PREPARATION OF ANTISERA TO α-FETOPROTEIN MAKING USE OF ESTRADIOL AFFINITY COLUMN

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1. Introduction

Alpha-fetoprotein (α-FP), a protein of fetal origin which is absent in sera of normal adults, has been recognized also as a tumor antigen associated with hepatoma and with malignant teratoma [1]. The detection of α-FP, and its evaluation as a serological test for primary liver cancer, have been attempted in several laboratories [2—4]. Recently, the development of a sensitive radioimmunoassay for α-FP has been reported [5]. One prerequisite for an immunoassay of any sort is, of course, a monospecific antiserum which will not react with any other serum component.

The methods generally employed for preparation of such a monospecific anti-α-FP serum utilize whole fetal serum [6] or serum from patients with hepatocellular carcinoma [7] for immunization, and exhaustive adsorption of the resultant antiserum with normal human plasma. This method, however, runs the risk that some serum components from normal human plasma may contaminate the antiserum preparation. For this reason, in preliminary experiments in which we tried to use the above approach, we chose for adsorption human plasma that has been cross-linked by glutaraldehyde. We did not find this method for preparing anti-α-FP serum satisfactory, as the repeated adsorptions with insolubilized normal plasma caused a drastic decrease in the anti-α-FP titer.

In a recent publication [8] it was reported that α-fetoprotein of rat has a very strong estrogen-binding capacity, thus enabling specific autoradiograph assay for the protein using labeled estradiol or estrone. It was, therefore, anticipated that the use of a preparation in which estradiol is bound to an insoluble matrix may serve as a means to adsorb α-FP from fetal serum or from the sera of hepatoma patients. We wish to report the preparation of such an insoluble estradiol-containing adsorbent, the specific binding of α-FP to it, and the use of the resulting material for preparation of monospecific anti-α-FP serum.

2. Materials and methods

Sepharose 4B was obtained from Pharmacia, Uppsala; diaminononane from Fluka, Switzerland; labeled estradiol (estradiol-17β-[4-^{3}H], 100 mCi/mM) was from New England Nuclear, Boston; estradiol-17β-monohemisuccinate from Ikapharm, Israel; and 1-ethyl-3-(3-diethylaminepropyl) carbodiimide (EDCI) from the Ott Chemicals Company, Muskagon, Michigan.

Plasma samples containing α-FP from patients with primary liver carcinoma were obtained through the courtesy of Drs. Smith and Osunkoya from the Medical School of the Ibadan University in Nigeria. Normal plasma was from the blood bank of the Kaplan Hospital, Rehovot. Anti α-FP serum samples were purchased from Instituto De Investigacion, Ulta, Spain, or from Meloy Laboratories, Springfield, Virginia.

2.1. *Determination of estradiol-binding proteins*

The estradiol binding proteins in plasma samples were determined by a radioassay using ^{14}C-labeled estradiol, according to the method described by Lindner et al. [9].

2.2. *Immunization procedures*

Immunization of a goat and two rabbits was performed by multisite intradermal injections of the

Sepharose-bound α-FP in complete Freund's adjuvant. 0.5 g of the insoluble material in a total volume of 2 ml adjuvant mixture was injected per goat or per rabbit. A booster injection of a similar amount was given 10 days later. The animals were bled weekly, starting one week after the booster injection.

2.3. *Adsorptions*

Whenever necessary, sera were adsorbed with insolubilized human plasma, obtained by polymerization with glutaraldehyde [10] (1 ml serum adsorbed with with polymerized material obtained from 1 ml plasma).

2.4. *Detection of α-FP or anti-α-FP*

Immunodiffusion according to Ouchterlony [11] or immunoelectrophoresis [12] were used for the detection of either α-FP or antibodies to it in the various samples.

3. Results

3.1. *Binding of estradiol to Sepharose*

The first stage in the preparation of this material was the binding of diaminononane, via one of its amino groups, to CNBr-activated [13] Sepharose. In the second stage, estradiol-17β-monohemisuccinate was bound to the diaminonane-Sepharose preparation via its carboxylic group, using a soluble carbodiimide reagent.

To 10 g of activated Sepharose 4B 20 mg of diaminononane were added in 0.1 M $NaHCO_3$. The reaction was allowed to proceed for 16 hr at 4° with stirring, after which the material was washed with 0.1 M $NaHCO_3$, followed by 50% dioxane/water solution. The estradiol-17β-hemisuccinate (9 mg) was activated in 50% dioxane/water solution with EDCI (5 mg) for 30 min at room temp. After centrifuging off the precipitate the material was added to the diaminononane-Sepharose. The reaction mixture was stirred for 16 hr at 4°. The amount of estradiol bound to diaminononane-Sepharose, 0.45 mg/1 g Sepharose, was determined by monitoring the optical density at 280 nm of the solution before and after the binding procedure.

3.2. *Binding of α-FP to estradiol-Sepharose*

The various plasma samples (5 ml) were passed either over columns made with estradiol-Sepharose beads (2 g), or — as a control — on columns of a Sepharose preparation to which only diaminonane was attached. The amount of estradiol-binding proteins in those samples before and after the adsorption was determined by radioassay (table 1). As seen from the table, both normal and α-FP-containing plasma showed similar levels of estradiol-binding capacity. This binding capacity was not decreased after passage through a column of diaminonane Sepharose. A drastic decrease was, however, observed after passage of α-FP-containing plasma through the estradiol-Sepharose column. In this case the most efficient adsorption of estradiol-binding proteins was obtained from fractions enriched in α-FP, obtained by precipitation between 40% and 70% saturated ammonium sulphate. Only such preparations were used in subsequent experiments. Normal plasma, which does not contain any α-FP as tested by immunodiffusion, showed only a slight reduction in its estradiol-binding capacity after adsorption on the estradiol Sepharose column. The solutions passed through the estradiol-Sepharose column still showed the presence of α-FP in immunodiffusion indicating that the adsorption was not complete.

3.3. *Attempts to elute α-FP*

After passage of the samples containing α-FP, the estradiol-Sepharose columns were washed repeatedly with 0.15 M NaCl—0.01 M sodium phosphate buffer (PBS), pH 7.0, followed by 1 M acetic acid. The acidic eluate, though containing protein (OD_{280} = 1.35), did not show the presence of α-FP. Washing with 0.05 M ammonia, or with 6 M urea, did not bring about the elution of the α-FP from the column.

3.4. *Immunizations*

Rabbits were immunized with the estradiol-Sepharose beads reacted with α-FP enriched fractions and treated as described above. One rabbit (R-1) was immunized with the beads washed only with PBS, whereas the second rabbit (R-2), and one goat (G) were immunized with the beads obtained after washing with both PBS and acetic acid. As shown in fig. 1, all three animals developed anti-α-FP antibodies, but whereas in the antiserum toward the first rabbit (R-1) several precipitin bands in immunodiffusion could be observed, the sera of either R-2 or the goat gave only two precipitin bands with α-FP containing plasma, of which one was identical with commercial anti-α-FP.

Table 1
Adsorption of estradiol-binding proteins by Sepharose-estradiol column.

Sample	Binding of ^{14}C-labeled estradiol (%)		
	Non-adsorbed	Adsorbed on diamino-nonvmane Sepharose	Adsorbed on estradiol Sepharose
Normal plasma	36	36	30
Plasma of hepatoma patient	43	38	23
α-FP-enriched* fraction of above plasma	40	38	17
α-FP-enriched* fraction of another hepatoma plasma	46	40	21
α-FP-enriched* fraction from normal plasma	39	35	33
Human serum albumin (40 mg/ml)	2	20	20

* The fraction precipitated with 70% saturated ammonium sulfate from the supernatant obtained after precipitating with 40% saturated ammonium sulfate.

The second precipitin band disappeared after one adsorption of these antisera with insolubilized whole human plasma (fig. 1). In contrast, a rabbit immunized with the whole serum of a hepatoma patient yielded antiserum giving many precipitin bands (fig. 2), which were very difficult to eliminate even by several adsorptions with insolubilized human plasma. Exhaustive adsorption caused a drastic decrease in the titer of anti-α-FP (fig. 2).

4. Discussion

We have described here a method by which a monospecific antiserum for α-fetoprotein can be prepared. The method is simple, does not require the tedious purification of the antigen, and yet brings about the formation of antibodies to α-FP which are contaminated by antibodies against only one other serum component. The contaminating antibodies can be easily removed in one adsorption step with insolubilized normal plasma.

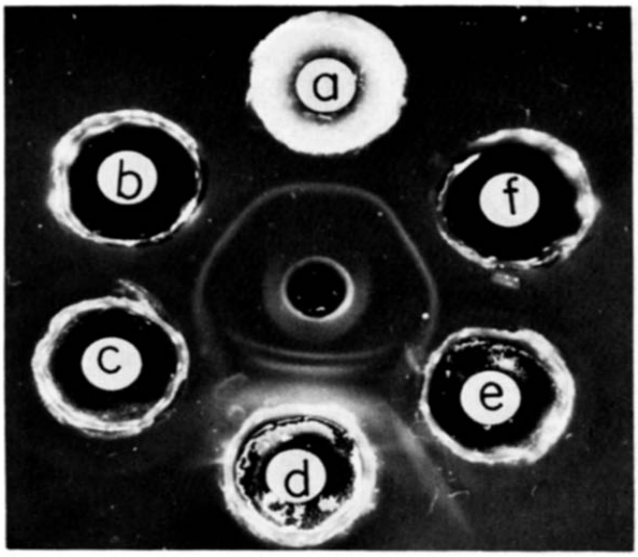

Fig. 1. Immunodiffusion on agar plate. Middle well: plasma of hepatoma patient. Peripheral wells: a) commercial anti α-fetoprotein; b) goat antiserum against α-fetoprotein after one adsorption with insolubilized human plasma; c) goat antiserum before adsorption; d) rabbit (R-1) antiserum against α-fetoprotein, non adsorbed; e) rabbit (R-2) antiserum against α-fetoprotein, non-adsorbed; f) rabbit (R-2) antiserum after one adsorption with insolubilized human plasma.

The method employed here is based on the principles of affinity chromatography – it takes advantage of the affinity binding of estradiol to α-FP, and used an estradiol column for the specific extraction of this protein from whole serum. There are several other proteins in the serum with binding affinity to estradiol [15] and they may also bind to the column. However, all these proteins with the exception of one, are removed from the column by acid elution. α-FP was not removed from the beads by either 1 M acetic acid or 0.05 M ammonia or 6 M urea. This may be due to the high affinity binding of estradiol to α-FP. The reported affinity binding constant of α-FP to estradiol-17β or estron is 10^8 M^{-1} [14], whereas the binding constant of these steroids to human serum albumin is 10^5 M^{-1} [15].

The normal plasma seemed to possess almost as much estradiol-binding capacity as the α-FP-enriched

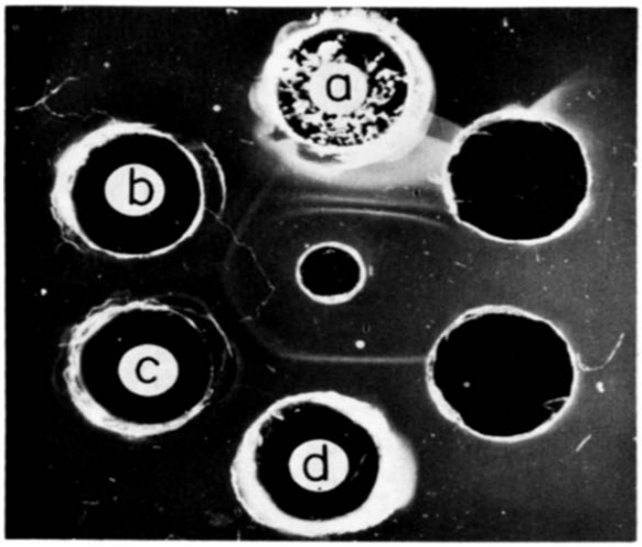

Fig. 2. Immunodiffusion in agar plate. Middle well: plasma of hepatoma patient. Peripheral wells: a) rabbit antiserum against whole plasma of hepatoma patient. b) The same rabbit antiserum after two adsorptions on insolubilized human plasma. c) The antiserum after three adsorptions. d) Commercial anti-α-fetoprotein.

fractions, even though it does not contain α-FP, indicating the presence of other estrogen-binding proteins. Such estradiol-17β-binding proteins were most probably present in the α-FP-enriched fractions as well. They were apparently either eluted from the modified Sepharose by elution with acid, or did not lead to antibody formation, as – with the exception of a single protein as mentioned above – no other antibodies were detected in our experimental antiserum that would react with another human serum protein.

α-FP could not be eluted, under the conditions used, from the estradiol-Sepharose column, and it thus seems that such a column may not be useful for the isolation of α-FP by affinity chromatography. The column procedure was, nevertheless, useful as an efficient and direct method for obtaining a monospecific anti-α-FP antisera.

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