AN ANTISERUM SPECIFIC FOR HUMAN SEX STEROID-BINDING PLASMA PROTEIN (SBP)

Jack-Michel RENOIR, Linda L. FOX*, Etienne-Emile BAULIEU and Christine MERCIER-BODARD

Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroides de l'Inserm and *ER 125 CNRS, Hôpital de Bicêtre, Université de Paris-Sud, 78, Rue du Général Leclerc, 94270 Bicêtre, France*

Received 25 November 1976
Revised version received 4 February 1977

1. Introduction

In human plasma, testosterone, estradiol and 17β-hydroxy-5α-androstan-3-one (androstanolone or 5α-dihydrotestosterone) are mainly bound to the 'Sex Steroid-Binding Plasma Protein', SBP [1,2]. SBP was purified to homogeneity using either biospecific adsorbents [3–5] or conventional purification methods [5]. Some physicochemical parameters of this protein have been determined, but a specific antiserum to SBP has not yet been reported.

Crude anti-SBP antisera can be used to check SBP preparations at different purification steps, but specific antisera are required to set up SBP immunoassays and radioimmunoassays, and for immunohistochemical investigations.

The present work will describe the preparation of anti-SBP antisera in rabbits or guinea-pigs. SBP showed high antigenicity but the resulting antisera contained contaminating antibodies. Immunoadsorption onto insolubilized contaminating antigens allowed us to obtain an anti-SBP antiserum, the specificity of which was verified by immunoelectrophoresis and double immunodiffusion.

Abbreviations: SBP Sex steroid-binding plasma protein, PBS 0.01 M phosphate buffered saline, 0.15 M NaCl, pH 7.3, IgG γG immunoglobulin, PAGE polyacrylamide gel electrophoresis, SDS–PAGE Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, R-TeBG Rabbit testosterone-binding globulin

*Present address: Biomedical Division, The Population Council, The Rockefeller University, York Avenue and 66th Street, New York, NY 10021, USA

Some preliminary information was presented in a report to the Vth International Congress of Endocrinology [6].

2. Materials and methods

2.1. *Chemicals*

5α-[1,2-^{3}H]androstanolone (47 Ci/mM) was obtained from Radiochemical Centre Amersham, pure transferrin and pure IgG from Behringwerke, (Marburg, FRG) agarose (Indubiose A 37) from l'Industrie Biologique Française (Paris), Ultrogels ACA 34 and 44 from LKB (Uppsala, Sweden), acrylamide (Biogel P300–400 mesh) from Biorad (Richmond, Calif.), Freund's Complete and Incomplete Adjuvants from DIFCO (Detroit, Mich.).

2.2. *Preparation of purified SBP*

Partially purified SBP was obtained from late pregnancy human plasma after ammonium sulfate precipitation (42% saturation), affinity chromatography on 3 *O*-succinyl 17β-estradiol ethylene-diamine acrylamide, ACA 44 gel-filtration and isoelectric focusing. The purified protein showed a single band in PAGE (7.5% total acrylamide concentration and 2.6% cross-linking agent) according to Davis [7]. It was known that SBP and transferrin are not separated in such a system [8]. Even in those SBP preparations giving only one stained band in PAGE at different acrylamide concentrations, four protein bands were detected in SDS–PAGE [5].

The purification of SBP was completely achieved by repeated ACA 44 gel-filtrations and by an additional isoelectric focusing. Only one stained protein band was then obtained in SDS–PAGE, and one precipitin arc in immunoelectrophoresis against crude antiserum [5].

2.3. *Production of antisera*

Four rabbits (adult males, Fauve de Bourgogne breed) and two guinea-pigs (EVIC-CEBA, conventional tricolor guinea-pigs) were immunized according to the procedure used by Vaitukaitis [9].

Partially purified SBP (100 μg/rabbit, 50 μg/guinea-pig) was emulsified in 2 ml Freund's Complete Adjuvant and was injected at 10–20 dorsal intradermal and two subscapular sites. Booster injections of the same amount of partially purified SBP, emulsified in 2 ml of Freund's Incomplete Adjuvant were given eight weeks later. Ten days later, blood was removed by puncture of the marginal ear vein for rabbits and by cardiac puncture for guinea pigs. The sera were filtered on Millipore (0.45 μm) filters. One ml aliquots of antisera and of preimmune sera were stored at −38°C. Subsequent booster injections of highly purified SBP were given monthly for eight months.

2.4. *Double immunodiffusion and immunoelectrophoretic analyses*

Agarose, 1.4%, in PBS with 0.01% sodium azide was used in double immunodiffusion plates according to Ouchterlony [10]. Immunoelectrophoretic analysis, using agarose-gel concentration of 1.3% in 0.1 M barbital buffer, pH 8.6, were performed according to Grabar [11] as modified by Scheidegger [12]. The electrophoresis was run at 4°C at 250–260 V for 3 trays of agarose-gel on microscope slides under 10 mA. The tank buffer was 0.4 M barbital buffer, pH 8.6, with bromophenol blue as tracking dye. The plates were stained with Coomassie Brilliant Blue for 75 s at 37°C and were destained according to Weber [13].

2.5. *Detection of antibodies against SBP*

Human late pregnancy plasma was precipitated at 42% ammonium sulfate saturation and the pellet was redissolved in 0.05 M Tris/0.05 M $CaCl_2$/0.2 M NaCl, pH 7.4 buffer. Samples containing 40–80 μg of SBP/ml or pure SBP (275 μg/ml) were incubated at 4°C for 3 h with 2 nM [^{3}H]androstanolone alone or in presence of a 1000-fold excess of unlabelled androstanolone.

Preimmune and immune sera were centrifuged at 1500 × *g* for 20 min at 4°C to remove cold precipitated proteins. Aliquots of 20 μl were incubated at 4°C for 24 h with 100 μl of [^{3}H]androstanolone-labelled SBP preparation.

The total incubation media were filtered on ACA 34 columns (17 × 0.5 cm) previously equilibrated with 0.05 M Tris/0.05 M $CaCl_2$/0.2 M NaCl, pH 7.4 buffer. The flow-rate was 3–3.5 ml/h, 0.3 ml fractions were collected and counted in a mixture of 3 ml ethanol and 10 ml Omnifluor (New England Nuclear) in toluene (4 g/litre). All radioactivity measurements were made in an Intertechnique SL 30 liquid scintillation spectrometer in the precalibrated channel for [^{3}H]. Counts were corrected to 100% efficiency by external standardization.

2.6. *Preparation of human IgG and transferrin immunoadsorbents*

Polyacrylamide beads were activated by glutaraldehyde and proteins (IgG or transferrin) were then linked batchwise on the gel as described by Ternynck and Avrameas [14]. Pure IgG, 6.8 mg, or transferrin were dispersed in 6 ml of PBS and mixed with 3 ml of glutaraldehyde activated polyacrylamide beads. An adsorbent containing 1.1 mg of insolubilized IgG or transferrin/ml gel was thus obtained. The adsorbent was then extensively washed and 1 ml of crude anti-SBP antiserum was added to 1 ml of adsorbent and rotated during 48 h at 4°C. The gels were allowed to settle and the supernatants were carefully removed. The beads were then thoroughly washed with PBS and the antibodies adsorbed to the gels were eluted by HCl–glycine 0.1 M, pH 2.8 and pH 2.2 [14].

2.7. *Protein measurements*

The proteins were measured according to Lowry [15] using bovine serum albumin as standard.

3. Results

3.1. *Detection of antibodies against SBP*

The elution profile of anti-SBP antiserum previously incubated with [^{3}H]androstanolone–SBP is

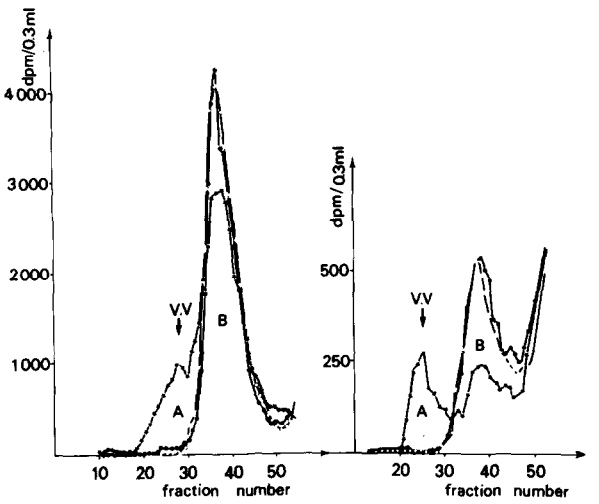

Fig.1. Effluent profiles of Ultrogel ACA 34 gel-filtration. Left panel, 100 μl of 42% $(NH_4)_2SO_4$ human late pregnancy plasma precipitate (about 4–8 μg SBP) incubated with 2 nM of [^{3}H]androstanolone, were filtered (full dots) on a 17 × 0.5 cm Ultrogel ACA 34 column. Effluent profiles of the same antigen(s) after incubation with preimmune serum or antiserum against calf uterus estradiol receptor (empty dots – identical curves) and anti-SBP antiserum (stars) are shown. Right panel, same incubates, with pure SBP (27.5 μg/100 μl) instead of human late pregnancy plasma were filtered. V.V, Void Volume measured by filtration of Blue Dextran.

shown on fig.1. Two radioactive macromolecular complexes were observed, the first one (peak A) in the void volume, the other one (peak B) in the second column volume. [^{3}H]Androstanolone labelled SBP, when filtered alone, was eluted in the peak B. After incubation of anti-SBP antiserum with labelled partially purified or pure SBP, 20% or 50% of the radioactivity of peak B appeared into peak A. This peak A was not observed when other antisera such as antiserum against calf uterus estradiol receptor was used [16].

The two macromolecular entities A and B displayed saturable [^{3}H]androstanolone-binding, since no radioactivity was observed in both peaks when SBP was previously incubated with 2 nM [^{3}H]androstanolone plus a 1000-fold excess of unlabelled androstanolone before exposure to antiserum (data not shown).

In other control experiments using rabbit antisera alone after incubation with [^{3}H]androstanolone, a small but saturable binding of [^{3}H]androstanolone due to R-TeBG [17] was observed in peak B. It represented less than 10% of the binding observed in SBP gel-filtration experiments and did not modify the effluent profiles (data not shown).

3.2. *Specificity of antisera*

In double immunodiffusion experiments, at least four precipitin bands were obtained when human plasma was reacted with anti-SBP antiserum, while one precipitin band was seen with pure SBP (fig.2). These results show that the SBP which had been used for the first and second immunizations (see Materials and methods) was not pure.

The major contaminating antibodies were characterized by immunoelectrophoresis as anti-transferrin and anti-IgG (fig.3) in rabbit and guinea-pig antisera. After several booster injections with highly purified SBP, the precipitin arcs intensity of contaminating

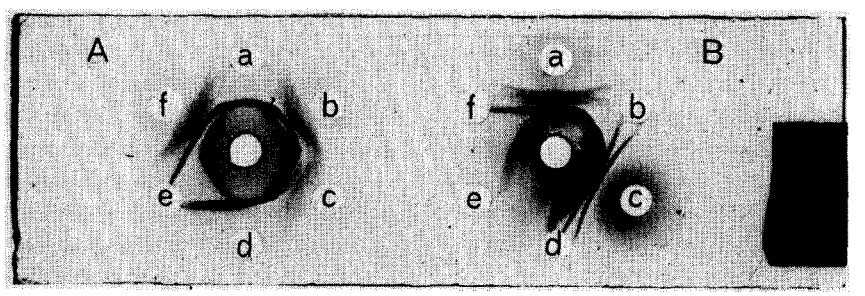

Fig.2. Reactivity of pure SBP and transferrin with rabbit anti-SBP antiserum. Central wells contained 10 μl crude rabbit antiserum (third bleeding). A, in ⓐ and ⓓ 10 μl of pure SBP (640 μg/ml), in ⓑ, ⓒ and ⓕ 10 μl of human late pregnancy plasma diluted, 1/32, and in ⓔ 10 μl of human transferrin (65 μg/ml). B, 10 μl human late pregnancy plasma diluted 1/32 were applied in well ⓐ, pure SBP (640 μg/ml) in ⓑ, human male plasma diluted 1/8 in ⓒ and human transferrin (65 μg/ml) in ⓕ.

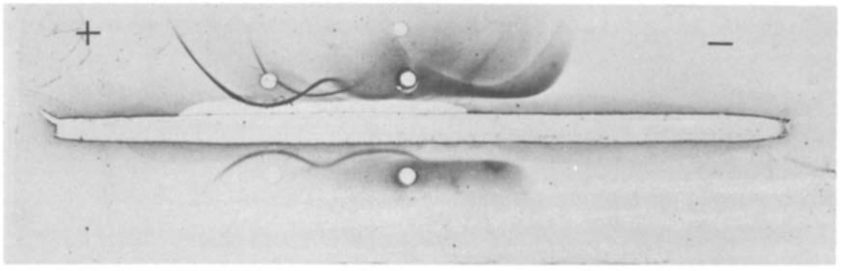

Fig.3. Identification of the major contaminants in rabbit antiserum. 2 μl human late pregnancy plasma diluted 1/4 (right upper well) and 2 μl of 'pure' IgG at 100 μg/ml (right lower well) were separated electrophoretically. Then 2 μl of pure transferrin at 65 μg/ml (left upper and lower wells) were added. At the same time 80 μl of rabbit antiserum were immediately added to the trough and the precipitin arcs were formed at 25°C within 24 h.

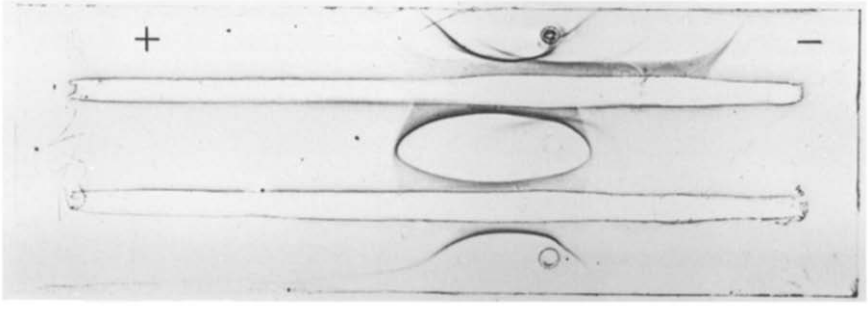

Fig.4. Evidence for a specific anti-SBP antiserum. Rabbit antiserum at tenth bleeding (upper trough) was allowed to react as described in fig.3 against human late pregnancy plasma diluted 1/4 (upper well) and pure SBP (50 μg/ml) (middle well). Part of this antiserum immunoadsorbed on insolubilized IgG (lower trough) was allowed to react against SBP (middle well) and whole human late pregnancy plasma (lower well).

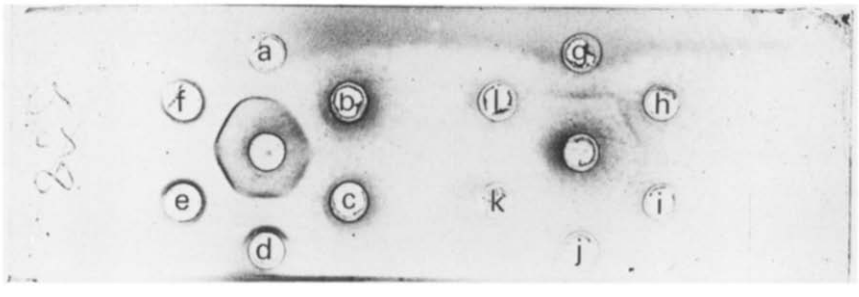

Fig.5. Anti-SBP antiserum efficiency measured by double immunodiffusion. 10 μl specific anti-SBP antiserum were applied in central well. Serial doubling dilutions of human late pregnancy plasma from (b) to (1). (b) = 10 μl of non diluted plasma (c) = 1/2. . . to (1) = 1/1024, 10 μl of pure SBP (160 μg/ml) were applied in (a).

antibodies was decreased while that of anti-SBP antibody was unchanged. Nevertheless, both anti-transferrin and anti-IgG contaminating antibodies were still observed.

3.3. *Purification of rabbit anti-SBP antiserum*

Polyacrylamide–transferrin was ineffective for the total purification of anti-SBP antisera. Since the IgG preparation used contained traces of transferrin (fig.3), the IgG adsorbent was used to remove both IgG and transferrin anti-bodies. After immunoadsorptions, immunoelectrophoresis of the non-adsorbed part of the anti-SBP antiserum showed a single precipitin arc with whole human late pregnancy plasma and with pure SBP (fig.4).

Only one precipitin arc was still observed in the SBP zone with whole human late pregnancy plasma and/or male plasma when immunoelectrophoresis were performed with different concentrations of purified adsorbed anti-SBP antiserum (diluted 10-fold, 4-fold or concentrated 3-fold).

The contaminating antibodies, after elution from IgG-adsorbent formed three strong precipitin arcs in the IgG zone of human plasma and in the transferrin and SBP zones. The latter could be due to a contamination of IgG by SBP and/or to a non-specific trapping of anti-SBP antibodies on the gel.

3.4. *Anti-SBP antiserum efficiency*

Using double immunodiffusion and serial doubling dilutions of whole human late pregnancy plasma against specific anti-SBP antiserum, SBP was detected at a dilution of 1/64 (fig.5) and a total identity reaction was observed between pure SBP and human late pregnancy plasma (fig.5(a) and (f)).

4. Discussion

The preparation of partially purified SBP was used to obtain antibodies against SBP which were detected by gel-filtration and immunodiffusion in agarose-gel.

Gel-filtration experiments showed that a large macro-molecular complex was formed when SBP labelled with [^{3}H]androstanolone was previously incubated with non purified anti-SBP antiserum and was eluted into the void volume of the column (fig.1, peak A). Appropriate controls indicated that the formation of this complex was due to an antibody–SBP interaction.

However, these crude antisera were not specific. This was largely due to the difficulty to obtain absolutely pure SBP. Transferrin and IgG were the main contaminating proteins. The presence of IgG in SBP preparations has been already reported by Bohn [18] who showed that IgG was the major contaminant of his SP_2 preparation, which he suspected to be SBP. Recently, Zöller [19] confirmed that mainly transferrin and IgG of human plasma were non-specifically bound to affinity columns and retained by hydrophobic retention or trapping. These findings could explain why IgG and transferrin were contaminating our SBP preparation because of the use of affinity resin.

The presence in the partially purified SBP preparations of trace amounts of IgG and transferrin were not detected in PAGE since transferrin was not separated from SBP in such a system [8] and the amount of IgG was too slight to be visualized. Both proteins were only detected by immunoelectrophoresis. Immunogenicity of IgG is very strong and, consequently, significant amounts of anti-IgG were produced by the animals when trace amounts of IgG were present in the SBP preparations. Therefore these crude antisera are very useful to monitor the purification of SBP.

Booster injections made with immunologically and electrophoretically pure SBP had improved the specificity of anti-SBP antisera, but contaminating anti-transferrin and anti-IgG were still present.

Immunoadsorption of rabbit anti-SBP antiserum on a synthetic IgG-adsorbent removed both anti-transferrin and anti-IgG and a specific anti-SBP antiserum could then be obtained. This anti-SBP antiserum gives one precipitin band against either human late pregnancy plasma or pure SBP with immunoelectrophoresis and double immunodiffusion.

Work is now in progress on the immunological titration of SBP with glucose-oxidase-linked antibodies and will be correlated to values previously obtained by steroid-binding. Specific anti-SBP antiserum will also be used in immunohistochemical studies.

Acknowledgements

The authors thank Dr P. Robel and Dr E. Eigenmann for valuable discussions, S. Bellorini and F. Boussac for secretarial assistance. The help of the Ford Foundation the Délégation Générale à la Recherche Scientifique et Technique and Roussel-Uclaf are greatfully acknowledged. Linda L. Fox was a post-doctoral fellow of the Population Council.

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