Studies of the human testis. XIX
Preparation of an antibody to human testosterone-oestradiol-binding globulin and its application to the study of testicular androgen-binding protein

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Abstract. Human testosterone-oestradiol-binding globulin (hTeBG) was purified from pregnancy serum by ammonium sulphate precipitation, preparative eluted electrofocussing, Concanavalin A-Sepharose affinity chromatography, Sephadex G-150 gel filtration, DEAE-Sephadex chromatography and preparative polyacrylamide gel electrophoresis. The yield was 0.3 mg of hTeBG with a specific activity of 1.1 nmoles DHT bound per mg. An antiserum to TeBG was raised in rabbits. Anti-hTeBG IgG was separated from rabbit TeBG by DEAE-Affi-Gel-Blue chromatography. Anti-hTeBG was titrated using protein A-Sepharose which quantitatively binds IgG and therefore bound [3H]DHT-hTeBG-anti-TeBG complexes.

The androgen binding components from human testis were separated on Concanavalin A-Sepharose columns into excluded and retained fractions. The antibody bound both testis fractions with titration curves which paralleled that of TeBG, indicating that these androphilic proteins share common immunodeterminants with hTeBG. The possibility that these testicular proteins are identical in amino acid sequence to TeBG and differ only in carbohydrate content will require further verification. Finally, these results indicate that antibodies to TeBG can be used to study human testicular androgen-binding protein.

An androgen-binding protein (ABP) is found in the testis and epididymis of a variety of species (Hansson et al. 1975). Studies in rats indicate that ABP is produced by Sertoli cells and is secreted into the seminiferous tubule and possibly into the blood (Gunsalus et al. 1978; Bardin et al. 1981). ABP has been purified from the rat (Musto et al. 1980), in which no other extracellular high affinity androphilic protein is found, and from the rabbit (Cheng & Musto 1982).

ABP found in the human testis (hABP) is very similar in physical and chemical properties to human TeBG (hTeBG) (Vigersky et al. 1976; Hsu & Troen 1978), leading to difficulties in its purification and study. These two proteins differ in their interaction with Concanavalin A (Hsu & Troen 1978; Lee et al. 1980); a portion of testis androgen-binding activity does not interact with Concanavalin A-Sepharose, and is identified as hABP (Hsu & Troen 1978; Lee et al. 1980; Cheng et al. 1984), whereas hTeBG is quantitatively retained by this lectin. The excluded protein is unlikely to represent a form of hTeBG modified by our purification scheme since a known amount of hTeBG added to a testis preparation and processed concomitantly was retained quantitatively by Concanavalin A-Sepharose (Lee et al. 1980).

In the present study hTeBG was partially purified, an antibody to the partially purified hTeBG...
was prepared in a rabbit, and the immunoglobulin G (IgG) fraction was obtained. The cross-reactivity of the anti-hTeBG with hABP was examined using a protein A-Sepharose separation procedure combined with a Dextran-coated charcoal (DCC) binding assay, and was found to cross-react completely.

Materials and Methods

Purification of human TeBG

The precipitate obtained from pregnancy serum by 30–50% ammonium sulphate was subjected to preparative flatbed isoelectric focusing using a LKB multiphor unit (LKB Instruments, Inc., Gaithersburg, MD). The TeBG binding activity was located from pH 4.9 to 7.7. The eluate was concentrated to a small volume by ultrafiltration, incubated with 60 nM DHT ([PH]DHT: non-labelled DHT, 1:20) and applied to a Concanavalin A-Sepharose affinity column. The bound material, containing TeBG, was eluted with 10% α-methyl-d-glucoside. Gel filtration of the eluted TeBG was performed on a column (2.5 × 50 cm) of Sephadex G-150 followed by DEAE-Sephadex column (1.5 × 45 cm) chromatography. hTeBG, which eluted at 155 mM NaCl, was concentrated, dialyzed and then applied to a Shandon Southern preparative polyacrylamide gel electrophoresis. The purity of the final preparation was determined in 6% non-denaturing disc polyacrylamide gels prelabelled with 3 nM radioactive DHT (Ritzen et al. 1974). The radioactivity in the gel slices was measured and a parallel gel was stained for protein with 0.1% Coomassie brilliant blue (Davis 1964).

Assay for TeBG activity

TeBG binding activity was measured with a Dextran-coated charcoal (DCC) assay. Since all buffers used during the column chromatographic purification of hTeBG contained DHT, free DHT was first removed from each column fraction with DCC. Each sample was incubated with 3 nM [PH]DHT, with or without 1.2 μM non-labelled DHT, in a total volume of 400 μl for 1 h at 20°C. With more highly purified protein fractions the conventional procedure underestimated steroid binding activity due to charcoal absorption of protein. This underestimation was prevented by adding 0.1% gelatine to each sample.

Examination of hTeBG antibody

Rabbit anti-human TeBG was obtained from one New Zealand white female rabbit immunized with 0.24 mg of hTeBG obtained after the preparative gel electrophoresis purification step. hTeBG, partially purified by ammonium sulphate precipitation, was incubated with [PH]DHT, mixed with diluted normal rabbit serum or anti-hTeBG serum overnight at 4°C and then passed through a column of Sephadex G-150 (1.5 × 50 cm). The [PH]DHT-hTeBG-antibody complex was eluted in the void volume. Protein A-Sepharose CL-4B has the capacity to bind 10 mg of h-IgG per mg. Protein A-Sepharose (0.1 ml) was incubated with antibody and antigen mixtures at 4°C for 1 h to separate the complex of [PH]DHT-TeBG-anti-TeBG from [PH]DHT-TeBG, free [PH]DHT and other proteins, and was thus utilized for the titration of anti-TeBG antiserum and anti-TeBG-IgG. To eliminate the influence of rabbit TeBG, anti-hTeBG-IgG and normal rabbit serum IgG were separated from rabbit TeBG by DEAE-Affi-Gel-Blue chromatography. Two peaks of protein were eluted, the first peak with 70 ml of the buffer alone, and the second peak with 70 ml of buffer containing 1.5 mM sodium chloride. The loss of binding activity of anti-hTeBG-antibody to hTeBG during purification was 40%.

Human ABP

Testicular tissue was obtained from patients undergoing orchidectomy as treatment for prostatic cancer who had received no prior hormone therapy. hABP was prepared from the testis as previously described (Hsu & Troen 1978). Testicular androgen-binding activity was separated into two binding species using Concanavalin A-Sepharose affinity chromatography: the excluded component was eluted in the void volume, whereas the retained component was eluted with 10% α-methyl-d-glucoside (Hsu & Troen 1978; Lee et al. 1980).

Results

Purification of TeBG

The sequential purification scheme of ammonium sulphate precipitation, preparative flatbed electrophoresing, Concanavalin A-Sepharose affinity chromatography, Sephadex G-150 gel filtration, DEAE-Sephadex chromatography and preparative polyacrylamide gel electrophoresis yielded 0.3 mg of hTeBG with a specific activity of 1.1 nmoles DHT bound per mg protein (Bradford 1976). The increase in specific activity over the starting material was 236-fold. This purified preparation was subjected to disc polyacrylamide gel electrophoresis on two separate gels prelabelled with DHT. The gel slices from one gel showed one peak (Rf = 0.38) of activity indicating [PH]DHT bound to hTeBG. The second gel stained for protein revealed two bands of contaminating proteins. The mobility of the most prominent band, however, was similar to that
of radiolabelled hTeBG. A significant loss (70\%) of DHT binding activity was observed during electrophocussing and chromatography. Only 10–20\% loss of binding activity was noted during all other purification steps.

Study of the TeBG antiserum

Gel filtration on Sephadex G-150 was used for the detection of the antibody to hTeBG. In the presence of anti-TeBG antiserum the radiolabelled peak was found in the void volume, representing the formation of \[^3H\]DHT-hTeBG-anti-hTeBG complexes. When mixture of \[^3H\]DHT-hTeBG and anti-hTeBG antibody was adsorbed to protein A-Sepharose prior to chromatography, this peak disappeared from the supernate because the antigen-antibody complex bound to protein A-Sepharose.

![Graph]

**Fig. 1.**

Titration of anti-hTeBG antibody. TeBG previously saturated with \[^3H\]DHT was incubated with 100 \(\mu\)l IgG from normal serum ( ), anti-hTeBG-IgG separated from antiserum ( — — ), or crude anti-hTeBG antiserum ( — — ), in the presence of excess \[^3H\]DHT. Each serum preparation added was diluted from 40- to 5120-fold. After overnight incubation at 4°C, all samples were treated with protein A-Sepharose and the supernatant was assayed by the DCC method. The ordinate represents the per cent hTeBG binding activity remaining in the supernatant after removal of protein A-Sepharose. TeBG alone specifically bound 25 000 CPM of \[^3H\]DHT/0.1 ml.

Titration of anti-hTeBG antibody with hTeBG using protein A-Sepharose

\[^3H\]DHT-hTeBG was incubated with dilutions (40 to 5120) of either anti-TeBG antiserum, anti-TeBG-IgG or normal rabbit IgG and the \[^3H\]DHT-hTeBG-anti-TeBG-IgG complexes were precipitated with protein A-Sepharose. The amount of radioactivity in the supernatant was proportional to the dilution of anti-hTeBG antiserum or anti-TeBG-IgG (Fig. 1). In contrast, normal rabbit serum IgG did not affect \[^3H\]DHT-hTeBG in the medium.

Immuno-cross-reactivity of human ABP with anti-hTeBG

The affinity of the anti-TeBG antiserum for testicular ABP was examined using pooled excluded and retained fractions following Concanavalin A-Sepharose affinity chromatography. Fig. 2 shows that the titration curves of both components of testicular androgen-binding activity were parallel to that of hTeBG, suggesting that each of these steroid-binding proteins has immunodeterminants which are similarly recognized by the anti-TeBG.

Discussion

The sequential purifications used yielded a purified hTeBG, although the preparation still contained one or two bands of contaminating proteins, as judged by gel electrophoresis. An antibody to this purified hTeBG was produced in rabbits and the anti-serum IgG was purified by DEAE-Affi-Gel-Blue chromatography. The antibody-bound radiolabelled hTeBG was detected by Sephadex gel filtration.

Binding of the antigen-antibody IgG complex by protein A-Sepharose offered a simple and sensitive method for quantitation of the immunoreactivity of the anti-TeBG to hTeBG and hABP. In this system, the antigen-antibody complex is quantitated by the reduction in hTeBG bound to radiolabelled DHT present in the supernatant. This indirect labelling assay method may be of use for any antigen for which a labelled cofactor or ligand is readily available and obviates the need to label the antigen directly.

Both the excluded and retained Concanavalin A fractions of human testis androgen-binding activity cross-reacted completely with the rabbit anti-

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TeBG-IgG. This finding agrees with the recent observation of Cheng et al. (1984). Together, these results suggest that hABP and hTeBG share a common immunodeterminant having, in part, a common polypeptide chain structure, and are consistent with a recent report in which peptide maps of hTeBG and hABP promoters were compared (Cheng et al. 1983). Therefore, the similarities in physicochemical (Vigersky et al. 1976; Hsu & Troen 1978) and immunological properties between hTeBG and hABP are compatible with the presence of a common monomer, or a portion thereof, shared by these two proteins. From the currently available data, however, it cannot be determined whether the component of testis androgen-binding protein which adheres to Concanavalin A-Sepharose is hABP and/or hTeBG.

Our finding that hABP can be detected by immunologic methods using an antiserum to hTeBG is consistent with the results of Egloff et al. (1982) who used immunofluorescence techniques and localized to the Sertoli cell an antigen which cross-reacted with anti-hTeBG. These results provide further support for the concept that ABP is a product of Sertoli cells (Sanborn et al. 1975; Louis & Fritz 1977). In contrast, Bordin & Petra (1980) reported that monkey Leydig cells, as well as adrenal cortex, were immunofluorescent positive after application of an anti-hTeBG. Whether there also exists a substance immunoreactive to anti-hTeBG within steroid producing cells which is not merely attached extracellular protein remains to be clarified.

Acknowledgment
The excellent technical assistance of Mrs. Sandy Seguiti is acknowledged.

References


Received on March 1st, 1984.