

Characterization of a monoclonal antibody to human sex hormone binding globulin

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We have produced a monoclonal hybridoma cell line (S₁B₅) that secretes an IgG_{2a} immunoglobulin with a high affinity (K_d 0.38×10^{-11} M) for ¹²⁵I-labelled sex hormone binding globulin (SHBG), and which will specifically immunoprecipitate SHBG from serum. The antibody is produced in high titre in culture medium and ascites fluid, and can be purified to apparent homogeneity by protein A affinity chromatography. When examined by isoelectrofocussing, a characteristic series of bands, which bind ¹²⁵I-SHBG, are observed at pH 8.5–9. Competitive, superimposable, displacement of ¹²⁵I-SHBG from the antibody is achieved with dilutions of human, chimpanzee and gorilla sera at comparable SHBG concentrations. Partial cross-reaction is observed with sera from other Old World primates, but not with sera from New World monkeys or any other vertebrate species studied.

Monoclonal antibody

Sex hormone

Binding globulin

Phylogeny

1. INTRODUCTION

Sex hormone binding globulin (SHBG) is a plasma glycoprotein that is considered to be an important modulator of sex-steroid hormone entry into target cells. The protein has been purified to apparent homogeneity, and antisera of high quality have been produced. These have been used to quantify the protein by various immunochemical methods [1–4], and to study its cellular and subcellular localization [5,6]. Some of the antisera produced to date appear to exhibit monospecific qualities, but the possibility exists that subpopulations of antibodies directed against minor contaminants may elude detection by conventional immunoelectrophoretic techniques. It is mainly for this reason that immunochemical evidence for target cell internalization of SHBG [5] has been received with scepticism.

Abbreviation: SHBG, sex hormone binding globulin; also known as testosterone-estradiol binding globulin (TeBG) and sex-steroid binding protein (SBP)

The generation and careful characterization of monoclonal antibodies to SHBG will undoubtedly help resolve questions to specificity in immunocytochemical studies, and may also clarify the controversy associated with estimates of SHBG molecular mass and subunit structure [7]. In addition, they will also facilitate the development of immunometric assays and obviate the requirement for pure SHBG as either standard or labelled ligand.

2. MATERIALS AND METHODS

2.1. Purification of SHBG

Human SHBG was purified from pooled pregnancy serum, essentially as in [8]. Briefly, 0.5 l serum was applied to a 30 × 5 cm DEAE-Sephacel CL6B (Pharmacia, Uppsala) ion-exchange column, which was washed with 0.1 M Tris-HCl (pH 8.5), 0.02 M KCl and then eluted with a 0.02–0.40 M KCl gradient, developed in the same buffer. Fractions containing SHBG binding activity [9] were pooled, and ap-

plied to a 10×2.6 cm affinity column of 3-oxo-17 β -hydroxy-5 α -androstane-17 α -(6-hexyn-1-ol) covalently linked to diaminoethyloxirane Sepharose CL4B. The column was washed with 0.1 M Tris-HCl (pH 7.5), 0.5 M KCl, 10% dimethylformamide (TKD) buffer until absorbance at 280 nm was negligible, incubated at 25°C for 1 h with a column volume of TKD buffer containing 40 μ g 5 α -dihydrotestosterone (DHT)/ml, and then eluted with TKD buffer. Fractions containing SHBG were pooled, concentrated by ultrafiltration using an Amicon PM10 filter (Amicon Corp., MA), and subjected to preparative polyacrylamide gel electrophoresis (PAGE) (BRL Inc., Bethesda). A 3100-fold purification of SHBG was obtained from the starting material, at a yield of ~20%. The protein was judged pure by analytical PAGE and SDS-PAGE, and a molecular mass of 50.5 kDa was estimated [10].

2.2. Iodination of SHBG

Purified SHBG was radioiodinated with *N*-succinimidyl-3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate (Amersham, England) as in [11]. The ¹²⁵I-SHBG was separated from small molecular mass radioiodinated complexes on a Sephadex G-100 column (20 \times 1 cm), pre-equilibrated with 50 mM phosphate buffer (pH 7.5), containing 0.25% gelatin as carrier protein, and then eluted with the same buffer. This method yields preparations of ¹²⁵I-SHBG with specific activities of 13–21 μ Ci/ μ g protein which appear to be >98% pure when examined by agarose gel electrophoresis (see fig. 3a), and which are stable during storage at –20°C for up to 2 months in 2% horse serum.

2.3. Immunization and cell fusion

Balb/C mice were immunized subcutaneously with 100 μ g SHBG in Freund's complete adjuvant. Antiserum titres were determined by incubation (16 h at 20°C) with 28000 dpm ¹²⁵I-SHBG, precipitation of antibody-bound complexes with 4% rabbit anti-mouse IgG (RAM) antiserum in 10% polyethylene glycol (PEG) 4000 by centrifugation (30 min at 3000 \times g), and aspiration of supernatants to waste. In this way, titres of 1:2000 at 50% of maximum binding (B_{\max} = 18500 dpm) were achieved 2 weeks after a single intraperitoneal (i.p.) booster (10 μ g SHBG in incomplete Freund's adjuvant). Three days after an intravenous injection

of 10 μ g SHBG in physiological saline, spleen cells were isolated from the mouse with the highest serum titres, and fused [12] in the presence of 50% PEG 4000 with equal numbers (1.5×10^7 cells) of X63-Ag8.653 myeloma cells which lack any expression of immunoglobulin synthesis [13]. After fusion, cells were seeded onto 48 \times 1 ml feeder layers containing 5×10^5 spleen cells in HAT selection medium. On day 24 after fusion, 69% of wells exhibited vigorous hybridoma cell growth, 59% of which exhibited anti-SHBG antibody titres (as measured above) equivalent to 10–65% of the maximum binding recorded for the immune serum.

2.4. Cloning and monoclonal antibody production

Wells containing hybridoma colonies secreting the highest titres of anti-SHBG antibodies were cloned and subcloned twice by the limiting dilution technique, using 2×10^5 non-immune spleen cells as feeder layers. One particular clone (S₁B₅) exhibited expansive growth and produced a high antibody titre in culture media (1:8000 or 10 mg antibody/l). Highly concentrated antibodies (1:500000 or 0.8 g antibody/l) were also produced by collecting ascites fluid from pristane (2,6,10,14-tetramethylpentadecane) primed (1 ml i.p. 2 weeks before use) Balb/C mice injected (i.p.) with 10^7 S₁B₅ cells.

2.5. Demonstration of monoclonality

Analytical isoelectrofocussing (IEF) was performed on prefocussed (1 h) thin-layer (0.5 mm) agarose gels containing 2% (w/v) polyampholytes (Serva, Heidelberg), pH 4–9 [14]. Samples of culture media (20 μ l) from several S₁B₅ sub-clones or ascitic fluid (5 μ l) were applied and subjected to IEF for 1 h at 6 W constant power (fig. 1a–c). The agarose gel was then blotted (90 min) with cellulose acetate paper (Cellagram II, Shandon Southern, USA) pre-soaked in a 1:2 dilution of RAM in 50 mM barbitone buffer (pH 8.6). After immunofixation, the gel was washed (16 h in 0.9% NaCl), blotted and dried prior to staining with Coomassie brilliant blue R250, and destained in ethanol–glacial acetic acid–water (35:10:55, v/v/v). In some experiments ¹²⁵I-SHBG (10⁶ dpm/ml) was added to the immunofixation buffer to identify protein bands with binding affinity for

SHBG (fig.1c). After fixation these gels were autoradiographed (96 h at 25°C) using Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

2.6. Identification of Ig class and subclass

The identity of the S₁B₅ antibody immunoglobulin class and subclass was ascertained by Ouchterlony type double immunodiffusion, using specific antisera against mouse IgA, IgE, IgM, IgG, IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ (Miles Laboratories, Slough, England and Nordic Immunological Laboratories, Maidenhead, England). Immunoprecipitation arcs were observed only between S₁B₅ and the IgG or IgG_{2a} antisera.

2.7. Purification of monoclonal antibody

Culture medium (500 ml) containing S₁B₅ antibody was applied at pH 8 to an affinity chromatography column (8 × 0.9 cm) of protein A-Sepharose CL4B (Pharmacia) at a flow rate of 100 ml/h. The column was washed (20 ml/h) with 50 ml of 0.14 M phosphate buffer (pH 8.0), followed by 10 ml of 0.1 M citrate buffer (pH 5.7), and then eluted with 10 ml of 0.1 M citrate buffer (pH 4.5). Eluate fractions (2 ml) were collected in tubes containing 0.4 ml of 1 M Tris-HCl (pH 9.0) and anti-SHBG antibody activity eluted as a single peak at pH 4.5.

The purified S₁B₅ antibody was subjected to analytical SDS-PAGE (T = 7.5%, C = 2.5%). After staining with Coomassie brilliant blue R250 and destaining with ethanol-acetic acid-water (30:10:60, v/v/v), only two protein bands (corresponding to molecular masses of 24.5 and 57.5 kDa) were evident, and these are characteristic of the heavy and light chains of the IgG class of immunoglobulins.

2.8. Antibody affinity and specificity

Dilutions of S₁B₅ culture medium (1:800 and 1:8000) were incubated with ¹²⁵I-SHBG (38000 dpm) for different time periods (0.25–4 h) at 20°C. Separation of bound and free ¹²⁵I-SHBG was accomplished by addition of an equal volume of 4% RAM in 10% PEG 4000, sedimentation of the insoluble antibody-bound complexes by centrifugation (3000 × g, 30 min) and aspiration of the free fraction to waste. Under these conditions, equilibrium between antibody and ¹²⁵I-SHBG was achieved within 2 h at both antibody dilutions

(fig.2a). The affinity of the S₁B₅ antibody was determined as in [15]. A 1:800 dilution of S₁B₅ culture medium was incubated with 0.15–8.5 ng ¹²⁵I-SHBG (13 μCi/μg protein), for 3 h at 20°C, and separation of antibody-bound ¹²⁵I-SHBG was accomplished as described above. The Scatchard plot indicates the presence of a single class of saturable high-affinity binding sites with a dissociation rate constant (*K_d*) of 0.38×10^{-11} M (fig.2b).

To demonstrate that the S₁B₅ antibody recognizes active SHBG, 100 μl of a 1:25 dilution of human pregnancy serum were incubated (16 h at 20°C) with 100 μl of dilutions (1:100) of an S₁B₅ ascitic fluid or a control non-immune ascitic fluid, and the DHT binding capacities of the supernatants were measured after immuno-precipitation of antibody-bound complexes with 4% RAM and 10% PEG, as described above. In this way it could be shown that the S₁B₅ antibody specifically removed >95% of the SHBG binding activity present in the sample.

To assess further the specificity of the S₁B₅ antibody, a 1:50 dilution of human pregnancy serum was ¹²⁵I-labelled as described above (section 2.2), and ~7 × 10⁵ dpm of ¹²⁵I-SHBG were added to a similar amount of the ¹²⁵I-labelled serum proteins. This was incubated (16 h at 20°C) with a 1 ml slurry of S₁B₅ antibody immobilized on carbodiimidazole-activated, microparticulate cellulose (S₁B₅-Ab-cellulose, 40 mg/ml) [16], and then washed sequentially with 0.9% NaCl (3 × 1 ml), 50 mM barbitone buffer (pH 8.6; 2 × 1 ml), 0.9% NaCl (3 × 1 ml), 0.14 M phosphate-buffered saline (pH 7.4, 1 ml), and 0.9% NaCl (2 × 1 ml), at 20°C. Antibody-bound complexes were then competitively displaced by incubation (16 h at 20°C) with 20 μl of the diluted serum used for iodination plus 10 μg SHBG. After centrifugation (3000 × g for 5 min) a sample (5 μl) of the supernatant (~100 μl) was analysed by SDS-PAGE (7.5% T, 2.5% C) after heating at 100°C for 5 min in 1% SDS + 1% mercaptoethanol, using ¹²⁵I-SHBG and the ¹²⁵I-labelled serum protein mixture as reference material. After electrophoresis was completed (3 h), gels were sliced (3 mm) and counted for ¹²⁵I (fig.3).

A study was also conducted to assess the phylogenetic specificity of the S₁B₅ epitope, in which 100 μl of a 1:2 dilution of S₁B₅-Ab-cellulose

(20 mg/ml) and ^{125}I -SHBG (42000 dpm) were incubated (16 h at 20°C) together with serial dilutions of human sera or plasma/serum samples from non-human primates and representative examples of other mammalian and sub-mammalian species. The 5α -dihydrotestosterone (DHT) binding capacities of human and primary samples were determined taking into account species differences in the 'off-rate' of DHT at 0°C [9], and used to evaluate their immuno-crossreactivity relative to DHT binding capacity (fig.4).

3. RESULTS AND DISCUSSION

Established cell fusion and cloning techniques [12] have been used to produce a monoclonal hybridoma cell line (S_1B_5) that secretes an antibody with high affinity and specificity for sex hormone binding globulin. The selection of S_1B_5 was based on the criteria that it exhibited vigorous growth, and produced high titres of an antibody capable of immunoprecipitating $>50\%$ of the maximum amount of ^{125}I -SHBG bound by the immune

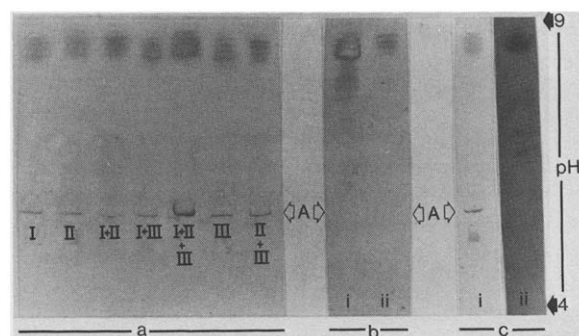


Fig.1. Isoelectrofocussing (IEF) of S_1B_5 culture media and ascitic fluid. (a) Culture media from several sister subclones of S_1B_5 (I-III) were subjected to IEF (pH 4-9) individually or as mixtures, and then immunofixed with rabbit anti-mouse IgG antiserum (RAM); (b) ascitic fluid from a pristane-primed mouse injected with S_1B_5 cells (i) and S_1B_5 antibody purified from this ascitic fluid by protein A-Sepharose affinity chromatography and immunofixed with RAM (ii); (c) S_1B_5 antibody immunofixed with RAM (i) and the same antibody immunofixed with a mixture of ^{125}I -SHBG and RAM (ii). A, application point. See section 2.5 for additional details.

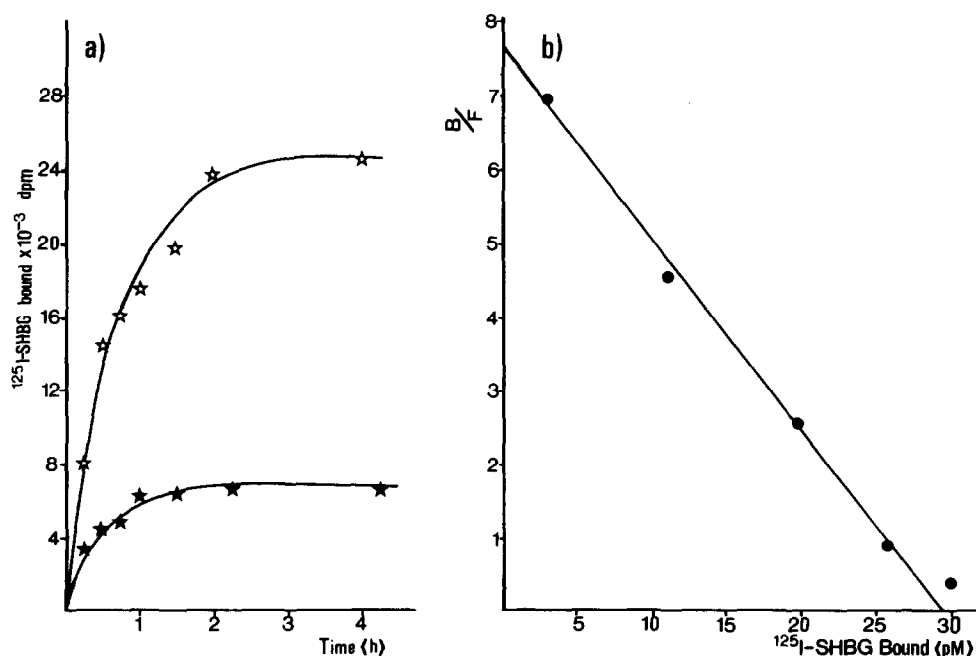


Fig.2. Equilibrium time course (a) and Scatchard analysis (b) of the binding of ^{125}I -SHBG to S_1B_5 antibody. The equilibrium time course was determined at two different culture medium dilutions [(☆) 1:800, (★) 1:8000], while Scatchard analysis was performed using a 1:800 dilution of culture medium; B/F represents the bound/free ratio. See section 2.8 for additional details.

serum. Several other cell lines were derived by repeated cloning and selection, but the antibody titres produced by these clones were rarely $>1:100$. Although these antibodies bound $\sim 20\%$ of the ^{125}I -SHBG used in the screening assay, and could be displaced by the SHBG used as antigen or a 1:10 dilution of pregnancy serum, they were unable to immunoprecipitate SHBG from serum. Further characterization of these clones and their antigenic determinants is in progress. It is, however, pertinent to note that although the SHBG used for immunization and antibody detection was judged to be pure by PAGE and SDS-PAGE, subsequent immunoelectrophoretic analysis of a polyvalent antiserum raised in a rabbit against this antigen revealed the presence of several other contaminating antigens [17]. These contaminants must therefore have been present in very low amounts relative to SHBG, but at sufficient concentrations to evoke an immune response.

Despite rigorous sub-cloning, it was considered essential to demonstrate monoclonality by an alternative method. This was done by comparing the isoelectric spectra of antibodies produced by several sister sub-clones of S_1B_5 . As shown in fig.1a, the same characteristic, microheterogeneous, isoelectric spectrum was found when these antibodies were examined individually or as mixtures. Moreover, each band was capable of binding ^{125}I -SHBG (fig.1c). This phenomenon of microheterogeneity of antibodies produced by monoclonal cell lines is well known, and is thought to be generated by post-synthetic modification of the protein, resulting in changes such as the loss of amide groups from glutamine or asparagine, or differences in acidic charge associated with variable amounts of sialic acid residues [18]. These data, together with the observation that high titres of S_1B_5 antibody were identified in all (24/24) wells which contained apparently clonal S_1B_5 hybridoma colonies after repeated cloning, indicate that the S_1B_5 cell line is monoclonal.

Several other discrete isoelectric spectra were observed in ascitic fluid produced by tumours of S_1B_5 (fig.1b), and undoubtedly reflect contamination by endogenous mouse immunoglobulins. However, S_1B_5 antibody can be conveniently purified to apparent homogeneity (see section 2.7) from culture media or ascitic fluid (fig.1b) by protein A-Sepharose affinity chromatography [19],

and its potential as an immunochemical probe may be anticipated by virtue of its particularly rapid rate of association (fig.2a) and high affinity for SHBG (fig.2b) which are superior to any of the polyvalent rabbit antisera we have obtained [17]. The S_1B_5 antibody not only recognizes a discrete epitope on human SHBG, but unlike the

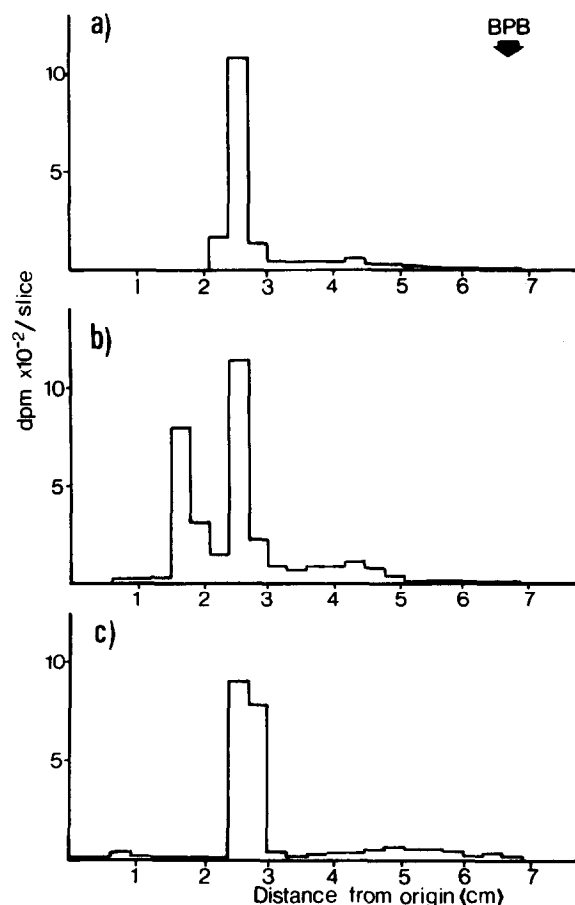


Fig.3. Demonstration that S_1B_5 antibody specifically recognizes SHBG in a diluted serum sample. (a) Pure ^{125}I -SHBG, (b) mixture of ^{125}I -labelled proteins in a 1:50 dilution of human pregnancy serum + pure ^{125}I -SHBG and (c) ^{125}I -labelled protein immunoabsorbed by S_1B_5 and then displaced with a mixture of 1:50 dilution of human pregnancy serum + $10\text{ }\mu\text{g}$ pure SHBG (see section 2.8 for details). The ^{125}I -labelled proteins were analysed by SDS-PAGE after heating the samples (100°C for 5 min) in the presence of 1% SDS + 1% mercaptoethanol. The size of the gel slices was 3 mm and the position of the bromophenol blue (BPB) used as tracking dye is shown.

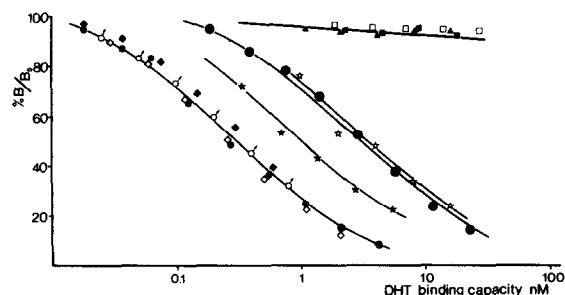


Fig.4. Competitive displacement curves of serum samples from humans and various primates. The relative amounts of SHBG in various samples are expressed in terms of DHT binding capacity (see section 2.8 for details). Human pregnancy (●), human male (○), male chimpanzee (◆), gorilla (◇), gibbon (★), *Macaca arctoides* (●), *Erythrcebus patas* (☆), woolly monkey (■), common marmoset (▲), and ruffled lemur (□). Other species tested include: horse, wallaby, polar bear, tapir, deer, sheep, camel, bison, guinea pig, flamingo, tortoise, bullfrog, iguana and anaconda.

polyvalent rabbit antisera we have generated it also does not interact with heat-denatured SHBG [17], and this implies a high degree of recognition for the tertiary structure of the molecule rather than a particular sequence of amino acids. In terms of species specificity, the exact molecular configuration of human SHBG, that is recognized by the S_1B_5 antibody, is also characteristic of SHBG in the chimpanzee and gorilla. The data also demonstrate that SHBGs in other Old World primates exhibit partial identity with the human S_1B_5 epitope, and it is interesting that SHBG in Old World monkeys cross-reacts less well than SHBG in the gibbon, which is evolutionarily more closely related to the great apes and man. However, in contrast to previous studies of the phylogenetic specificity of polyvalent anti-human SHBG antisera [4,20], it is clear that the S_1B_5 antibody does not cross-react with SHBG in New World monkeys or the lemur.

In conclusion, the characteristics of the S_1B_5 antibody indicate that it may represent an invaluable reagent for use as a labelled antibody in immunometric assays and immunocytochemical localization studies and may also reveal information about the molecular structure of SHBG.

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