chromatography.- The *Ricinus communis* agglutinin RCA_I (M.W., 120.000) was obtained by affinity chromatography of Castor Bean extracts on a Sepharose-N-ε-amino-caproyl-β-D-galactopyranosylamine column prepared according to Gordon *et al.* (22). Two lectins RCA_I and RCA_{II} were eluted with 0.1 M galactose and subsequently separated on a G-150 Sephadex column equilibrated with a pH 7.2 phosphate buffered saline. The RCA_I lectin (100 mg) was coupled to Sepharose 4B (20 ml packed volume) previously activated by the CNBr procedure of March *et al.* (23). AFP samples were applied on the RCA_I column (1x10 cm) which was washed with 20 mM phosphate buffer (pH 7.2)-0.15 M NaCl until the A_{280nm} returned to the baseline. The bound RCA_I-reactive material was eluted with the same buffer containing 0.1 M D-galactose.

Con A-Sepharose affinity chromatography.- Concanavalin A was purchased from two different manufacturers (Pharmacia Fine Chemicals and Industrie Biologique Française) and was coupled to the CNBr-activated Sepharose 4B. AFP (2 mg) or amniotic fluid (2 ml) were passed through the Con A column (1.2x10 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.6) containing 1 M NaCl and 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$. The non-reactive and the weakly reactive material (see "Results") were removed by washing the column with the equilibration buffer and the Con A-reactive material was eluted with 0.1 M O-methyl-α-D-glucose in the same buffer.

AFP quantity determination.- The AFP concentration in the fractions eluted from the lectin columns was determined using unidirectional immunoelectroassay (24) on anti AFP impregnated agarose plates.

RESULTS AND DISCUSSION

The original feature of this work follows from the observation that, in adequate conditions, affinity chromatography on Con-A-Sepharose can resolve three fractions instead of the two classical bound and unbound fractions. As illustrated in figure 1, the elution pattern of amniotic proteins is composed of two peaks (*a* and *b*) eluted with the washing buffer and of a third one (*c*) specifically eluted with the methyl-α-D-glucose containing buffer. Whereas albumin is present only in peak *a*, AFP is distributed in peaks *a*, *b* and *c*. Since albumin and AFP are eluted in a single fraction upon chromatography on free Sepharose 4B in identical size column and elution conditions, the retarded fraction (peak *b*) is rather due to its slight affinity towards the Con A lectin than to a possible sieving effect

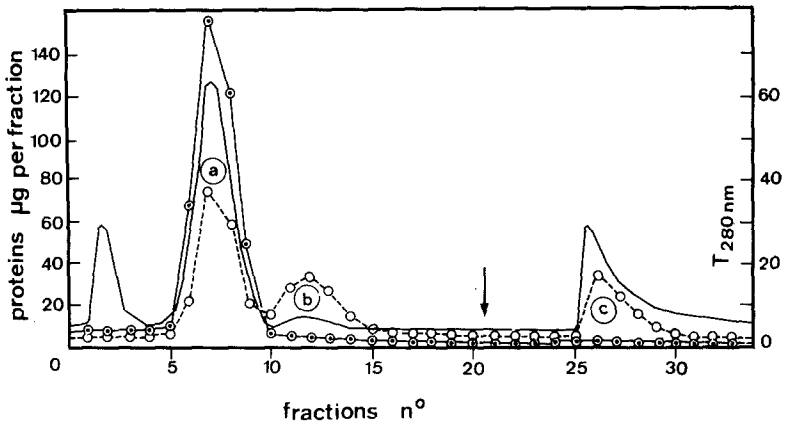

Figure 1.- Con A-Sepharose chromatography of rat amniotic fluid (2 ml). The column (1.2x10 cm) was washed with 60 ml (flow rate : 9 ml/h) of 0.05 M Tris-HCl (pH 7.6)-1 M NaCl-1 mM $MnCl_2$-1 mM $MgCl_2$-1 mM $CaCl_2$. The Con A-reactive material was eluted with 0.1 M methyl-α-D-glucoside in the same buffer (the start of this elution is indicated by the arrow). Fractions of 3 ml were collected. Aliquots were assayed for AFP (-o---o-) and albumin (-o—o-) content by immunoelectroassay. (————) : Transmittance at 280 nm.

or to non-specific interaction with the Sepharose gel. Control experiments with fresh amniotic fluid samples or with each isolated *a*, *b* or *c* fractions have ruled out the artefactual nature of the Con A elution pattern. It is noteworthy that the separation degree of peak *a* from peak *b* depends on the column size. With short column the Con A-weakly reactive AFP is eluted together, or as a peak shoulder, with the Con A-non reactive AFP. With too long columns, peak *b* falls down flat by trailing and can escape notice. These reasons which probably explain why only two AFP Con A-variants have been described in different previous reports (11, 18-20), have prompted us to reconsider the problem of the heterogeneity of rat AFP.

In a first step whole rat AFP obtained by immunosorbent chromatography, has been divided in AFP_A (60 %) and AFP_B (40 %) by preparative gel electrophoresis (16). In a second step, only AFP_A has been further resolved in AFP_{A1} (90 %) and AFP_{A2} (10 %) by affinity chromatography on RCA_I-Sepharose (21). Finally, AFP_{A1}, AFP_B (RCA_I-non reactive) and AFP_{A2} (RCA_I-reactive) were subjected to Con A-affinity chromatography. As shown in figure 2, all the three variants are again heterogeneous, each yielding three Con A-variants. The Con A-non reactive AFP are termed A_{1a}, A_{2a}, B_a ; the Con A-weakly reactive, A_{1b}, A_{2b}, B_b ; the Con A-reactive, A_{1c}, A_{2c}, B_c. The relative amounts of each variants are indicated in figure 3.

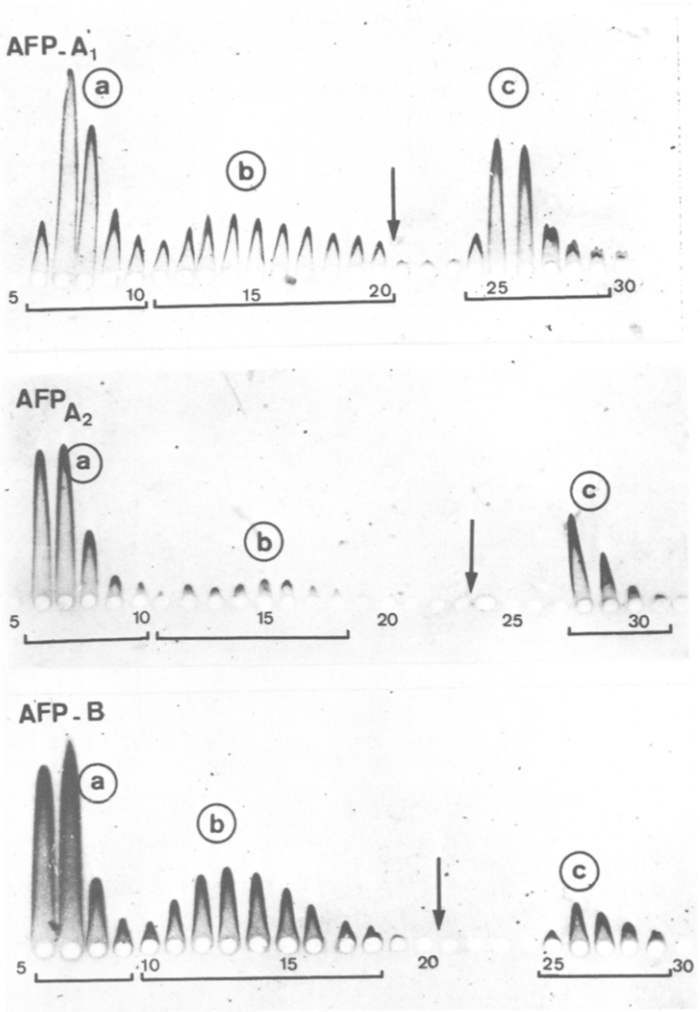

Figure 2.- Fused rocket elution pattern of AFP_{A1}, AFP_{A2} and AFP_{B} on Con A-Sepharose chromatography. The elution conditions of each AFP variant (2 mg) were the same as in figure 1.

From these findings and earlier works (16, 21) it appears that (i) the rat AFP microheterogeneity is more extended than previously described ; (ii) the nature of the microheterogeneity is multiple. Indeed, two or three kinds of heterogeneity can be considered. The first, observed by gel electrophoresis (13-17) can be attributed to differences in the polypeptide chain of the two electrophoretic variants as revealed by their molecular weight and CNBr-cleavage products comparison (21). Then, multiple slight differen-

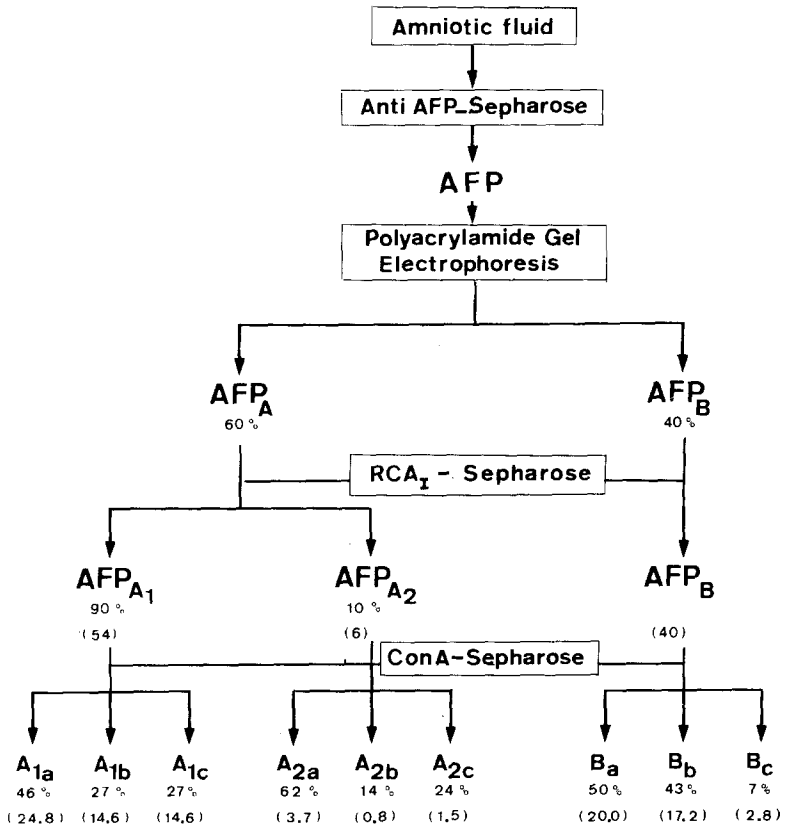

Figure 3.- Scheme of the isolation of the nine AFP variants and their relative amounts in each separation step (values in brackets are expressed as percent of the whole starting AFP).

ces in carbohydrate probably occur to explain the AFP heterogeneity on the lectin columns. The RCA$_I$ lectin is known to be specific for glycoproteins containing non reducing terminal galactose residues (25, 26). Whereas the Con A specificity which is not so strict and well-defined, seems to be related to the presence of partially substituted mannosyl residues in the glycoprotein carbohydrate chains (27, 28). Conformational differences in the polypeptide region carrying the carbohydrate chains may also be responsible for the slight Con A affinity of some AFP variants. Further comparative structural analyses are therefore required to elucidate this problem and in another hand, the estrogen-binding and immunosuppressive abilities of AFP from different sources could be reexamined taking account of these different kinds of molecular heterogeneity.

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