Monoclonal antibodies to the pituitary growth-hormone receptor by the anti-idiotypic approach

Production and initial characterization

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We obtained 10/192 and 3/384 antibody-secreting hybrids after immunization of Balb/c mice with either human growth hormone or affinity-purified rabbit anti-(human growth hormone) respectively. Radiolabelled rabbit anti-(human growth hormone) antibodies, but not human growth hormone, were specifically bound by supernatants from the 13 hybrids. The binding was completely inhibited by human-growth-hormone serum binding protein. However, anti-(human growth hormone antibodies) were detected in the sera of all the mice immunized with human growth hormone. In an independent fusion, which was carried out after immunization with fewer doses of human growth hormone, anti-(human growth hormone) antibodies were also obtained. Five hybrids, where the starting antigen was human growth hormone, were selected for ascites production, and the corresponding monoclonal antibodies were partially purified and characterized with respect to their immunoglobulin isotype and their interaction with human-growth-hormone receptors. These antibodies were found to enhance the binding of radioiodinated human growth hormone to human-growth-hormone serum binding protein from human and rabbit plasma by 40%. Scatchard analysis of the effect of one of the monoclonal antibodies showed that this enhancement was due to an increased number of binding sites. All of the partially purified antibodies but one (F12) inhibited the binding of human growth hormone to rat but not rabbit, liver microsomes to various extents, as well as to H-4-II-E rat hepatoma cells. Monoclonal antibody F12 enhanced the binding of radiolabelled human growth hormone to rat liver microsomes and H-4-II-E hepatoma cells. This enhancement was found to be due to an increase in the number of binding sites.

INTRODUCTION

The importance of pituitary growth hormone for promoting normal body growth is well established. The mechanism, however, by which this action is brought about is not yet known, but it is well documented that the hormone binds to a cell-surface receptor (Isaksson *et al.*, 1985). Pituitary growth hormone belongs to a family of immunologically and structurally related peptide hormones including prolactin and placental lactogen, the receptor interactions of which overlap to various extents (Hughes & Friesen, 1985). Human growth hormone (hGH) binds with high affinity to all known receptors for growth hormone and prolactin throughout the vertebrates (Nicoll *et al.*, 1986).

Binding sites for growth hormone have been demonstrated in several subcellular fractions and in various tissues in different species (Simpson *et al.*, 1983; Hughes *et al.*, 1985). Different receptor classes appear to be present within a given tissue (Barnard *et al.*, 1985). Recently, a putative growth-hormone receptor from rabbit liver membranes was purified and partly sequenced together with a rabbit-growth-hormone serum binding protein (Spencer *et al.*, 1988). The two proteins were

shown to have identical *N*-terminal sequences. Rabbit and human receptor sequences were also determined from the corresponding cDNA clones (Leung *et al.*, 1987).

Previously, polyclonal antisera (Waters & Friesen, 1979) and, recently, monoclonal antibodies (Simpson *et al.*, 1983; Barnard *et al.*, 1984, 1985) have been produced to provide tools for growth-hormone-receptor studies. The monoclonal antibodies were raised against growth-hormone receptors of rabbit and rat liver and were shown to influence hormone binding to different liver receptor preparations to various extents (Barnard & Waters, 1986a). Other monoclonal antibodies were found to be directed against epitopes outside the hormone-binding sites of the receptors.

The theory of the idiotypic network and the internal image of the antigen has provided a new approach for the study of membrane-bound receptors (Jerne, 1974; Strosberg, 1984). Antibodies (anti-idiotypic) can be raised against the binding sites of other antibodies from a different species and from another individual of the same species. According to the idiotypic-network theory, such antibodies can also appear within the same individual as part of an immunoregulatory mechanism

Abbreviations used: bGH, bovine pituitary growth hormone; HAT, hypoxanthine/aminopterin/thymidine; hGH, human pituitary growth hormone; hGH-SBP, human-growth-hormone serum binding protein; oPrl, ovine prolactin; r.i.a., radioimmunoassay; PBS, phosphate-buffered saline (composition and pH are given in the text).

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(autoanti-idiotypic antibodies) (Erlanger *et al.*, 1986). If the starting antigen is a receptor ligand, the anti-idiotypic antibodies may mimic the ligand's interaction with the receptor. Both heterologous and autologous anti-idiotypic antibodies have been obtained after immunization with several hormones and receptor ligands, and they have been shown to interact with the corresponding receptors (for references, see Ku *et al.*, 1987).

We applied the anti-idiotypic approach for the study of growth-hormone receptors with the hope of obtaining monoclonal antibodies that could mimic the universal growth-hormone-receptor-binding activity of hGH. Balb/c mice were immunized with either hGH or affinity-purified rabbit anti-hGH. Spleen cells of the mice were then fused with sp 2/0 myeloma cells. Screening of the resulting hybrids gave several monoclonal antibodies which were found to interact with growth-hormone-receptor preparations as described in the present paper.

EXPERIMENTAL

Reagents

Recombinant hGH (Somatorm) was generously given by KabiVitrum AB, Stockholm, Sweden. Bovine pituitary growth hormone (bGH) was purchased from Miles Laboratories, Stoke Poges, Slough, Berks., U.K. Ovine prolactin (oPrI) and bovine serum albumin fraction V were from Sigma Chemical Co., St. Louis, MO, U.S.A. Rabbit anti-mouse immunoglobulin subfractions kit and single-reaction Enzymobead radioiodination reagent were bought from Bio-Rad, Richmond, CA, U.S.A. Rabbit anti-(human haptoglobin) and rabbit anti-(mouse immunoglobulin) antisera were from Dakopatts, Copenhagen, Denmark. Mouse monoclonal IgG₁ (BN 11.6) has already been described (Nilson *et al.*, 1987). Goat anti-(rabbit immunoglobulin) antiserum was prepared in this laboratory (Björck *et al.*, 1977). Monoclonal rat anti-(mouse immunoglobulin light chain) antibodies were prepared as described by Brodin *et al.* (1989). Na¹²⁵I was from Amersham International. CNBr-activated Sepharose 4B, which was used for coupling hGH according to the instructions of the manufacturer, DEAE-Sephadex A-50 and Sephadex G-25 were purchased from Pharmacia, Uppsala, Sweden. Novacell ultra-filter cells were bought from Filtron Technical Corp., North Borou, MA, U.S.A., and Centricon 10 microconcentrators were purchased from Amicon, Danvers, MA, U.S.A.

Cell cultures and animals

Sp 2/0 myeloma cells, fetal-calf serum, HAT and hypoxanthine/thymidine solutions were bought from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. All other culture medium constituents were bought from Gibco, Paisley, Renfrewshire, Scotland, U.K. H-4-II-E rat hepatoma cells (Benedict *et al.*, 1973) were bought from the American Type Culture Collection (A.T.C.C. no. CRL 1548), Rockville, MD, U.S.A. Sterile culture dishes, flasks and tubes were from Nunc, Roskilde, Denmark, and flexible microtitre plates were from Becton-Dickinson Labware, Oxnard, CA, U.S.A.

Balb/c mice (6–7 weeks of age) were bought from Charles River Breeding Laboratory, Margate, Kent, U.K., and Sprague-Dawley rats (35–40 days of age, 140–150 g) were purchased from Alab, Sweden. The rabbits were from a local supplier.

Calculation of binding data

Binding data were subjected to Scatchard (1949) analysis to give the number of binding sites and the dissociation constants. The data presented are means for triplicate determinations. In Figs. 2–7 (below) the s.d. for each data point is expressed as the percentage in relation to the amount of specifically bound radiolabelled hGH in the absence of other additions.

Production of antibodies

Rabbits were injected subcutaneously with 50–100 µg of hGH in phosphate-buffered saline [PBS; 15 mM-sodium phosphate (pH 7.4)/0.15 M-NaCl] and Freund's complete adjuvant. Similarly, the animals were boosted every 4 weeks with hGH in PBS and Freund's incomplete adjuvant.

Mice were injected intraperitoneally and in the footpads with 50–100 µg of hGH or affinity-purified rabbit anti-hGH in PBS, together with Freund's complete adjuvant. Booster doses in Freund's incomplete adjuvant were given after 4, 7 and 11 weeks. The last booster dose was given intraperitoneally without adjuvant 3–4 days before fusion. The booster schedule used for the study of the time course of appearance of antibodies in mouse serum is described in the legend of Fig. 1 (below). Blood was sampled from the orbital vessels of the mice with a capillary Pasteur pipette.

Fusion of sp 2/0 myeloma cells with spleen cells from immunized mice was carried out as described by Oi & Herzenberg (1982). After distributing the fused cells into 96-well plates in HAT medium with 20% (v/v) fetal-calf serum, the medium was changed every 3–4 days. At 10–15 days after fusion, growing hybrids could be seen in more than 95% of the seeded wells. Cloning was done by limiting dilution. In order to get large amounts of antibodies, monoclonal-antibody-producing hybrids were injected into Balb/c mice as described by Oi & Herzenberg (1982).

Purification and characterization of antibodies

Polyclonal rabbit anti-hGH antibodies from serum and monoclonal antibodies from ascites fluid were purified by ion-exchange chromatography on DEAE-Sephadex A-50 as described by Nilson *et al.* (1986). The purity of the immunoglobulins was evaluated by SDS/PAGE (Laemmli, 1970) on 10% gels. After staining with Coomassie Blue G-250 and drying, the gels were scanned in a Joyce-Loebl Integrating Chromoscan 3 densitometer.

On the basis of the scanning results, the purity of the immunoglobulins from ascites fluid was estimated to be 30% for the various preparations. None of the monoclonal antibodies showed binding of radiolabelled hGH in the solid-phase radioimmunoassay (r.i.a.) described below or in an ordinary r.i.a. The concentration of these partly purified monoclonal antibodies was determined from their A₂₈₀, an A₂₈₀^{1 mg/ml} value of 1.25 (Hardy, 1986) being used.

The fractions from the DEAE-Sephadex chromatography of rabbit serum which showed high binding of radiolabelled hGH in the r.i.a. (see below) were pooled, and samples corresponding to an original amount of 20 ml of rabbit serum were loaded on to an hGH-Sepharose column (3.9 mg of hGH coupled to 0.5 g of Sepharose) at 4 °C. The column was washed with 15 mM-

Tris (pH 8.0)/0.15 M-NaCl/0.02% NaN_3 /0.1% Triton X-100 (column buffer) until the A_{280} of the eluate was below 0.010. Anti-hGH antibodies were then eluted with 15 ml of 3 M-KSCN in the column buffer and immediately dialysed twice against 2 litres of 15 mM-Tris (pH 7.4)/0.15 M-NaCl. The dissociation constant for the binding of these antibodies to hGH was 4×10^{-9} M as determined from r.i.a. data, where 0.5 ng of radiolabelled hGH had been incubated with 0.8–1.6 $\mu\text{g/ml}$ of antibodies and dilutions of hGH.

Immunoglobulin samples were concentrated to the desired concentration in the Novacell and the Centricon 10 concentrators.

The monoclonal antibodies were typed using the class- and subclass-specific rabbit anti-(mouse immunoglobulin) subfractions kit in a solid-phase r.i.a. as suggested by the manufacturer.

R.i.a.

The binding of radioiodinated hGH to rabbit anti-hGH antiserum or purified preparations of rabbit anti-hGH was determined in an r.i.a. in the following way. Samples of serum or samples of purified rabbit anti-hGH were diluted to various extents with r.i.a. buffer [100 mM-sodium phosphate (pH 7.4)/0.1% bovine serum albumin/0.02% NaN_3]. A 0.5 ng portion of ^{125}I -labelled hGH was added to a final volume of 0.4 ml. After the mixture had been incubated overnight at 4 °C, 0.3 ml of ox serum and 1.6 ml of 15% (w/v) poly(ethylene glycol) in r.i.a. buffer were added. After centrifugation at 1900 g for 15 min, the pellets were counted for radioactivity in a γ -radiation counter. As a control, dilutions of purified polyclonal rabbit anti-haptoglobin were run in parallel; these antibodies did not exhibit binding of labelled hGH.

For screening of supernatants of hybrids, 96-well microtitre plates were coated overnight with 600 ng of monoclonal rat anti-(mouse immunoglobulin light chain) antibody/well in PBS ('first layer'). Then the plates were washed three times with PBS/0.05% Tween. Mouse antibodies or the mouse hybridoma supernatants were added diluted in PBS/0.05% Tween ('second layer') and the incubation was continued for 2 h. The plates were then washed with PBS/0.05% Tween and 30000–50000 c.p.m. of radiolabelled hGH or affinity-purified rabbit anti-hGH were added ('third layer'). In some instances partly purified rabbit hGH serum binding protein (-SBP) was included in the third layer at a final dilution of 1:1000. After 1 h the plates were washed and the wells cut and counted for radioactivity in a γ -radiation counter. All the incubations were kept at 4 °C, and the volume added per well was 50 μl .

The specificity of this screening method for mouse antibodies directed against the hGH-binding site of the rabbit immunoglobulins was estimated in the following way. With radioiodinated rabbit anti-hGH added in the third layer, the fraction of the added radioactivity which was bound was 10–15% with serum from mice immunized with rabbit anti-hGH and 3–5% with serum from mice immunized with rabbit anti-(mouse immunoglobulins) in the second layer. Only 0.12% was bound with normal mouse serum in the second layer. There was no binding between the rat antibody in the first layer and the radiolabelled rabbit anti-hGH. The binding of radiolabelled rabbit anti-hGH antibodies could be displaced almost completely by the addition of unlabelled rabbit

anti-hGH at a protein concentration of 10 $\mu\text{g/ml}$ (results not shown).

Purification of the hGH-SBP

Serum from male rabbits was diluted 4-fold with ice-cold PBS. Then an equal volume of 30%-satd. $(\text{NH}_4)_2\text{SO}_4$ solution at 4 °C was added, with stirring, on an ice bath, and after 1 h the suspension was centrifuged at 5000 g for 10 min. The pellet was dissolved in 15 mM-Tris (pH 8.0)/0.15 M-NaCl/10 mM- CaCl_2 /0.02% NaN_3 and then dialysed against 2×2 litres of the same buffer. This resulted in a 33-fold purification of the SBP with a yield of 50%, as determined by its ability to inhibit the binding of radiolabelled rabbit anti-hGH to monoclonal mouse anti-(anti-hGH antibody M8) (see below). The dialysed sample was then subjected to hGH-Sepharose affinity chromatography as described above, except that 10 mM- CaCl_2 was added to the buffer, the detergent was excluded and the KSCN concentration was increased to 4 M. The hGH-SBP was purified 5000-fold by the precipitation and affinity-chromatography steps. hGH-SBP from man was prepared in a similar way. A similar approach has been developed by Herington *et al.* (1987).

Binding to hGH-SBP

The affinity-purified hGH-SBP preparations were coated on flexible microtitre plates overnight at 4 °C at a protein concentration of 0.03 mg/ml in 15 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/10 mM- CaCl_2 /0.02% NaN_3 . In parallel control experiments we coated flexible microtitre plates in the same way with rabbit anti-hGH and anti-(human haptoglobin) antiserum. After washing the plates three times with PBS/Tween, radioiodinated hGH was added with dilutions of unlabelled hGH or unlabelled monoclonal antibodies in 25 mM-Tris/HCl (pH 7.5)/10 mM- CaCl_2 /0.1% bovine serum albumin/0.02% NaN_3 (radioreceptor assay buffer). The incubation was continued for 2 h at 4 °C and then the plates were washed three times with PBS/Tween, the wells being cut out and their radioactivity measured in a γ -radiation counter.

Preparation of liver microsomes

Microsomal membranes were prepared from the livers of male and female rats and of male or female rabbit by the method of Thomas *et al.* (1987). Aliquots of the resulting microsomal preparations suspended in radioreceptor assay buffer were frozen and stored at –20 °C. For binding studies, samples of microsomal membranes [$(15\text{--}150) \times 10^{-15}$ mol of high-affinity hGH binding sites/tube] were incubated with 0.2–0.5 ng of radioiodinated hGH and dilutions of unlabelled hGH or monoclonal antibodies in radioreceptor assay buffer in a total volume of 0.5 ml. The tubes were incubated overnight at 4 °C. Ice-cold 25 mM-sodium acetate, pH 5.4 (1.5 ml) was then added and, after 30 min, the tubes were centrifuged at 1500 g for 15 min. The supernatant was discarded by suction and radioactivity in the tubes was measured in a γ -radiation counter. For data presentation the specific binding was calculated by subtracting from the total radioactivity in the pellets the residual radioactivity in the pellets in tubes incubated with 1 μg of hGH.

Hepatoma cells

H-4-II-E hepatoma cells were plated at a density of 15000 cells/cm² in plastic Petri dishes (100 mm diameter). The cells were grown in Dulbecco's modified Earle's

medium supplemented with 10% (v/v) foetal-calf serum and 10% newborn-calf serum, 2 mM-sodium pyruvate, 2 mM-glutamine, penicillin (50 i.u./ml) and streptomycin (50 µg/ml). The cultures were incubated at 37 °C in 7% CO₂, and the medium was changed every 3–4 days. When the cell cultures became confluent, the dishes were washed with 10 ml of PBS. The dishes were washed once more with 3 ml of 25 mM-Hepes (pH 7.5)/120 mM-NaCl/5 mM-KCl/1 mM-KH₂PO₄/1 mM-MgSO₄/2.5 mM-CaCl₂/0.04% glucose/0.1% bovine serum albumin (KRH buffer). The cells were then incubated in the latter buffer at 37 °C on a shaker with various dilutions of unlabelled hGH or monoclonal antibodies. After 30 min, 0.5 ng of radioiodinated hGH was added to a final volume of 2.5 ml and after another 1.5 h the dishes were washed once with 10 ml of ice-cold PBS. In order to harvest the cells, 1 ml of 1% trypsin in PBS was added for 5 min and the resulting cell suspension was transferred to a tube. The dishes were rinsed again with 1 ml of 0.5 M-NaOH and 0.1% Triton X-100, and this was combined with the previous cell suspension. The radioactivity in the tubes was measured in a γ -radiation counter.

Radioiodination

Proteins were iodinated with 0.5 mCi of Na¹²⁵I by the use of the Enzymobead radioiodination reagent. The specific radioactivity of the labelled hormones was 30–50 mCi/mg of protein and of the affinity-purified rabbit anti-hGH, 80–120 mCi/mg of protein.

RESULTS

After injecting into mice four doses of antigen, fusion of spleen cells with sp 2/0 myeloma cells was carried out. Screening was started 10–15 days later. From three successive screenings of hybrids from mice injected either with purified rabbit anti-hGH or with hGH we obtained 3/384 and 10/192 stable hybrids respectively, which produced antibodies that bound radioiodinated rabbit anti-hGH (Table 1). The binding was completely inhibited by the addition of unlabelled rabbit anti-hGH or (NH₄)₂SO₄-precipitated rabbit hGH-SBP. In these fusions, which took place 11 weeks after the first immunization, we did not obtain any hybrids producing antibodies which bound radioiodinated hGH. Such antibodies were obtained, though, in an earlier fusion where only the hGH-binding capacity was sought. In that case the mice were given three doses of hGH and the fusion took place only 7 weeks after the first immunization and we obtained 26/192 positive hybrids (M. I. Elbashir, unpublished work).

Along with the mice injected with hGH, from which we obtained 10/192 hybrids, five other mice were treated similarly. These five mice were not used for fusion, but instead they were bled at time intervals and their serum was tested for the binding of radioiodinated hGH or rabbit anti-hGH. Bleeding was done 34, 64, and 97 days after the first injection with hGH. Antibodies binding radioiodinated hGH were already present in the sera at the time of the first bleeding, whereas a response resulting in antibodies binding radioiodinated rabbit anti-hGH was not obvious until the second bleeding (Fig. 1).

After cloning by limiting dilution and expansion of the hybridomas secreting antibodies binding radioiodinated rabbit anti-hGH and where the binding could be inhibited by hGH-SBP, five clones were selected for ascites pro-

Table 1. Monoclonal antibodies obtained from the screening of stable hybridomas from two fusions

The antigen used for generating these antibodies was hGH, except for the antibodies marked '*', in which case affinity-purified anti-hGH was used.

Monoclonal antibody	Isotype	Binding of ¹²⁵ I-labelled rabbit anti-hGH (%)
2B5*		4.0
E11		14.0
F12	IgG ^{2b} κ	14.2
F5	IgG ^{2a} κ	23.7
G5*		11.6
G9		12.3
M2		13.9
M8	IgM κ	15.6
M10	IgG ^{2a} κ	15.9
M11	IgG ^{2b} κ	12.6
M13		18.9
M14		21.8
M16*		20.0

duction, antibody purification and determination of immunoglobulin isotypes. The starting antigen for these five clones was hGH (Table 1). The results reported below refer to these five purified monoclonal antibodies, namely M8, M10, M11, F5, and F12.

The relation of the five purified monoclonal antibodies to the growth-hormone receptor was studied by investigating how they affected the binding of radioiodinated

Fig. 1. Time of appearance of anti-hGH and anti-anti-hGH in the pooled sera from five mice

The mice had been immunized on day 1 and boosted on days 27, 50 and 78. The ordinates show the percentage bound of the total amount of labelled hGH (●) or labelled rabbit anti-hGH (○) added in the screening assay described in the Experimental section. The sera were added at a dilution of 1:5 in the assay. Control values (obtained with normal mouse serum) have been subtracted.

Fig. 2. Enhancement of radiolabelled hGH binding to affinity-purified hGH-SBP from human serum in the presence of monoclonal antibodies (MAbs)

The ordinate shows the percentage of radiolabelled hGH specifically bound at various concentrations of unlabelled hGH (Δ), monoclonal antibodies M8 ($\square$), M10 ($\blacksquare$), M11 ($\blacktriangle$), F5 ($\circ$) or F12 ($\bullet$) or the control mouse monoclonal IgG₁ antibody ($\blacklozenge$). In all, 18% of the total amount of the radiolabelled hGH added was bound to the hGH-SBP, and 90–95% of the bound hGH was specifically bound. The s.d. for each point varied between 6 and 13%.

hGH to various growth-hormone membrane-bound receptor preparations or hGH-SBP. When affinity-purified hGH-SBP from male rabbit or man serum was coated on flexible microtitre plates, we were able to detect binding of radioiodinated hGH by up to 50% of the added radioligand. The K_D for the interaction of the hGH and the rabbit hGH-SBP was 0.6×10^{-12} M. Various concentrations of monoclonal antibodies were added with a fixed amount of radioiodinated hGH to the hGH-SBP-coated plates. All the five purified monoclonal antibodies enhanced hGH binding by approx. 40% to hGH-SBP from human and rabbit serum, as exemplified in Fig. 2 for human hGH-SBP. This enhancement was verified further by investigating the effect of unlabelled hGH on the binding of radioiodinated hGH to rabbit hGH-SBP in the absence or presence of purified monoclonal antibody M10 (0.48 mg/ml). When M10 was present, the binding of the radiolabelled hGH increased (Fig. 3). Scatchard (1949) analysis revealed an increase of the number of binding sites by 40%, with no effect on the K_D of the binding. The mouse monoclonal IgG₁ had no effect.

In contrast with the results seen with the rabbit hGH-SBP, the five purified monoclonal antibodies did not enhance, nor did they inhibit, the binding of radioiodinated hGH to male or female rabbit liver microsomes (results not shown). The concentration of the monoclonal antibodies was varied up to 0.8 mg/ml.

Of the five purified monoclonal antibodies, all but F12

Fig. 3. Effect of monoclonal antibody M10 on the interaction of radiolabelled hGH with rabbit hGH-SBP

The Figure shows the percentage of specifically bound radiolabelled hGH at various concentrations of unlabelled hGH in the absence of any additions ($\bullet$) or in the presence (0.48 mg/ml) of M10 ($\blacksquare$) or control mouse monoclonal IgG₁ ($\circ$). The s.d. for each point varied between 2 and 5%.

inhibited the interaction of radioiodinated hGH with the microsomes from male rat liver to various extents. M10 and F5 showed stronger inhibition than M8 and M11,

Fig. 4. Effect of the five partly purified monoclonal antibodies (MAbs) on the binding of radiolabelled hGH to male-rat liver microsomes

The Figure shows the percentage of specifically bound radiolabelled hGH at various concentrations of hGH ($\blacklozenge$), monoclonal antibody M8 ($\blacksquare$), M10 ($\bullet$), M11 ($\square$), F5 ($\circ$) or F12 ($\blacktriangle$) or the control mouse monoclonal IgG₁ antibody ($\triangle$). About 10–20% of the total amount of radiolabelled hGH was bound, and 65–75% of that binding was specific. The s.d. for each point varied between 1 and 10%.

Fig. 5. Effect of two of the partly purified antibodies on the binding of radioiodinated hGH to H-4-II-E hepatoma cells

The Figure shows the binding of radiolabelled hormone at various concentrations of unlabelled hGH in the absence (Δ) or in the presence of monoclonal antibody (MAb) M10 ($\circ$), M11 ($\bullet$) or control mouse monoclonal IgG₁ ($\blacktriangle$). The total amount of radiolabelled hGH bound was 5%, and 40–60% of that binding was specific. The s.d. for each point varied between 2 and 9%.

Fig. 6. Effect of monoclonal antibody (MAb) F12 on the specific binding of radioiodinated hGH to male-rat liver microsomes

The ordinate shows the percentage of specifically bound radioiodinated hGH in the presence of unlabelled hGH ($\circ$) or monoclonal antibody F12 ($\bullet$). The s.d. for each point varied between 3 and 10%.

Fig. 7. Effect of monoclonal antibody F12 on the titration of male-rat liver microsomes with hGH

The specific binding of radioiodinated hGH was measured at different concentrations of unlabelled hGH in the absence ($\circ$) or presence (0.8 mg/ml) of monoclonal antibody F12 ($\blacksquare$) or monoclonal mouse IgG₁ ($\bullet$). The s.d. for each point varied between 3 and 10%.

although not of the same magnitude as that seen with unlabelled hGH (Fig. 4).

Similar experiments were performed with female-rat microsomes. In this case, partial inhibition of the binding of radioiodinated hGH was seen, but only with M10, M11 and F5 (results not shown). The extent of inhibition was lower, about 20–30% of the effect of unlabelled hGH, than that seen with male-rat liver microsomes, although comparable with the effect of bGH, which was also less efficient an inhibitor with female rat liver microsomes. Purified monoclonal antibody M8 had no effect on the binding of radioiodinated hGH to female-rat liver microsomes.

Radioiodinated hGH bound to H-4-II-E hepatoma cells in monolayer culture with the KRH buffer used in those experiments. Binding of radioiodinated hGH was not seen with the radioreceptor-assay buffer. The inhibitory effect of purified monoclonal antibodies M8, M10, M11 and F5 was the same, and was similar to that seen with male-rat liver microsomes as shown for M10 and M11 in Fig. 5.

Purified monoclonal antibody F12 had no effect on the binding of radioiodinated hGH to male- or female-rat liver microsomes up to a concentration of 0.05 mg/ml (Fig. 4). Above that concentration the binding of radioiodinated hGH was enhanced 5-fold (Fig. 6). Such enhancement was also seen with H-4-II-E hepatoma cells (results not shown). The titration of male-rat liver microsome hGH-binding sites with unlabelled hGH was investigated in the absence or presence of excess amounts (0.8 mg/ml) of F12 and control monoclonal IgG₁ (Fig. 7). With F12 present there was a 4-fold increase in the number of specific binding sites for radioiodinated hGH, with no effect on the dissociation constant. Repeated

testing of F12 for the binding of hGH in the ordinary r.i.a. or the solid-phase r.i.a. was negative.

DISCUSSION

Monoclonal antibodies have been found to be useful as tools for the study of hormone receptors for gaining information about receptor structure and function not accessible previously by the use of polyclonal antibodies. In the present paper we have described the attempts to obtain monoclonal antibodies directed against the growth-hormone receptor after immunization with the hormone or anti-hormone antibodies from a different species, rather than with the receptor itself.

After immunization with the hormone, we could detect anti-hGH and anti-(anti-hGH) antibodies in the sera of the mice. The time-dependence of their appearance was different (Fig. 1). In the fusion which was carried out in a parallel experiment, we only obtained anti-(anti-hGH) antibodies (Table 1), although anti-hGH antibodies were also obtained in an earlier fusion where the animal received only three doses of the hGH. The results can be explained by the cyclic pattern of antibody response as part of an immunoregulatory mechanism, as was suggested by Jerne (1974). It has been shown that activated B-cells fuse more efficiently than non-activated B-cells (Goding, 1980). A likely explanation for our findings is that anti-hGH-producing B-cells, which were activated after injections with hGH, readily fused with myeloma cells 7 weeks after the first injection, but became less favoured as fusion partners after 11 weeks in comparison with the complementary anti-(anti-hGH)-producing B-cells, which were activated at a later stage in response to the appearance of anti-hGH antibodies.

From the 13 monoclonal antibodies obtained which bound rabbit anti-hGH and where the binding was inhibited by rabbit hGH-SBP, five were selected for partial purification and characterization. Of these five antibodies, at least three (M8, M11 and F12) represented antibodies from different clones as judged by the isotyping and subsequent binding studies, whereas the other two, although different from the others, were indistinguishable.

The interaction of growth hormone with various receptor structures can be classified as being either 'somatogenic' or 'lactogenic' (Cadman & Wallis, 1981). Binding sites from which growth hormone can be displayed by bGH are called 'somatogenic', and binding sites from which growth hormone can be displayed by human placental lactogen or oPr1 are called 'lactogenic' (Hughes *et al.*, 1985). Male- and female-rat liver microsomes contain somatogenic and lactogenic binding sites for hGH in different proportions (Haldosén & Gustafsson, 1988). None of the four competing antibodies (F5, M8, M10 and M11) was capable of displacing the hGH completely from the rat liver microsomes or the H-4-II-E hepatoma cells, a finding which could indicate some specificity for one subtype of hGH receptor. With male- (in contrast with female-) rat liver microsomes, the bGH is almost as efficient in displacing radiolabelled hGH as is hGH itself. The four competing monoclonal antibodies were less competitive when female-rat liver microsomes were used, indicating somatogenic binding specificities. More thorough specificity studies are needed, though, before the affinity of these antibodies for the somatogenic or lactogenic hGH binding sites can be established.

However, none of them affected the binding of radio-labelled hGH to male- or female-rabbit microsomes. An explanation for this finding is that rabbit anti-hGH antibodies were used for the identification and selection of the monoclonal mouse antibodies. The rabbit anti-hGH antibodies are likely to be specific for epitopes in the hGH not found in the rabbit growth hormone. They can preferentially bind to, and detect, such monoclonal antibodies which mimic hGH epitopes not found in the rabbit growth hormone. Consequently, these antibodies will bind poorly to the rabbit growth-hormone receptor.

We found the dissociation constant for the interaction of the affinity-purified hGH-SBP from the rabbit (when coated to microtitre plates) with hGH to be a few orders of magnitude lower than the value reported previously (Barnard & Waters, 1986b). This increased affinity could be the result of conformational changes in the binding protein upon adsorption to the microtitre-plate walls. The interaction was specific for hGH, however. With the same microtitre-plate assay, none of the binding proteins from the species investigated would bind radiolabelled bGH or oPr1, nor did these hormones at a concentration of 80 µg/ml inhibit the binding of hGH (results not shown).

Enhanced binding of radiolabelled hGH to human and rabbit hGH-SBP coated to microtitre plates was seen after addition of any of the five partly purified monoclonal antibodies. Such enhancement could, of course, be an artifact due to changes in the hGH-SBP upon adsorption to the microtitre plates. However, enhancement of the binding of radiolabelled affinity-purified rabbit hGH-SBP to monoclonal antibody M8 was also seen in the presence of hGH. In these experiments rat anti-(mouse immunoglobulin light chain) monoclonal antibody was coated on the plates and then monoclonal antibody M8 was added. Finally, the radio-labelled SBP was added with or without hGH (results not shown). It was shown for monoclonal M10 that the enhancement of the binding of hGH to the hGH-SBP was due to an increased number of binding sites. Enhanced binding of oPr1 (Katoh *et al.*, 1987), epidermal growth factor (Fernandez-Pol, 1985) and nerve growth factor (Chandler *et al.*, 1984) to their respective receptors has been reported in the presence of monoclonal antibodies against the receptors. In the latter two cases the enhancement was due to a change in the affinity.

Some of the findings presented here are not in agreement with the assumption that the hGH-SBP is totally identical with the extracellular portion of the liver hGH receptor (Leung *et al.*, 1987), but instead suggest that there is a distinct difference between the hGH-SBP and the liver hGH receptor, despite the *N*-terminal amino-acid sequence similarity. First, the concentration of antibodies which gave half-maximal enhancement of the binding of radiolabelled hGH to the serum binding protein was 70 times higher than the concentration which caused half-maximal inhibition of the binding of hGH to liver microsomes. Secondly, the antibodies enhanced the binding of radiolabelled hGH to the human and rabbit hGH-SBP, but inhibited the binding to the rat microsomal hGH receptor preparations and had no effect on the binding to the rabbit microsomal liver receptor preparations. In addition, Ymer & Herington (1985) have found that the binding of radioiodinated hGH to rabbit serum is only in part dependent on the presence of Ca²⁺. For the binding of hGH to various liver membrane

preparations, there is an absolute requirement of Ca^{2+} [for references, see Ymer & Herington (1985)]. It may be reasonable to assume the presence of different post-translational modifications of two proteins with different final destinations in spite of identical primary structures. Even such small differences could result in changes in affinity between monoclonal antibodies and the recognized epitopes.

In conclusion we have reported on the production and initial characterization of five monoclonal antibodies, four of which interfere directly with the specific binding of hGH to rat liver microsomes and directly or indirectly with such binding to human and rabbit hGH-SBP. These antibodies should be useful as tools for mapping and characterizing these binding sites.

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