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MONOSPECIFIC ANTIBODIES TO THE SEX STEROID-BINDING PROTEIN (SBP) OF HUMAN AND RABBIT SERA: CROSS-REACTIVITY WITH OTHER SPECIES

Sandra Bordin†, Judy Lewis, and Philip H. Petra

From the Departments of Obstetrics and Gynecology, and Biochemistry, University of Washington, Seattle, Washington 98195

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INTRODUCTION

The existence of a sex steroid-binding protein (SBP) in the plasma of many species including man is now well-established. Understanding of the physiological role of this protein is however far from complete. It has been suggested that SBP is involved in sex steroid hormone action by controlling the concentration of unbound hormone available for target tissues (for review see Reference 1). However, more recently, it has also been suggested that in addition to this function SBP has a direct effect on the sex steroid uptake and metabolism in the prostate (2). We have chosen an immunological approach to study the role of SBP in sex steroid hormone function. The idea is to prepare monospecific antibodies against hSBP and rSBP and perform experiments in appropriate animal model systems. This approach is now possible since homogeneous human SBP (3,4) and rabbit SBP

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All correspondence should be addressed to: Dr. Philip H. Petra.

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(5) can be obtained in relatively large amounts. In this report we describe the preparation of purified monospecific rabbit hSBP-antibodies and guinea pig rSBP-antibodies with their immunochemical properties against the serum of different animals species.

MATERIALS AND METHODS

Reagents and Chemicals.

Human serum collected at eight months of pregnancy was obtained from the University Hospital, Department of Laboratory Medicine. Macaca nemestrina and Papio cynocephalus sera were obtained from the Regional Primate Research Center, at the University of Washington, and the rest of the animal sera were obtained from the Division of Animal Medicine, University of Washington. Freund's Complete and Incomplete Adjuvant was purchased from Difco Laboratories (Detroit, MI). (1,2-^{3}H)-5α-dihydrotestosterone (DHT) (44 ci/mmo) and Omnifluor were purchased from New England Nuclear (Boston, MA); barbital-Na and barbital from Sigma Chemical Co. (St. Louis, MO); agarose from BioRad Laboratories (Richmond, CA); DEAE-Sephadex A50 from Pharmacia Fine Chemical, Inc. (Piscataway, NJ). All buffers were prepared from sodium salts.

Antigen

The hSBP[1] and rSBP[1] used for immunization were purified to homogeneity from late pregnancy human serum (3,4) and immature rabbit serum (5). The pure proteins were stored at 4° C in a solution containing 10 mM Tris, 0.1 M NaCl, ^{3}H-DHT (10^{-5} M - 0.18 ci/mmole), and 10% glycerol. Protein concentration was approximately 1-2 mg/ml as calculated by filter assay (7).

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1. Abbreviations: SBP, sex steroid-binding plasma protein; hSBP, human SBP; rSBP, rabbit SBP; nSBP, Macaca nemestrina SBP; bSBP, baboon (Papio cynocephalus) SBP; DHT, 5α-dihydrotestosterone; PBS, phosphate buffered saline.

Preparation of Antisera

Antisera to hSBP: New Zealand white female rabbits (approximate weight 3 kg) were injected intradermally in the subscapular region with 1 ml PBS containing 50 µg of purified hSBP and 50% Freund's Complete Adjuvant. Three weeks later the animals received a series of three intradermal injections of hSBP at three or four day intervals using increasing dosages of antigen (20, 30, 50 µg of hSBP in 1 ml of PBS and 50% Freund's Incomplete Adjuvant on each succeeding injection). One week after the last injection the rabbits were bled by puncture of the ear central artery. Subsequent booster injection of 50 µg hSBP in 1 ml of PBS were given intradermally every 8-10 weeks.

Antisera to rSBP: Sexually mature female guinea pigs were injected intradermally at several sites in the subscapular region with 0.2 ml of PBS containing 20 µl of purified r SBP and 50% Freund's Complete Adjuvant. The inoculation protocol was repeated twice in the following two weeks. Two weeks after the last injection the animals were bled by heart puncture.

Isolation of Immunoglobulins from Antisera.

The anti-hSBP and anti-rSBP immunoglobulin fractions (IgG, IgA) were prepared from a pool of rabbit antisera and guinea pig antisera according to the procedure of Harboe and Ingild (8). The antibody titer of the anti-hSBP immunoglobulin fraction was estimated by "rocket" immunoelectrophoresis using purified hSBP (9). The specificity of the anti-hSBP and anti-rSBP immunoglobulin fraction was analyzed by "crossed" immunoelectrophoresis (10) using human late pregnancy serum and immature rabbit serum, respectively, as antigens (Figure 1).

Immunoelectrophoretic Analyses.

The immunoelectrophoretic analyses were performed in agarose 1% (w/v) barbital glycin/Tris buffer (pH 8.8) according to Svendsen (11). The immunoelectrophoresis was performed at 10^{o} - 15^{o} C, at 2 V/cm in the gels for 16-18 hours. Bromophenol blue was used as tracking dye. The immunoprecipi-

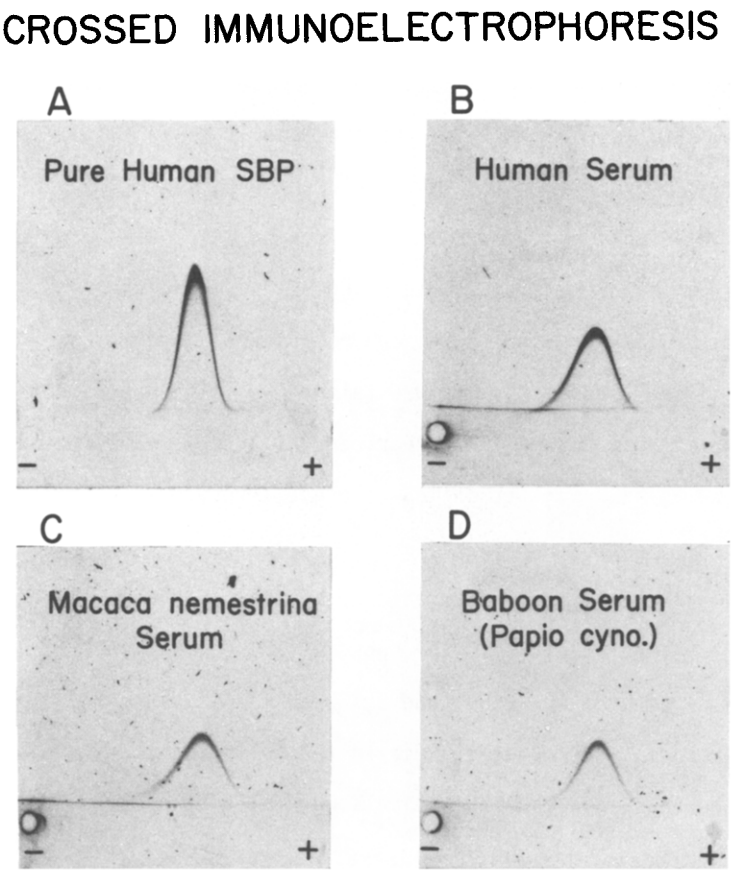

Figure 1. Cross-reactivity of human and non-human primate SBPs with hSBP-antibodies. One ml of the immunoglobulin fraction of rabbit antisera was present per cm^2 of 1% agar in 0.02 M barbital buffer, pH 8.6. Electrophoresis was performed 4 h at 4 v/cm at 10^{o} C in the first dimension and 18 h in the second dimension. The following were added in each well: A) 3 µg pure hSBP; B) 5 µl of undiluted human late pregnancy serum; C) 8 µl of Macaca nemestrina serum; D) 8 µl of baboon serum.

tates were stained with 0.5% (w/v) solution of Coomassie Brilliant Blue R250 in methanol-acetic acid water (45:10:45 by volume) for 15 minutes, followed by background destaining in dye-free solvent. The antigens used in the

immunoelectrophoretic analyses were represented either by total sera or by $(NH_4)_2$ SO_4 precipitated sera.

Assay of 5α-Dihydrotestosterone Binding Activity.

The binding affinity of (1,2,-^{3}H) 5α-dihydrotestosterone (^{3}H-DHT, 48 Ci/mmole) to SBP in the sera of the investigated animal species was performed by the filter assay previously described by Mickelson and Petra (7).

Analytical Slab-gel Electrophoresis of Rabbit Serum.

Agarose slab-gels (1 x 10 cm) were prepared (11) in the presence of 1 nM ^{3}H-DHT. Immature rabbit serum pretreated with charcoal and diluted 50-fold with the barbital glycin/Tris buffer, pH 8.8 was incubated overnight with 10 nM ^{3}H-DHT in the presence or absence of 1000 nM radioinert DHT. After treating the samples with charcoal, aliquots (20 µl) were applied to the gels and electrophoresed at 10° C for 4 hours at 4 volts/cm. Gels were cut into 1.3 mm strips and counted as previously described (19).

RESULTS AND DISCUSSION

The presence of specific hSBP and rSBP antibodies in the antisera of immunized rabbits and guinea pigs was shown by immunoelectrophoresis using pure hSBP and rabbit serum as antigens (Figure 1a and 3). The monospecificity of the hSBP-antibodies and rSBP-antibodies was demonstrated by crossed-immunoelectrophoresis against total human late pregnancy serum and rabbit serum, respectively. In the case of human serum, only one precipitate formed (Figure 1b), which appeared immunologically identical to the purified hSBP by "tandem-crossed" immunoelectrophoretical analysis (12)(Figure 2a). The titer of the hSBP antibodies isolated from a pool of several antisera was estimated to be 1350 µg/ml by "rocket" immunoelectrohporesis (9). Only one precipitate was also detected in the case of rabbit serum (Figure 3). These observations coupled with data on the steroid-binding specificity of pure hSBP (3) and pure rSBP (5) used as antigens in these studies strongly implies that the proteins we have purified have the same immunological and biochemical properties as the native hSBP and rSBP present in plasma; purification did not alter

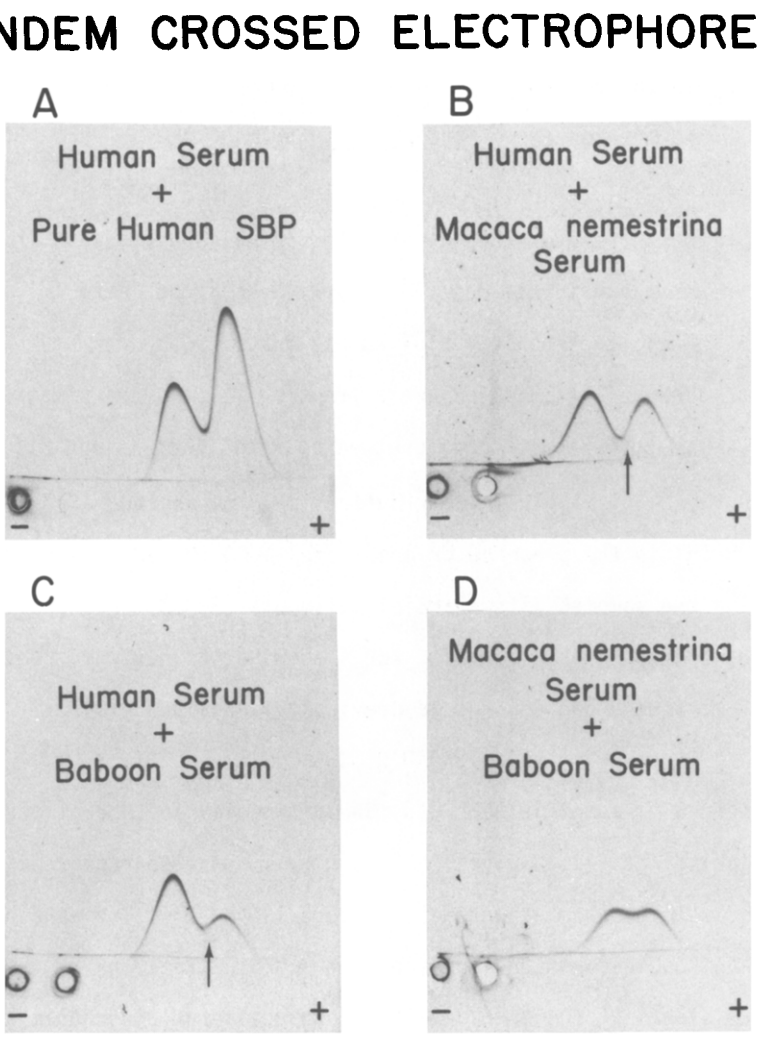

Figure 2. Tandem-crossed electrophoresis of human and non-human SBP with hSBP-antibodies. Same experimental conditions as in Figure 1. The following were added in the adjoining wells, respectively, from left to right: A) 5 µl of undiluted human late pregnancy serum and 3 µg pure hSBP; B) 5 µl of undiluted human late pregnancy serum and 7 µl of Macaca nemestrina; C) 5 µl of undiluted human late pregnancy serum and 7 µl of baboon serum; D) 5 µl of Macaca nemestrina serum and 5 µl of baboon serum. The arrow represents the position of "spurs".

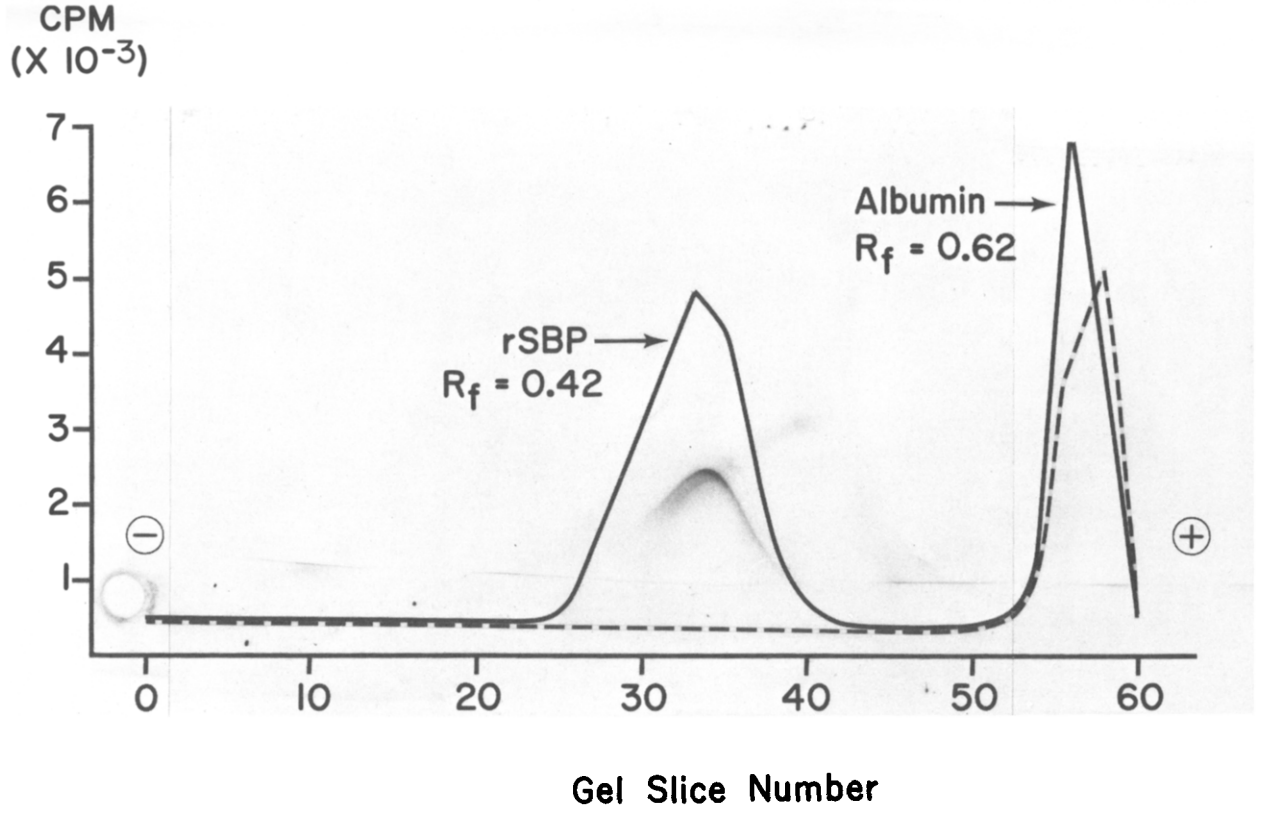

Figure 3. Crossed-immunoelectrophoresis of immature serum with purified rSBP-antibodies. The figure represents a superimposition of an analytical slab-gel electrophoresis of rabbit serum onto the crossed-immuno-electrophoretic pattern. The immunoprecipitate coincides with the Rf of 0.42 of rSBP. Rabbit serum was incubated with 10 nM ^{3}H-DHT in the presence of 1000 nM radioinert DHT (o---o) or in the absence (●——●).

the biological properties of these proteins. Recently, Renoir et al (6) have also prepared an antiserum against human SBP. However, the SBP preparation they used was not pure as shown by the presence of anti-transferrin and anti-IgG. Nevertheless, after purification of their antiserum, comparison of our monospecific hSBP antibody with theirs indicates that hSBP-Seattle and hSBP-Paris are immunologically identical[2].

Studies on the cross-reactivity of the hSBP and rSBP antibodies with sera from other mammalian species where SBP has been previously identified revealed noteworthy differences. The SBPs present in the plasma of rabbit (14,15,16), cat (16), dog (13), sheep (17), goat (17), cow (17,18), and calf[3] do not cross-react with hSBP-antibodies. However, the hSBP antibodies cross-reacted with the sera of the two nonhuman primates Macaca nemestrina (Figure 1c) and Papio cynocephalus (Figure 1d). These two primates are known to have SBP (19,20). The results mean that all three primate SBPs have common relationships in their three-dimensional structures. In tandem-crossed immunoelectrophoresis where two antigens can be analyzed for cross-reactivity with the same antibodies on one gel plate (12), partial immunological identity was demonstrated (Figure 2b and c). This is shown by the existence of "spurs" at the point of intersection of the immunoelectrophoretic patterns. The data imply that the antibodies have determinents directed toward specific areas in the structure of hSBP which do not exist in nSBP[1] and bSBP[1]. The absence of a "spur" when the sera of the two nonhuman primates are compared (Figure 2d) also suggests that the hSBP-antibodies cross-react only with structural domains which are common to both nSBP and bSBP. In the case of the rSBP-antibodies there is no cross-reactivity with SBP present in the plasma of humans, monkey, baboon, cat, dog, sheep, goat, cow, and calf. These results

2. Baulieu, Bordin, Lewis, Mercier-Bodard, Petra, Renoir - unpublished results.

3. Bordin and Petra - unpublished results.

further support that rSBP and hSBP are different immunologically and, as recently shown, biochemically (5).

The antigen preparations containing hSBP and rSBP injected in rabbits and guinea pigs contained a large excess of DHT (10^{-5} M) necessary to stabilize the proteins in the pure state. However, since the rate of dissociation of the hSBP-steroid and rSBP-steroid complexes are very rapid at 37^{o} C, the injected hSBP and rSBP would be expected to exist mainly in the unbound form in vivo. In fact, it can be calculated that about 80% of hSBP is testosterone-free in human plasma at equilibrium using the known concentrations of total testosterone and SBP in the plasma and the K_D for the complex at 37^{o} C (13). Consequently, we would expect the rabbits and guinea pigs to synthesize antibodies mainly against the unbound form of SBP. Our data of Figure 2a show that there is complete immunological identity between pure hSBP (99% saturated with DHT) and human serum where hSBP is mostly steroid-free. Therefore, it does not appear that specific antibodies have been produced against the portion of the polypeptide chain containing the steroid binding site. Although this is expected, since the steroid binding site represents a small fraction of the entire surface of the SBP molecule, the data indicate that the binding site does not have unusual immunological activity. The availability of specific antibodies directed toward the steroid binding site of hSBP would prove very valuable in the elucidation of the tertiary structure of the site using immunological methods.

In summary, we conclude that significant structural similarity exists between hSBP and both nSBP and bSBP. Although it is not possible at this time to correlate these findings in terms of biological function, we have reported, nevertheless, that significant similarities exist between hSBP and nSBP in terms of their steroid binding properties (19). Consequently, these subhuman primates should serve as useful animal models for the study of the physiological role of hSBP. Furthermore, the availability of rSBP-antibodies

should also serve to develop the rabbit model to study the function of SBP in that species and to compare it to the simian model.

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References

1. Westphal, U. (1971) in: Steroid Protein Interactions. Springer Verlag, New York.
2. Mercier-Bodard, C., Marchut, M., Perrot, M., Picard, M.T., Baulieu, E.E., and Robel, P. (1976) J. Clin. Endocrinol. Metab. 43, 374-386.
3. Mickelson, K.E., and Petra, P.H. (1975) Biochemistry 14, 975-963.
4. Mickelson, K.E., Teller, D.C., and Petra, P.H. (1978) Biochemistry 17, 1409-1415
5. Mickelson, K.E., and Petra, P.H. (1978) J. Biol. Chem., 253, 5293
6. Renoir, M., Fox, L.L., Baulieu, E.E. and Mercier-Bodard, C. (1977) FEBS Letters 75, 83-88.
7. Mickelson, K.E., and Petra, P.H. (1974) Fed. Europ. Biochem. Soc. Lett. 44, 34-38.
8. Harboe, N., and Ingfild, A. (1975) in: A Manual of Quantitative Immunoelectrophoresis (Axelsen, H., Kroll, Y. and Weeke, B. eds) pp. 161-164, Universitetforlaget, Olso, Bergen, Tramso.
9. Laurell, C.B. (1966) Anal. Biochem. 15, 45-52.
10. Ganrot, P.O. (1972) Scand. J. Clin. Lab. Invest. 29, Suppl. 124, 39-47.
11. Svedsen, P.Y. (1975) in: A Manual of Quantitative Immunoelectrophoresis (Axelsen, H. Kroll, Y. and Weeke, B. eds) pp. 15-35, Universtitetforlaget, Olso, Bergen, Tramso.
12. Kroll, Y. (1975) in: A Manual of Quantitative Immunoelectrophoresis (Axelsen, H., Kroll, Y. and Weeke, B. eds) pp. 57-59, Universitetforlaget, Oslo, Bergen, Tramso.

13. Tabei, T., Mickelson, K.E., Neuhaus, S. and Petra, P.H. (1978) J. Steroid Biochem. In press.

14. Mahoudeau, T.A., and Corvol, P. (1973) Endocrinology 92, 113-119.

15. Rosner, W., and Darnstadt, R.A. (1973) Endocrinology 92, 1700-1707.

16. Mickelson, K.E. (1977) Ph.D. Dissertation, University of Washington, Seattle, Washington

17. Corvol, P. and Bardin, C.W. (1973) Biol. Reprod. 8, 277-282.

18. Suzuki, Y., Itagaki, E., Mori, H., and Hosoya, T. (1977) J. Biochem. 81, 1721-1731.

19. Petra, P.H. and Schiller, H.S. (1977) J. Steroid Biochem. 8, 655-661.

20. Neuhaus, S., and Petra, P.H.