ANTIBODY PRODUCTION AGAINST UNUSUAL ESTROGEN-BINDING PROTEIN OF RAT LIVER AND INTERACTION BETWEEN ISOLATED IMMUNOPRECIPITATES AND ESTRADIOL

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UDC 612.351.1.015.348-063:612.621.31].017.1

KEY WORDS: unusual estrogen-binding protein; antibodies.

Unusual estrogen-binding protein (UEBP) occupies a unique place not only in the heterogeneous system of liver estrogen-binding proteins, but also among receptor and nonreceptor proteins specifically binding steroid hormones known at the present time. The unusual hormone-binding properties of this protein, and androgen-dependence of its induction, and its high sensitivity to the regulatory action of sex steroids all indicate that UEBP may play an essential role as one of the regulators or mediators of the action of sex steroids on the liver, and may also be a marker of androgenic influences on that organ [3, 5-7].

To study the functional role and structural properties of UEBP an essential stage is to obtain a highly purified preparation of this protein. Until now the use of the generally adopted physicochemical methods has resulted, judging from the estrogen-binding activity of the UEBP, in only 50-100-fold purification of the protein, whereas to obtain a homogeneous preparation of UEBP purification by approximately 5000 times would be necessary [3].

The object of the present investigation was to obtain a highly specific antiserum (AS) and to isolate antibodies against UEBP. This may be of great importance both for purification of UEBP by immunochemical methods and also for its immunologic testing in the process of isolation (UEBP is highly labile and quickly loses its hormone-binding properties during purification). The availability of antibodies against UEBP would also provide much wider opportunities for the investigation of its biological role *in vivo*.

EXPERIMENTAL METHOD

Mature male and female rats were used. A partially purified preparation of UEBP was obtained from liver cell cytosol from male rats [5] by salting out with $(NH_4)_2SO_4$ at 55-75% saturation, by gel filtration through Ultragel AcA-44, and by ion-exchange chromatography on DEAE-Sephadex A-50 [3]. A preparation of female liver proteins not containing UEBP was obtained under identical conditions. The preparations were dialyzed and concentrated by rechromatography on DEAE-Sephadex A-50 and precipitation by $(NH_4)_2SO_4$ at 75% saturation. The UEBP preparation obtained from 30 animals, used for immunization, contained 200-250 μg UEBP, on the basis of its estradiol (E_2)-binding activity, and 15-20 mg of total protein.

Male rabbits were immunized by the following scheme: first injection into surgically exposed popliteal lymph nodes and the surrounding tissue of both hind limbs in a dose of 0.2-0.3 ml of a mixture of the UEBP preparation with Freund's complete adjuvant (1:1); second injection of 0.4 ml of the UEBP preparation without adjuvant 1 month later, intramuscularly into the region of the lymph node in the hind limb and into the contralateral forelimb together with intravenous injection of 0.2 ml of the same preparation into the central vein of the ear. Blood was taken one week after the last injection 4 or 5 times on alternate days. The AS titer was determined by Ouchterlony's immunodiffusion method [1]. The AS was exhausted with liver protein preparations by incubation of the appropriate mixture at 36°C for 1 h and at 0-4°C overnight. The resulting precipitate was removed by centrifugation.

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(Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 92, No. 10, pp. 462-465, October, 1981. Original article submitted April 1, 1981.

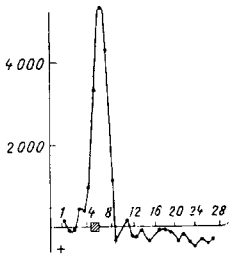

Fig. 1

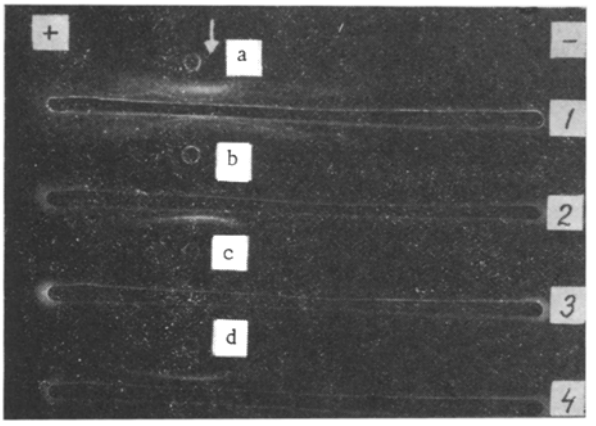

Fig. 2

Fig. 1. Comparison of values of specific ^3H-E_2 binding by precipitates formed during reaction of UEBP preparation with different amounts of mono-AS_{UEBP} (shaded columns) and correspondingly diluted sample of poly-AS_{UEBP} (unshaded column). Abscissa, ratio between antiserum and UEBP preparation; ordinate, specific binding of ^3H-E_2 (in cpm per sample), + and ± signs indicate degree of detection of UEBP preparation by immunodiffusion test with mono-AS_{UEBP} after exhaustion with UEBP preparation in different ways. Similar results were obtained in one or two other experiments.

Fig. 2. Immunoelectrophoretic analysis of interaction between UEBP preparation (a) and of preparation of female liver proteins not containing UEBP (b) with poly-AS_{UEBP} (1), with AS_{UEBP} exausted with proteins of a preparation from female liver (mono-AS_{UEBP}) (2), with mono-AS_{UEBP} exhausted with the UEBP preparation (3), with mono-AS_{UEBP} additionally exhausted with preparation of female liver used in the same quantity as the UEBP preparation (4). Arrow indicates position of UEBP on electrophoresis in agar.

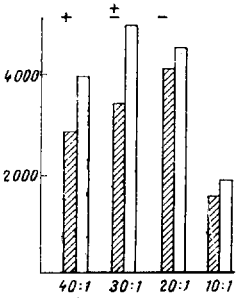

Fig. 3. Determination of position of UEBP after electrophoresis in agar on the basis of E_2-binding activity of extracted agar fractions. Abscissa, Nos. of agar fractions; ordinate, specific binding of ^3H-E_2 in 0.75-ml aliquots of agar fraction (in cpm).

Electrophoresis in 7.5% polyacrylamide gel (PAG) was carried out in Maurer's system 1 [2] in tubes (4.5 × 75 mm) with a current of 3 mA applied to the tube, without the use of concentrating gel. The test material (about 100 µg total protein per tube) was dialyzed beforehand against an eightfold dilution of buffer for concentrating gel. Electrophoresis and immunoelectrophoresis in 1% agar were carried out by Grabar's method [1] for 4-5 h with a voltage of 40-100 V and a current of 10-15 mA in 0.05 M Tris-HCl—0.001 M EDTA (TE) buffer, pH 8.35. After extraction of the agar fractions with 0.01 M Tris-HCl—0.01 M KCl—0.001 M EDTA (TPE) buffer, pH 7.5, containing 0.5 M NaCl, specific binding of E_2 was determined by the method of adsorption on dextran-coated charcoal [4]. The 2,4,6,7-^3H-E_2, with a specific radioactivity of 108 Ci/mmole, was obtained from the Radiochemical Centre, Amersham,

England. To determine specific binding of E_2 by immune precipitates, they were first washed with TPE buffer, suspended in the same buffer, and incubated for 15 min at 0°C with 300 pg ^3H-E_2 with or without 400 ng E_2. The precipitate was thrown down by centrifugation. The ^3H-E_2 bound with the precipitate was extracted with 1 ml 96% ethanol. Radioactivity was measured, specific binding of E_2 calculated, and values of the association constant (K_{assoc}). determined in the usual way [4, 8].

EXPERIMENTAL RESULTS

That it is possible to obtain a practically monospecific AS against UEBP (mono-AS_{UEBP}), only partially purified, is based in principle on the fact that this protein is present in the male but absent in the female liver.

Analysis of the partially purified UEBP preparation and the corresponding fraction of female liver proteins by electrophoresis in PAG showed that from 12 to 15 individual proteins were demonstrable in both preparations; moreover, the preparation of female liver evidently contained practically all ballast proteins present in the UEBP preparation. These findings indicate that the obtaining of a mono-AS_{UEBP} by the following scheme, as used in the present investigation, is a realistic possibility: 1) immunization of rabbits with the partially purified UEBP preparation and the production of polyspecific AS against the UEBP preparation (poly-AS_{UEBP}); 2) exhaustion of the poly-AS_{UEBP} with proteins of the female liver preparation and production of a mono-AS_{UEBP}; 3) exhaustion of the mono-AS_{UEBP} with the UEBP preparation and production of precipitating UEBP—antibodies against UEBP complexes (AB_{UEBP}).

The poly-AS_{UEBP} obtained after immunization with the UEBP preparation can interact not only with the UEBP preparation, but also with a preparation of female liver proteins not containing UEBP. In this case the overall titers of poly-AS_{UEBP} were very close in the first ($^1/_{32}$) and second ($^1/_{16}$-$^1/_{32}$) cases.

Testing the E_2-binding activity of the UEBP in agar fractions after electrophoresis of the UEBP preparation enabled the position of this protein to be determined after electrophoresis (1-3 mm from the start on the cathode side; Fig. 1). Immunoelectrophoresis, carried out under similar conditions, revealed a precipitation arc in the case of interaction between the UEBP preparation and poly-AS_{UEBP} in this same region that was absent in the case of interaction between the preparation of female liver and the same AS, and which evidently reflects complex formation by UEBP with AB_{UEBP}. Meanwhile, immunoelectrophoresis revealed antigens in the preparation of female liver which reacted with practically all seven or eight types of antibodies formed against the ballast proteins of the UEBP preparation (Fig. 2). Consequently, the female liver preparation can be used to exhaust poly-AS_{UEBP} and to obtain mono-AS_{UEBP}.

Poly-AS_{UEBP} exhausted by the female liver preparation in the ratio of 1.0:0.6-0.8 or more begins to give only one precipitation band detectable by the immunodiffusion method. Disappearance of the remaining precipitation bands was due to interaction between antibodies of the poly-AS_{UEBP} with proteins of the female liver preparation in the course of exhaustion, and not to simple dilution of the AS, because control replacement of the female liver preparation by TPE buffer did not affect the number of precipitation bands formed by interaction between poly-AS_{UEBP} and the UEBP preparation. Immunoelectrophoretic analysis of interaction of AS_{UEBP} exhausted by the female liver preparation and the UEBP preparation confirmed the presence of only one distinct precipitation arc in the region coinciding with the position of UEBP during electrophoresis in agar, and the absence of that arc during interaction of the same AS with proteins of the female liver preparation (Fig. 2). This arc evidently reflects the reaction of AB_{UEBP} with UEBP and the AS obtained after exhaustion can be regarded as practically monospecific for UEBP, at least within the limits of sensitivity of the methods used. Additional evidence of the high specificity of the resulting AS for UEBP is that it could be completely exhausted only by the UEBP preparation (exhaustion ratio 10:1, 20:1), but not by the female liver preparation (Fig. 2).

On exhaustion of mono-AS_{UEBP} with the UEBP preparation, for practical purposes only UEBP—AB_{UEBP} complexes will be precipitated. Evidence of the presence of UEBP in the resulting precipitate was given by the ability of precipitating complexes to specifically bind E_2. It was found that the E_2-binding activity of the precipitate depended on the ratio of mono-AS_{UEBP} and UEBP preparation which were mixed (Fig. 3). If there was an excess of mono-

AS_{UEBP}, little specific binding of E_2 took place, evidently on account of screening of the E_2-binding sites of the UEBP by antibodies. The maximum of E_2 binding was observed in the zone of equivalence or corresponding to a small excess of the UEBP preparation, i.e., it approximately coincided with the exhaustion ratio of mono-AS_{UEBP} and the UEBP preparation determined by immunodiffusion. In an excess of antigen binding of E_2 was reduced, probably as a result of incomplete precipitation of UEBP by antibodies (Fig. 3). Values of K_{assoc} for interaction of E_2 with UEBP—AB_{UEBP} complexes, determined in this investigation, were $(3.0-5.0)\cdot 10^8$ M^{-1} and were very similar to values of $K_{assoc.}$ for interaction between E_2 and UEBP itself [3].

The ability of the UEBP—AB_{UEBP} complexes to bind E_2 makes it possible to determine the presence of AB_{UEBP} in AS by the method of testing the E_2-binding activity of the precipitate. Comparison of the content of AB_{UEBP} in poly- and mono-AS_{UEBP} by this method showed that the greater part of the AB_{UEBP} found in poly-AS_{UEBP} remained in the mono-AS_{UEBP} (Fig. 3). This is evidence that the procedure used to obtain mono-AS_{UEBP} have no significant effect on the content of AB_{UEBP} in AS.

Immunochemical analysis of an AS highly specific for UEBP and investigation of the E_2-binding properties of the immunoprecipitates obtained from it thus show that this AS is virtually monospecific with respect to UEBP, and that the UEBP—AB_{UEBP} complexes formed can bind E_2. The precipitates thus obtained can evidently be used to prepare both pure UEBP and pure antibodies against it.

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