Heterogeneity of growth-hormone receptors detected with monoclonal antibodies to human growth hormone

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The specificity of hormone–receptor interactions has been examined with the aid of monoclonal antibodies (MABs) (EB1, EB2, QA68 and NA71) defining four non-overlapping antigenic determinants on human growth hormone (hGH). The results indicate that growth-hormone receptors in liver obtained from different sources differ with regard to their affinities and relative numbers; they may also differ with respect to the region of the growth-hormone molecule to which they bind. Antibody NA71 effectively inhibited hormone binding to all receptor preparations tested, although with various degrees of potency. Monoclonal antibody EB1 demonstrated a graded inhibition with respect to its ability to block ^{125}I -hGH binding to receptors from various sources, the maximum inhibition being seen in receptor preparations from mouse and ovine liver and the minimum in rat liver. MABs EB2 and QA68 also showed various abilities to inhibit hormone–receptor interaction, depending on the origin of the receptor preparation. Furthermore, the receptor-binding characteristics of hormone–antibody complexes were dependent on whether the binding-site preparation was derived from pregnant, lactating or ‘normal’ animals. A particularly striking difference between the ability of hormone–MAB complexes to bind to receptors from different sources was seen for microsomes (microsomal fractions) derived from livers of animals of the ‘Little’ mouse strain. These animals become progressively obese, and it was shown that MABs were considerably more effective in inhibiting ^{125}I -labelled hGH binding to microsomes from phenotypically obese mice than to those derived from their non-obese littermates. The results can be explained by the presence of multiple receptor types for GH, the relative proportions of which vary according to the physiological state of the animal, and possibly between species.

INTRODUCTION

Human growth hormone (hGH) possesses a single polypeptide chain (191 amino acids) sharing various extents of structural homology with non-primate GHs (Lewis *et al.*, 1980; Wallis, 1981). Structural overlap also exists with hPL and hPRL, which are phylogenetically related hormones (Wallis, 1981). As a result of the structural similarities between these hormones, considerable immunological cross-reaction has been observed between the non-primate GHs from different species (Hayashida, 1975). The functional relationships between different GHs are less clear. Human GH is active in many non-primate systems and also displays considerable lactogenic activity. In contrast, non-primate GHs are not active in man and fail to bind to lactogenic sites (Wallis, 1980). Both PL and PRL are primarily lactogenic in their characteristics.

Specific hGH-binding sites have been demonstrated in diverse tissues. One of the richest sources is the liver of the pregnant rabbit. Early work suggested that these sites were predominantly growth-promoting or somatogenic (Tsushima & Friesen, 1973), but more recent findings indicate that the sites in this tissue are capable of binding both GHs and PRLs from some, but not all, species (Cadman & Wallis, 1981). There appear to be very few sites in rabbit liver preparations which are exclusively specific for GH or PRL. Various authors have

suggested that there is a heterogeneity of GH receptors in pregnant-rabbit liver (Hughes, 1979; Cadman & Wallis, 1981), and a recent study by Barnard *et al.* (1985) has confirmed, with the aid of anti-receptor MABs, that there are at least three structurally distinct GH-binding sites in rabbit liver. The specificity of hGH-binding sites in the female rat liver is also complex (Herington *et al.*, 1976). In rabbit and rat mammary gland, on the other hand, only a single class of distinct lactogenic sites (characterized by their ability to bind prolactins and hGH but not non-primate GHs) is found.

Lymphoid cells also contain hGH-binding sites. Those found in the rat Nb2 cell line share some of the properties of the lactogenic sites in the mammary gland. However, several lines of evidence suggest that the hGH-binding sites on such lymphoma-derived cells may represent a distinct type of receptor; thus monoclonal antibodies (MABs) to hGH affect the binding of the hormone to lymphocytes (Ivanyi, 1982a; Aston & Ivanyi, 1983) differently from that to mammary gland (Cadman *et al.*, 1982). hGH-binding sites have also been found on the human lymphoid cell line IM9; cross-linking studies with bifunctional reagents suggest that the subunit molecular size of this hGH receptor differs from that of either rabbit mammary gland or liver (Hughes *et al.*, 1983).

We describe here the effects of MABs of diverse specificity on the binding of ^{125}I -hGH to liver receptors

Abbreviations used: GH, growth hormone (somatotropin); PL, placental lactogen; PRL, prolactin; MAB, monoclonal antibody; the prefixes h, r, b and o refer to the human, rat, bovine and ovine hormones respectively.

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derived from different species. The antibodies (EB1, EB2, QA68 and NA71) define four distinct antigenic determinants, of which two are hGH-specific (QA68 and NA71) and two cross-react equally well with hPL (EB1 and EB2) (Ivanyi, 1982*a,b*). The two hGH-specific MABs were the most potent inhibitors of hormone binding to receptors, irrespective of the source of the liver receptor preparation, although quantitative differences existed. In contrast, EB1 and EB2 were almost devoid of hormone-binding inhibitory activity in some receptor preparations, whereas in others they were almost as effective as the hGH-specific MABs.

MATERIALS AND METHODS

Materials

Human GH (2.0 i.u./mg) was chromatographically purified on Sephadex G-100 from outdated MRC clinical-grade material. Bovine GH was purified chromatographically as described by Wallis & Dixon (1966). oPRL was a gift from Dr. A. Parlow and the NIADDK, NIH, Bethesda, MA, U.S.A., and rGH was provided by Dr. I. Robinson (National Institute for Medical Research, Mill Hill, London, NW7 1AA, U.K.). BALB/c and Lit/Lit ('Little' strain) mice were bred at Wellcome Research Laboratories. The latter breed is characterized by a deficiency of pituitary GH, and some animals become obese with age (Eicher & Beamer, 1976). Lou/F rats and New Zealand White rabbits were obtained from Olac (Shaw's Farm, Blackthorn, Bicester, Oxon. OX6 0TP, U.K.) and Ranch Rabbits (Crawley Down, Crawley, West Sussex, U.K.) respectively.

MABs

The antibodies used in this study were derived by fusion of BALB/c mouse spleen cells with NS1 myeloma cells and cloned by standard techniques (Ivanyi & Davis, 1980, 1981); they have fairly recently been reviewed elsewhere (Aston & Ivanyi, 1985). Four MABs were examined (EB1, EB2, QA68 and NA71), of which two bind equally well to both hGH and hPL (EB1 and EB2). The binding of these MABs to proteolytic digests of hGH suggests that the determinants are all associated with the 15 kDa N-terminal fragment (Aston & Ivanyi, 1983). All the antibodies failed to bind to both hPRL and non-primate GHs and were non-precipitating in diffusion gels. The affinities of these MABs have been determined from Scatchard plots: NA71, 5.2×10^9 l/mol; QA68, 1.0×10^9 l/mol; EB1, 4.3×10^8 l/mol; EB2, 1.0×10^9 l/mol.

Iodination of hormones

Radioiodination of hGH was performed by the lactoperoxidase method, the modifications described previously (Aston *et al.*, 1985) being employed. Essentially, 10 μ g of hormone was labelled with 1 mCi of Na¹²⁵I in sodium phosphate buffer (300 mM, pH 7.2) before separation of the free iodide by Sephadex G-25 chromatography. Monomeric ¹²⁵I-labelled hGH was subsequently separated from any aggregated material by chromatography on Ultrogel AcA 34 in sodium phosphate buffer (50 mM, pH 7.2) containing bovine serum albumin (3%, w/v). The specific radioactivity of the tracer was generally $(50-60) \times 10^6$ c.p.m./ μ g of protein.

Receptor preparation

Crude membrane fractions were prepared from either frozen or freshly excised livers by the method of Tsushima & Friesen (1973). All preparative steps were carried out at 4 °C. The livers were rinsed in cold 0.9% NaCl and subsequently homogenized in 5 vol. of cold 0.3 M-sucrose with a Sorval Omnimixer at a speed setting of 10 for 1 min. The homogenate was filtered, under pressure, through eight layers of muslin and centrifuged for 20 min at 15000 g and at 4 °C. The supernatant was further centrifuged for 90 min at 100000 g at 4 °C. The resulting microsomal pellet was resuspended in assay buffer [0.025 M-Tris/HCl (pH 7.4)/10 mM-CaCl₂/0.06 mM-thimerosal/0.1% bovine serum albumin, w/v] by five strokes in an all-glass homogenizer. Aliquots of the microsomal preparations were subsequently frozen and stored at -20 °C. Protein concentration was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Binding studies

The incubation procedure was similar to that of Cadman & Wallis (1981), with some modifications. Each assay tube contained 200 μ l of assay buffer, 200 μ l of microsome preparation (200-400 μ g of protein), 100 μ l of diluted hormone or MAB (0-10 μ g/tube of hormone and/or 0-100 μ g of MAB/tube) and 50 μ l of ¹²⁵I-hGH (approx. 15000-20000 c.p.m. tube). Receptor preparations were rehomogenized after thawing and diluting by five strokes in an all-glass homogenizer. Tube content were mixed and incubated overnight at 4 °C. The incubation was terminated by addition of 1.5 ml of cold 'stopping' buffer (0.025 M-sodium acetate/acetic acid, pH 5.4). The tube contents were mixed and the tubes left at 4 °C for 30 min. Bound and free iodinated hormone were separated by centrifugation at 1500 g for 15 min at 4 °C. The supernatant was decanted and the precipitate counted for radioactivity in an LKB Multirack γ -radiation counter.

The total specific binding (100% value) was determined as the radioactivity bound to receptor which could be displaced in the presence of an excess of unlabelled hGH (10 μ g/ml). The displacement of specific binding at different concentrations of hormone or MAB was expressed as a percentage of the total specific binding. The total specific binding and non-specific binding ranged between 700 and 7000 c.p.m./tube (5-50% of total radioactivity) and 1000 and 5000 c.p.m./tube (5-25% of total radioactivity) respectively, depending on the source of the receptor-microsome preparation (see Fig. 1 below). None of the MABs blocked non-specific binding; however, a significant and unexplainable increase in non-specific binding with EB2 was observed at the higher concentrations of the MAB.

RESULTS

Binding sites for ¹²⁵I-labelled hGH in liver preparations from different species (pregnant mouse, rat or rabbit or non-pregnant sheep) were examined by displacement with unlabelled bGH, hGH or oPRL in order to identify whether the sites were predominantly somatogenic or lactogenic in nature (Wallis, 1980) (Figs. 1*a-1d*). oPRL failed to displace ¹²⁵I-labelled hGH from microsomal receptor preparations from sheep liver, whereas the human and non-primate GHs were of equal displacing

Fig. 1. Displacement of ^{125}I -labelled hGH bound to liver microsomal receptors from various species by unlabelled hGH, bGH and oPRL.

^{125}I -labelled hGH was incubated with liver microsomal receptors prepared from sheep (a), pregnant rabbit (b), pregnant mouse (c) and pregnant rat (d) in the presence of increasing concentrations of unlabelled hGH (■), bGH (○) or oPRL (□). Abbreviation: PBS, phosphate-buffered saline control (no unlabelled hormone).

potency, suggesting a somatogenic-like site. In contrast, with receptor preparations from rabbit (Fig. 1b), mouse (Fig. 1c) or rat (Fig. 1d) liver, labelled hGH could be displaced by both oPRL and bGH to various extents, indicating a mixed somatogenic/lactogenic receptor. The levels of specific and non-specific binding for rat, mouse and ovine liver microsomes were of similar magnitude, whereas in rabbit liver receptor preparations over 70% of the total binding was displaced with unlabelled hormone. It is noteworthy that rat GH failed to displace ^{125}I -labelled hGH from rat liver microsomes (results not shown), whereas bGH competed effectively with labelled hGH; this complicates the simplistic categorization of receptors as 'somatogenic' or 'lactogenic'.

The ability of four different MABs EB1, EB2, QA68 and NA71 to compete for binding of ^{125}I -labelled hGH with binding sites in mouse, rat, rabbit and sheep liver

microsomes was studied (Figs. 2a–2d). MAB EB1 showed significant differences with respect to its ability to block binding of ^{125}I -labelled hGH to receptors from different species. This antibody (EB1) inhibited binding of hormone to rabbit liver microsomes to a modest extent (30% at $100\text{ }\mu\text{g}$ of protein), but at the same dose gave almost 100% inhibition of specific binding to ovine liver receptors. The difference between ovine liver receptors and those from other species was most striking for antibody EB2 (Fig. 2b), where 50% displacement was achieved with only $1\text{ }\mu\text{g}$ of antibody. In contrast, displacement of specific binding in mouse, rat and rabbit liver was not observed beyond 40% at doses of EB2 of up to $10\text{ }\mu\text{g}$. Antibody QA68 showed a high efficacy of displacement of ^{125}I -labelled hGH from receptors from sheep, rat and mouse liver, but was comparatively poor at displacing the hormone from rabbit liver microsomes

Fig. 2. Effects of MABs of diverse specificity on the binding of ^{125}I -labelled hGH to liver microsomal receptors from various species

^{125}I -labelled hGH was incubated with liver microsomal receptors prepared from sheep (□), pregnant rabbit (●), pregnant mouse (■) and pregnant rat (○) in the presence of increasing concentrations of MABs EB1 (a), EB2 (b), QA68 (c) and NA71 (d). Binding of ^{125}I -labelled hGH was expressed relative to the maximum specific binding (total binding—non-specific binding determined in the presence of $10\text{ }\mu\text{g}$ of unlabelled hGH). Abbreviation: PBS, phosphate-buffered saline control (no unlabelled hormone).

under these conditions. Antibody NA71 was most effective in inhibiting binding to rabbit liver.

Receptor heterogeneity was also observed when rat liver microsomes derived from 'normal', pregnant and lactating animals were compared (Figs. 3a–3d). At $1\text{ }\mu\text{g}$ of MAB EB1 protein, over 50% displacement of specific binding was observed for microsomal receptors from normal rats, whereas no displacement was detected at the same antibody dose with receptors from lactating animals. Antibody EB2 failed to result in significant displacement of binding of ^{125}I -labelled hGH from any of these receptor preparations, except at very high doses. Both QA68 and NA71 were of considerably higher inhibitory potency (10–100-fold) than either EB1 or EB2,

but their inhibitory effects showed only minor quantitative differences between the different microsomal preparations.

Mice of the 'Little' strain (Lit/Lit) are characterized by a specific pituitary GH deficiency which results in animals of overall small size. Furthermore, some of these animals become progressively obese with age, depositing large amounts of subcutaneous fat. It is not known whether the progressive obesity of these animals is associated with their deficiency in GH. Binding sites for hGH in liver microsomes from this strain were analysed with the four MABs (Figs. 4a–4d). The binding of ^{125}I -labelled hGH to receptor preparations from phenotypically obese animals (Lit^{ob}) in the presence of EB1 was

Fig. 3. Effects of various MABs on the binding of ^{125}I -labelled hGH to liver microsomal receptors from 'normal', lactating and pregnant rats

^{125}I -labelled hGH was incubated with liver microsomal membranes prepared from normal female (■), pregnant (●) and lactating (○) rats in the presence of increasing concentrations of MABs EB1 (a), EB2 (b), QA68 (c) and NA71 (d). Binding of ^{125}I -labelled hGH was expressed relative to the maximum specific binding. Abbreviation: PBS, phosphate-buffered saline control (no unlabelled hormone).

significantly lower than the binding to non-obese animals. At $10\text{ }\mu\text{g}$ of MAB, over 90% of specifically bound ^{125}I -labelled hGH was displaced from microsomes from Lit^{ob} mice, whereas at the same antibody dose only 10% of bound ^{125}I -labelled hGH was displaced from receptors from non-obese animals. Both obese and non-obese Lit/Lit mice demonstrated equal amounts of total (6000 c.p.m.), specific (2000 c.p.m.) and non-specific (4000 c.p.m.) binding. Similar, although less marked, differences between obese and non-obese animals were observed for antibody EB2. Antibody QA68 was almost 1000-fold more effective in inhibiting binding to receptors from Lit^{ob} mice than from the corresponding

non-obese animals, whereas the difference with NA71 was modest.

DISCUSSION

Specific binding sites for hGH have been demonstrated in diverse tissues from various species. Indeed, even within a single organ such as liver, several putatively different hGH-binding sites, with different specificities, have been demonstrated by displacement with various GHs and PRLs (Ranke *et al.*, 1976; Wallis, 1980; Cadman & Wallis, 1981). The existence of different GH-binding sites, possibly with distinct specificity and

Fig. 4. Effects of various MABs on the binding of ^{125}I -labelled hGH to liver microsomal receptors from phenotypically lean and obese animals of the 'Little' mouse strain

^{125}I -labelled hGH was incubated with liver microsomal membranes prepared from phenotypically obese (○) and lean (■) animals of the 'Little' mouse strain in the presence of increasing concentrations of MABs EB1 (a), EB2 (b), QA68 (c) and NA71 (d). Binding of ^{125}I -labelled hGH was expressed relative to the maximum specific binding. Abbreviation: PBS, phosphate-buffered saline control (no unlabelled hormone).

Table 1. Summary of relative potencies of different monoclonal antibodies in displacing ^{125}I -labelled hGH from liver microsome receptors

MAB Tested	ID ₅₀ * (ng of MAB/tube)						
	Mouse			Rat			Sheep
	Balb/c	Lit/Lit	Lit ^{ob}	Normal	Pregnant	Lactating	
EB1	2×10^4	10^5	200	10^3	$> 10^5$	$> 10^5$	4000
EB2	2×10^4	$> 10^5$	4000	$> 10^5$	10^5	$> 10^5$	630
QA68	20	600	0.6	20	200	300	2500
NA71	80	250	40	5000	8000	130	8

* ID₅₀ (determined from the data of Figs. 2–4) represents the concentration of MAB which displaces 50% of bound ^{125}I -labelled hGH from liver microsome receptors.

function, is supported by studies employing techniques involving the cross-linking of the hormone to corresponding receptor subunits. Such approaches have revealed that GH receptors from different sources show different subunit M_r values (Hughes *et al.*, 1983). More recently, GH-receptor subtypes which differ in their hormone-binding regions have been demonstrated with the aid of MABs raised against the rabbit and rat liver receptors (Barnard *et al.*, 1985). We have shown previously that the panel of MABs employed here are all of high potency in the inhibition of binding of ^{125}I -labelled hGH to lymphoid cells (Ivanyi, 1982*a*; Aston & Ivanyi, 1983).

The question of receptor variation has been addressed here with MABs of distinct combining-site specificity. Two of the epitopes for MABs examined have been shown to be present on both hGH and hPL (EB1 and EB2), whereas a further two are specific for hGH (QA68 and NA71) (Ivanyi, 1982*a*). The ability of different MABs to inhibit binding of ^{125}I -labelled hGH to different microsome preparations is summarized in Table 1.

Of the four antibodies tested, EB1 showed the greatest variation with respect to its ability to inhibit binding of labelled hGH to receptors from different sources. Thus EB1 was much more effective in inhibiting binding to receptors from livers of pregnant mouse or non-pregnant sheep than to those from pregnant rat or rabbit (Fig. 2*a*). Within the same species, EB1 inhibited binding to liver microsomes from normal female rats much more effectively than to those from pregnant or lactating animals (Fig. 3*a*), and it was 500-fold more potent in inhibiting binding to receptors from obese 'Little' mice than to those from non-obese animals of the same strain. Similar variations in ability to inhibit binding to different receptors were seen with the other antibodies tested, though the detailed pattern of variation was different for each. For example, antibody EB2 failed to distinguish between the binding sites on microsomes from pregnant, lactating and normal rats, contrasting markedly with EB1 (cf. Figs. 3*a* and 3*b*).

These results confirm previous studies (e.g. Cadman *et al.*, 1982; Ivanyi, 1982*a*) showing that MABs may differ in their ability to block binding of ^{125}I -labelled hGH to 'receptors'. They also suggest that binding sites from various sources differ, since the pattern of inhibition by MABs differs for each source. In some of the cases studied the MABs were able, when added at sufficiently high concentrations, to inhibit receptor-hormone binding completely, indicating that the MAB-hormone complex could not bind to the receptor. In other cases (e.g. Fig. 2*c*) MAB gave partial inhibition, with a plateau of binding at high MAB concentrations, suggesting that the MAB-hormone complex could bind to at least some of the receptors present, possibly with decreased affinity. In many cases, however, the concentration of MAB used was not sufficiently high to determine whether inhibition of binding was complete or partial. The concentration of MAB used in these cases was limited by availability and solubility.

At least three factors may contribute to the various effects of different MABs on binding to different receptors: (a) concentration of receptor; (b) different affinities of receptors (binding to the same site on hGH) in different tissues; (c) different affinities of receptors for binding of hGH involving binding to distinct sites on the hormone molecule. Our results cannot be easily

explained simply by different concentrations of the same binding site in different tissues. However, different relative concentrations of two or more receptors of different affinities could be important. Where MABs gave complete inhibition of binding at high concentrations (i.e. the MAB-hGH complex did not bind to receptors), the different pattern of binding of different MABs to tissues could be due to the presence of receptors with different affinities for hGH (and therefore able to compete relatively well or poorly with MAB for binding of labelled hGH). Where the evidence suggests that MAB-hGH complexes could still bind to receptors, however, interaction of receptors with different sites on the hGH molecule is a likely explanation. However, the evidence available is insufficiently clear-cut to prove this in any of the cases studied here.

Our results thus indicate that receptors from different sources vary in terms of their affinity for hGH and possibly other properties. For receptors from different species the differences between receptor populations could be explained by the species differences themselves. For microsomes from animals of the same species, but in different physiological states, the results suggest that the receptors in liver vary according to the physiological state. Since there is considerable heterogeneity of the sites that bind labelled hGH in the liver (see above), a possible explanation for our results is that the proportions of the different types of binding sites vary very markedly according to the physiological state of the animal.

The use of a heterologous system, in which ^{125}I -labelled hGH is used for binding to 'receptors' from non-primate liver, clearly makes the interpretation of the biological significance of our results difficult. Nevertheless, the existence of clear-cut differences between binding sites from different sources has been demonstrated, and a physiological explanation presumably underlies this. It may be that the different receptors seen in various physiological states are involved in binding different hormones of the GH/PRL family, such as placental lactogen or proliferin (Linzer & Nathans, 1984). Alternatively, or in addition, the variation of receptors may be associated with the different biological actions of GH (somatogenic, lactogenic, diabetogenic, insulin-like, lipid-mobilizing; Wilhelmi, 1982), which may be mediated by different receptors and indeed by different regions of the GH molecule. In this respect the marked differences between the receptors from livers of obese and non-obese 'Little' mice (Fig. 4) are particularly notable, since the overall metabolic balance in these animals is clearly very different.

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