MOLECULAR BIOLOGY OF GROWTH HORMONE

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I. INTRODUCTION

In 1921 Evans and Long[1] discovered that growth-promoting subtances were present in an emulsion of the pituitary anterior lobe by producing gigantism in rats after repeated intraperitoneal injections of the material. A quarter of a century elapsed, though, before Li and Evans[2] isolated a highly purified growth hormone (GH) of bovine origin.

The administration of GH to young animals promotes its growth but this action is more notorious in hypophysectomized animals where growth is arrested. The hormone produces an increase in body weight without appreciable change in chemical composition.

Hypersecretion of GH in the adult causes the enlargement of the bones of the face and extremities originating the clinical condition known as acromegaly. Hyposecretion of GH leads to a special dwarfism where the animal remains proportionately small.

One of the specific effects of GH consists in stimulating longitudinal bone growth, although this is quite probably an indirect effect mediated through the synthesis of a family of peptides collectively known as somatomedins (SM).

GH also exerts a general metabolic action with changes in the utilization of proteins, lipids, and carbohydrates. The physiological effects of GH are so varied that it is difficult to express an unifying theory. Growth and development are complex, integrated processes which involve, besides GH, all the endocrine system.

Since 1921, GH has kept busy many laboratories throughout the world trying to explain its chemical nature and the molecular mechanisms of its multiple actions.

It was thus discovered that GH is not a single entity but a collection of similar proteins with variant forms which share functions and are immunologically related, yet distinct. Human beings are exquisitely specific and do not react but to its own GH. Nevertheless, the remote paths of evolution indicate that all GHs have a common ancestry and this fact is brought to light by the weak effect on man of some fragments of nonhuman GHs.

Present GHs are a fascinating example of evolutionary development, still not completely understood, but undoubtedly related with the origin of man on earth.

GHs belong to a family of protein hormones whose other members are the prolactins (PRL), also derived from the anterior pituitary gland, and the placental lactogens (PL).

There is a plentiful supply of recent reviews on different facets of this complex group of substances, clearly involved in the control of several growth and metabolic processes. Restricting the enumeration to GHs, they cover their chemistry,[3-7] immunochemistry,[8] biology and biochemistry,[9-12] their evolution[13-16] and clinical aspects,[17-18] and include books and symposia[19-23] and a most illuminating lecture by Friesen.[24]

In what follows we have attempted a rather one-sided review of what is known in molecular terms about GH, instead of surveying its many physiological effects. We are aware that both approaches are, of course, intimately connected and will merge in the future.

Our efforts will be focused on the evolutionary relationships between these hormones and on the genetic studies that have started to clarify the origin of the puzzling molecular heterogeneity of GH. The biological activities of the multiple forms of GH, of the somatomedins, and of fragments of GH will also be discussed as well as some significant advances on its immunochemistry and on the biology of its cellular receptors.

II. MOLECULAR EVOLUTION

All the members of the GH family are proteins formed by a single polypeptide chain of approximately 200 amino acids with 2 or 3 intrachain disulfide bonds and free N- and C-terminal ends.[7] Comparison of their sequences shows that human GH (hGH) and human PL (hPL) are quite close relatives (85% identity); considerably more than what is found among hGH and bovine (bGH), ovine (oGH), and equine GHs (eGH) themselves (about 65% identity); or between hGH and the PRLs (about 27%). If homologous amino acids are taken into consideration, the percentages cited before raise to 95%, 91%, and 65%, respectively.[25]

These proteins are all globulins with molecular weights in the neighborhood of 22,000, soluble in dilute acids and alkalies, which have similar sedimentation and diffusion coefficients, elipticity, etc. Furthermore, they share many biological actions.[5,7]

It is presently accepted that all the members of this family have an ancestral gene in common which, by duplication and divergent evolution, gave rise to the separate genes coding the synthesis of the GHs and PRLs as we know them today. This duplication must have occurred early in the evolution of the vertebrates (approximately $3.5 - 4.0 \times 10^8$ years ago).[26] Considerable divergence took place from this point onward as evidenced by the low degree of identity between GHs and PRLs.

The second well-established gene duplication occurred in the GH gene and gave rise to the PL. This information has been obtained in man but quite probably is also true for other primates. These events happened during the evolution of the primates, after the separation of the main orders of placental mammals. Similar but independent gene duplications within these later groups very likely originated the respective PLs but information is needed to time more accurately these events.

Wallis[16] has constructed an evolutionary tree for the GH-PRL family (Figure 1) using the available amino acid sequences, including those deduced from nucleotide sequences, and a simplified common ancestor method. Branch lengths are accepted point mutations per 100 residues and are corrected for multiple mutations at any 1 site.

According to Wallis,[16] the rates of evolution within this family have not been constant: a very rapid change of GH occurred during the evolution of the primates and, still further, when hPL diverged from hGH, mutations appear to have been accepted about five times more rapidly in the placental hormone than in the pituitary hormone.

The enhanced rate of evolution of primate GHs is suggestively correlated with peculiar characteristics of their biological properties: only this class of hormones is active in man and hGH has definite lactogenic activity which is absent in the hormone of nonprimates.

III. GENETIC STUDIES

Seeburg et al.,[27] in 1977, determined the primary structure of a cDNA for rat GH (rGH) mRNA. The amino acid sequences for rGH and its precursor (pre-hormone), were deduced from the nucleotide sequences. This cDNA was soon used to synthesize a hybrid gene and expressed in bacteria to produce rGH sequences.[28]

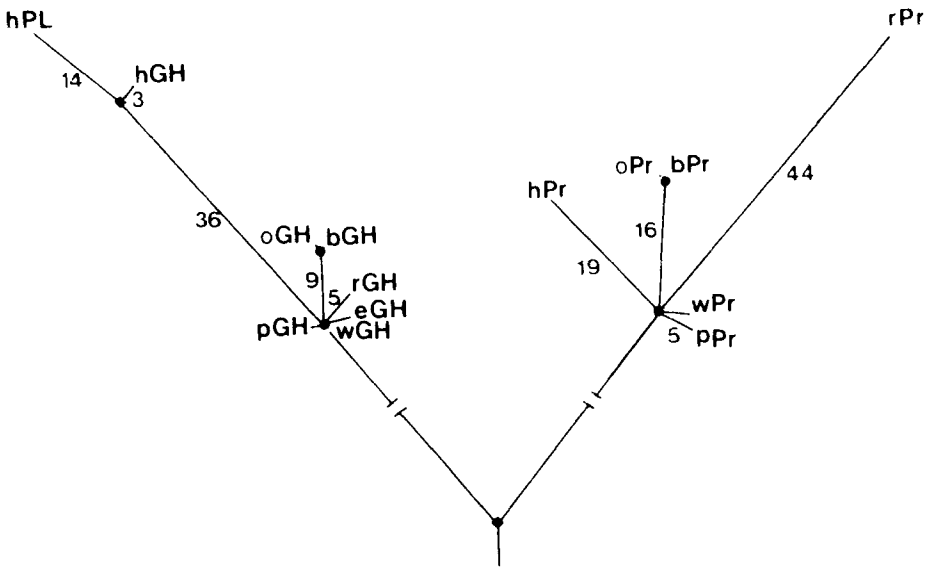

FIGURE 1. Evolutionary tree for the growth hormone-prolactin family. Branch lengths are accepted point mutations/100 residues. ♦, indicates a gene duplication; ●, marks a point of divergence of one or more different species. Abbreviations: wGH, sei whale GH; Pr, prolactin; bPr, bovine Pr; oPr, ovine prolactin; pPr, porcine Pr; rPr, rat Pr; wPr, fin whale Pr; hPr, human Pr. (From Wallis, M., *J. Mol. Evol.*, 17, 10, 1981. With permission.)

The cloning and sequencing of the cDNA for hGH mRNA was achieved by Martial et al.[29] in 1979 and found to contain 29 nucleotides in its 5′ untranslated region, 651 nucleotides coding for the pre-hormone, and 108 nucleotides in the 3′ untranslated region. This cDNA was linked in phase to a fragment of the D gene of Escherichia coli in a plasmid vehicle and a fusion protein was synthesized under the control of the regulatory protein of the Trp operon. The chimeric protein obtained reacted specifically with antibodies against hGH.

Goeddel et al.,[30] using a novel combination of chemically synthesized DNA and cDNA, were able to produce large amounts of hGH in bacteria with all the characteristics of the native hormone.

Almost complete sequences corresponding to the coding regions of the genes for hPL and rPRL have also been described.[31,32] Wallis[16] has used this information to deduce ancestral sequences and elaborate the evolutionary tree for the GH family (Figure 1). The hGH and hPL genes have been sequenced by Fiddes et al.[33] A 2.6 kilobase Eco RI restriction endonuclease fragment containing hGH gene sequences and a 2.8 kilobase Eco RI fragment containing hPL gene sequences have been identified by hybridization to cloned cDNA. Restriction endonuclease mapping showed that the hGH gene contains at least three intervening sequences interrupting the coding sequence. Partial DNA sequence analysis confirmed the location of the intervening sequences and the identity of the fragment.

A detailed restriction enzyme map has not yet been determined for the hPL gene, but the preliminary evidence suggests that the patterns of intervening sequences may be similar to that of the hGH gene.

A most interesting variant of hGH among the several classes isolated and identified by Lewis and his colleagues[34] in the pituitary gland (see Section IV) is the 20,000 dalton GH (20 K) which is found in all human pituitaries. It has a molecular weight about 2000 less than normal hGH (22 K) because it lacks 15 amino acid residues between positions 32 and 46 of the chain. The question is: how is this remarkable variant formed? As indicated by Wallis,[35] a clue is provided by examining the structure of the GH gene.[33] Of the 3 intervening sequences mentioned before, 1 occurs between the codons specifying residues 31 and 32 in

the mRNA. This is precisely the position of the start of the deletion in the 20 K variant. The end of the deleted sequence that occurs between residues 46 and 47 does not correspond to the position of another intervening sequence.

It is difficult to believe that this partial correspondence between a deleted sequence in the protein and an intervening sequence in the corresponding gene is a coincidence. It seems likely, therefore, that

1. Either 20 K represents a product of a duplicated gene in which an intervening sequence (intron) has been lengthened to include part of a coding sequence (exon) or
2. That the mRNA for 20 K is produced from the same primary RNA transcript (and therefore the same gene) as normal hGH, which would therefore be processed in two different ways

In the second case, a region of the primary RNA transcript that is a coding region for normal hGH would be treated as part of an intron for the 20 K variant, and different ways of processing a single primary RNA transcript would lead to production of 2 different proteins.

Whichever of the two explanations is correct, it is difficult to avoid the conclusion that the occurrence of an intervening sequence in a gene has allowed the formation of a remarkable protein variant.

The human genes for GH, PL, and a third GH-like gene have been located on the long arm of chromosome 17 in humans.[36,37] It is estimated that these genes diverged approximately 50 million to 60 million years ago,[36] whereas, as mentioned before, the PRL and GH genes diverged approximately 400 million years ago and it is not yet known if they are contiguous on chromosome 17. It is also unclear how many copies of each gene exist per genome.[37] Although the arrangements of genes in the hGH gene cluster and a regional restriction map have not been established, comparison of different genomic clones containing hGH or hPL genes, or both, has shown that there are at least two types of hGH and three types of hPL genes with different nucleotide sequences. In the case of hGH, one normal hGH gene (hGH-N) encodes the known protein sequence, whereas the other variant gene (hGH-V) differs at multiple sites (cited by Reference 37).

The first use of recombinant DNA technology to investigate the molecular basis of defective production of pituitary GH was described by Phillips et al.in 1981.[37] These authors were able to study four of the six children discovered by Illig et al.[38,39] with familial isolated growth hormone deficiency type A. By using ^{32}P-labeled hGH cDNA sequences as a probe, patterns seen after various digestions with restriction endonucleases indicated that these individuals were homozygous for a deletion of at least 7.5 kilobases of DNA. This deletion includes the gene that encodes the normal GH but does not include the variant GH gene. Their phenotype of extremely short stature, the absence of GH production in immunoassay studies, and the formation of hGH-antibodies in high titers after hGH treatment suggest that the product of their hGH-V genes is deficient or poorly functional, or both.

Wallis[40] has commented that the complexity of the organization of hGH and hPL gene families contrasts with the situation in nonprimate mammals. In rat, for example, cDNA to rGH cross-hybridizes with only a single GH gene, the structure of which has been elucidated in detail. The gene contains four introns. PRL is also represented by a single gene in the rat genome and again, the gene contains four introns the positions of which corresponds to those in GH, but the sizes of which are far greater (on the average by a factor of five). It seems likely that the genes of the GH family underwent a period of rapid change, including gene duplication and multiple substitutions during the evolution of the primates.

Human dwarfism is not always a simple consequence of lack of GH: many children are very short despite normal levels of immunoreactive GH in their blood and the absence of any other obvious cause. It has recently been proposed[41,42] that many of these children have

a defective, immunoreactive GH. Growth may be inducible in such children by exogenous normal hGH. It is difficult to obtain GH in adequate amounts from human pituitaries, but the expression of hGH in Echerichia coli using recombinant DNA technology provides an inexhaustible source of this protein. The bacterially synthesized hGH is methionyl-hGH, the extra amino acid arising from the AUG start codon inserted at the beginning of the gene.[30,43] This cloned product has been purified to homogeneity, and its specific activity, as determined by radioimmunoassay (RIA), is comparable to that of pituitary GH. Clinical trials with such material are in progress.

IV. MULTIPLE FORMS OF GH

Highly purified GH preparations, as conventionally isolated from the pituitaries, are still heterogeneous mainly due to the multiplicity of different GH components rather than to the presence of other protein impurities.

Multiple forms of GH were detected in several preparations of hGH, rGH, and bGH.[44] These forms could be isolated by electrophoresis and gave markedly different bioassay to RIA ratios. Skyler et al.,[45] by the same methodology, were able to detect up to 12 variants of hGH. Similarly, Schleyer et al.[46] isolated five subfractions of porcine GH (pGH) which had slightly different amino acid compositions.

A rigorous analysis of the molecular nature of variant GH forms found in the pituitary gland has been made only for bGH, oGH, and hGH. In the bovine hormone, one heterogeneity was traced to the existence of two N- terminal residues, namely, Ala- and Phe-.[47,47a] Peña et al.[48] showed that the origin of this variation was not allelic and it is now considered probable that it may arise by ambiguous processing of a GH precursor.[7] A second variation in bGH was found in position 127 of the chain,[49,50] where a Leu residue is replaced by Val. Seavey et al.[51] proved that it is the result of an allelic polymorphism in the population of cows from which the GH was obtained.

The N- terminal heterogeneity was also found in oGH.[52] The most extensive and detailed investigations have been performed in the human pituitary gland by Lewis and his co-workers[53] where they characterized several single- and two-chain forms of hGH as well as an interchain S-S dimer.

One two-chain form named 24K (24,000 mol wt) is originated by a single cleavage point at Phe 139. The apparent higher molecular weight than normal hGH (22K), is attributed to the increased Stokes radius of this form. The other 2 chain forms are α_2, that lacks residues 135—140 and α_3, that lacks residues 135—145. The α_2 form shows a marked increase in PRL activity and some potentiation of growth stimulating ability, but α_3 is a superactive GH. Both were indistinguishable from intact GH by RIA.

Another post-translational modification of hGH that occurs in the gland leads to the formation of a disulfide dimer which is essentially inactive as a growth promoter, but, if reduced and carbamidomethylated, becomes fully active. The location of the S-S bridge, or bridges, has not been determined. This form is most likely the "big" form detected in plasma by Soman and Goodman.[54]

Perhaps the most interesting of the many GH variants is the 20K form. It is found in all human pituitaries and comprises about 15% of the total GH content, making it 1 of the most abundant of all hormones of the pituitary gland. The precise position of the deletion of the 15 amino acids residues has been shown to be between residues 32 and 46.[53,55] The variant possesses growth-promoting activity with potency similar to that of normal GH, but lacks the insulin-like activity and free fatty acids-mobilizing activity normally associated with the hormone. It was interesting to find that the variant does stimulate SM production;[55a] this gives additional support for the view that SM production is an integral part of the growth process and also indicates that the insulin-like activities of GH are not a prerequisite.

The ability of 20K to increase the concentration of SMs in blood is interesting also in light of the finding that 20K competes only poorly with hGH for the somatotropic receptors of rat liver. Different preparations of 20K were found to be only 3 to 20% as potent as hGH to displace ^{125}I-hGH from liver receptors and 22 to 53% as effective in the mammary gland receptor assay systems. These findings suggest that 20K and hGH may have separate receptors or that the binding characteristics of the two hormones may be quite different.[56]

The question of how the 20K variant is probably formed has been discussed in Section III. Other single-chain variants that are recognized by their unique electrophoretic characteristics have been referred to as fast, slow, slow-slow, and deamidated GH.

The slow forms do not show any chemical differences with the normal, 22K hGH. The fast hGH has the N- terminal end blocked and represents about 5% of the total GH content of the gland. Very little is known of the biological properties of these forms besides that they show somatotropic and lactogenic activities. Finally, 2 deamidated forms of hGH have also been described by Lewis et al.,[57] 1 of them lacks the amide in Asn 152 and the other in Gln 137.

It is important to summarize, quoting Lewis et al.,[53] the physiological activities that have been eliminated from the major form of hGH (22K). Their purest, but still heterogeneous hGH, was inactive in producing hyperglycemia and hyperinsulinemia in the dog, and it failed to stimulate insulin release from isolated islet cells. The material lacked in vitro lipolytic activity in the rat fat pad, with or without dexamethasone, but did exhibit an early in vivo hypoglycemic effect and a lowering of serum-free fatty acids at a dosage of 50 μg per rat. Even though the "pure" material used in these tests was not homogeneous, by isoelectric focusing[53] it was free of diabetogenic peptide, two-chain forms, 20K form, disulfide dimer, amino-terminus blocked form; and was low in, but not free of, the slowly migrating forms.

The substance responsible for the diabetogenic activity of the pituitary has been purified by Lewis et al.[53] and it has been suggested that it is a fragment of GH, but still further work is needed to clarify the divergent conclusions reached by a number of investigators.

V. CELLULAR RECEPTORS FOR GROWTH HORMONE

Over many years the primary site of action of growth hormone was indirectly studied by following a functional response induced in the intact animal or in whole tissues. When radioactively labeled hormones with high specific activity became available, it was possible to demonstrate binding of GH to its presumptive cellular receptors in a saturable and reversible process with structural specificity and high affinity, dependent on time, temperature, pH, and ionic environment.[58-62]

The three classical sites of GH action in the rat (diaphragm, adipose tissue, and skeletal muscle) show little binding activity[63] but this does not mean that functional GH receptors are absent in them.

A similar situation occurs in the kidney, which is known to intensely concentrate injected GH,[64] but where only recently[65,66] have hGH receptors been well characterized.

Most workers have tended to study the liver GH receptor largely because the liver content of GH receptor has been found greater than that of any of the other tissues in the species so far examined (see 67 for review and 68).

The liver has also been extensively studied as a target organ for GH in the rat because of its role as the major producer of SM. In addition to this role, GH increases protein and RNA synthesis in the liver[69] and the activity of a number of enzymes such as ornithine decarboxylase, tyrosine amino transferase, and tryptophan pyrrolase.[70] GH also has various metabolic effects on the liver.[71]

A characteristic property of hGH distinguishes this hormone from those of nonprimates: in addition to the growth and metabolic activities in common with them, the human hormone promotes growth in primates, and has lactogenic activity in both primate and nonprimate species.[72,73,73a,73b] The study of the interaction of ^{125}I-hGH with liver receptors of nonprimates has led to classification of the receptors in 2 categories: somatotropic[61,74-77] and lactogenic.[65,76,77] The binding of ^{125}I-hGH to the somatogenic receptors is specifically inhibited by the growth hormones of both primates and nonprimates. The binding of ^{125}I-hGH to lactogenic receptors is specifically inhibited by lactogenic hormones like prolactins and placental lactogens.

A third receptor type, first described in cultured human lymphocytes[78,60] and subsequently in human liver,[79] binds human growth hormone specifically and with high affinity, while only a very weak binding (approximately 0.03% compared to hGH) was detected for hPL. Carr and Friesen[79] have also shown that placental extracts of ovine, bovine, and caprine origin bind strongly to the hGH receptor in the human liver and Chan et al.[80] have confirmed these findings with a purified preparation of ovine PL (oPL), which was also found as active as bGH in the body weight assay in the rat.

Lesniak et al.,[81] using human IM-9 lymphocytes and the oPL prepared by Chan et al.,[80] confirmed that it was as potent as hGH in binding to the hGH receptor while bGH, oGH, and oPRL were as weakly bound as hPL. All these hormones were also capable of inducing the loss of GH receptors when incubated with the cells for 18 to 24 hr at 37°.[81] Their activity in this respect did not correspond exactly with their activity in competing with ^{125}I-hGH for the receptors. This induced receptor-loss was interpreted as an indication of biological activity of the various preparations, and the relative potencies thus derived were shown to be of a similar order of magnitude to those previously found from minimal effective doses in man. No data are yet available on the biological activity of oPL in man, but from its receptor activity one must anticipate that it may be considerable. Thus the active sites in oPL and hGH are sterically more similar than those in the nonprimate growth hormones. This raises a question of considerable evolutionary interest if we consider the enhanced rate of evolution of hGH and hPL compared to that of nonprimate hormones (see Figure 1).

Comparative studies on GH amino acid sequences have thrown much light on the structural relationships within this family of protein hormones and receptor-binding studies are beginning to give more detail of their three-dimensional conformation than has previously been possible. There are, however, many questions that still remain unanswered. Thus we do not understand why hPL has more lactogenic than growth-promoting activity when its primary structure is more like hGH than hPRL. The lactogenic activity of hGH has been known for a long time and this activity is in keeping with recent estimates of its potency for competing with ^{125}I-hPRL for lactogenic receptors, but it is not clear why nonprimate GHs are devoid of lactogenic activity.

As a result of their own work and from previously published data, Lesniak et al.[81] proposed a speculative classification of hormones and receptors shown in Table 1. However, the values marked have been challenged by more recent investigations as will become clear from the discussion that follows.

Type and specificity of receptors are not yet completely clear and perhaps the reason for conflicting results lies in the heterogeneity of the assays employed which include different preparations (isolated cells, microsomes, detergent solubilized receptors, etc.) from differnt tissues (liver, mammary gland, blood) of different animals (man; female, male, or pregnant rat; rabbit, etc.), and hormones from different species of animals assayed under various experimental conditions. At a variance with what is found for the mammary gland receptors,[82], which are strictly lactogenic, the hepatic sites that bind hGH in rat and rabbit appear to be more complex. Cadman and Wallis[83] have attempted to clarify this situation through a detailed characterization of the hGH-binding sites in the liver of the late pregnant rabbit

Table 1
CLASSIFICATION OF HORMONES AND RECEPTORS ACCORDING TO LESNIAK ET AL.[81]

Group	Hormones	Receptors for primate GH	Receptors for nonprimate GH	Receptors for lactogenic hormones
I	Primate GH	100	100	100
	Bovine PL	100	100	100
II	Nonprimate GH	0.03	100	0[a]
III	Human PL	0.03	0[a]	100
	Ovine PRL	0.03	0[a]	100
	Human PRL	0	0[a]	100

Note: The numbers indicate the extent of reactivity (shown in percentage) of each group of hormones with the different classes of receptors.

See text.

and their results suggest that there are very few specific somatotropic or specific lactogenic sites in this organ since bound hGH could be displaced almost completely by somatotropic or lactogenic hormones. Thus, bGH and oGH both effected considerable displacement of ^{125}I-hGH and pig, rat, and rabbit GHs also competed for binding sites but with a low potency compared with hGH; bPRL and oPRL both competed effectively for binding sites with ^{125}I-hGH but rat and rabbit PRLs showed no competition and hPL displaced ^{125}I-hGH with only 0.1% of the potency of hGH itself. oPRL was capable of considerable displacement of ^{125}I-bGH and bGH was almost as effective as oPRL at displacing ^{125}I-oPRL.

The results obtained by Cadman and Wallis[83] suggest that the rabbit liver contains very few specific lactogenic receptors of the type found in the mammary gland[82] where bound ^{125}I-oPRL or ^{125}I-hGH could be displaced by unlabeled lactogenic hormones but not by unlabeled somatotropic hormones like bGH. This contrasts with the situation in rabbit liver where bound ^{125}I-oPRL or ^{125}I-hGH can be almost completely displaced by bGH.

The bulk of the binding of hGH to the hepatic receptor preparation used by Cadman and Wallis[83] appears to be due to the presence of one or more receptors than can bind both somatotropic and lactogenic hormones. The physiological significance of such a receptor is not clear. A receptor that mediates the growth-promoting actions of GH would be expected to have a much greater affinity for this hormone than for prolactin. The inability of rabbit PRL to displace ^{125}I-hGH suggests that the receptor can in fact distinguish between the homologous GH and PRL. However, the fact that the endogenous (rabbit) GH binds with much lower affinity than many other GHs casts some doubts on the liver receptor as a mediator of the biological actions of the hormone.

Cadman and Wallis[83] suggest that if the bulk of the hGH-binding sites in these rabbit liver preparations are not mediating the specific biological action of GH, other biological functions for them must be considered. They may be involved in mediating the actions of other hormones of the GH-PRL family, such as PL, or modified forms of GH or PRL. Alternatively, they could be involved in mediating the degradation of GH or PRL and their removal from the circulation. However, Waters and Friesen[67] have been able to separate two types of binding sites from the liver of the pregnant rabbit: a somatotropic one with high affinity for GH and low affinity for oPRL, and another lactogenic with a high selective affinity for oPRL. The molecular characteristics of these binding sites are not definitively established and some discrepancies, even originated in the same research group,[84] are still unsolved. The hGH-binding protein has an apparent molecular weight between 200,000 and

300,000; is probably composed of subunits, and has an acidic pI. Chemically it is a sialoglycoprotein, or is closely associated with a molecule of those characteristics. An antiserum against this protein has been obtained[85] and will be a most useful probe in answering the question whether the hepatic hGH-binding site really represents the hGH receptor.

VI. SOMATOMEDINS

Many of the GH peripheral effects, especially those on bony growth, seem to be mediated through the secondary factors, somatomedins, already mentioned.[86] They constitute a family of polypeptides with cartilage stimulating activity and insulin-like properties on insulin target tissues such as fat and muscle.[87]

At present, several such factors have been purified from human serum: SMs A_1 and A_2,[88] SM C,[89,90] insulin-like growth factor 1 (IGF-1),[91] and insulin-like growth factor 2 (IGF-2).[92] Similar peptides, grouped under the generic name of multiplication stimulating activity (MSA), have been purified from a conditioned medium in which rat liver cells had been cultured.[93]

All these peptides, although of similar molecular size (7 to 8000), are not identical; they differ in electrical charge and chemical structure. Recent sequencing of IGF-1 and IGF-2 showed their remarkable homology with insulin. It was also found that IGF-1 and SM C are quite closely related in their primary structure.[94]

Another SM, also regulated by GH but lacking any insulin-like activity, was named SM B.[95] The examination of its structure indicated a close homology with the trypsin binding site of the bovine pancreatic trypsin inhibitor. In fact, weak antitrypsin activity has been found in SM B and it is easy to suggest a role for a protease inhibitor in growth. It may well be a modulator of prime importance in the regulation of tissue growth, in the conversion of prohormones to active hormones, and perhaps even in the unmasking of hormone receptors.

Somatomedins are produced mainly by the liver, although limited studies indicate that other tissues, such as kidney,[87] muscle and cultured fibroblasts may also release them. The liver may respond to available nutrients and hormones and channel calories toward or away from growth by altering the levels of circulating SMs and SM inhibitors.

SMs circulate noncovalently bound to a specific carrier protein (2 different have already been isolated); less than 1% SM in serum is present in the free form. Whether this represents the biologically active fraction, or whether variations in affinity to the binding protein or to the tissue receptors determine the biological effect is not known.

SM actions may be characterized in terms of binding to receptor sites on tissue plasma membranes, effects on tissues in vitro, and consequences of administration in vivo.[87]

The extensively studied insulin-like actions of SMs suggest that they are not responsible for the diabetogenic actions of GH which may be due to direct actions of the hormone or may be mediated by other factors.[7]

Less is known about SM growth-related actions. The difficulty in obtaining sufficient quantities of purified SMs has slowed appreciation of their effects in intact animals. Initial evaluation of growth-promoting effects of SMs was largely dissappointing. Administration of SM A produced little increase in body weight in normal rats or tibial width in hypophysectomized rats;[96] it stimulated the incorporation of sulfate in the skin but not in costal cartilage[97] nor increased the longitudinal bone growth[98], both in hypophysectomized rats.

In 1978, data from Van den Brande's laboratory suggested that SM could produce growth in vivo: a SM preparation (containing no GH, insulin, testosterone, or thyroxine) administered to Snell's dwarf mice induced increase in body weight, body length, and cartilage uptake of sulfate comparable to those produced by GH.[99]

Van Wyk et al.[94] found vigorous DNA synthesis and mitotic activity in hypophysectomized frogs injected with pure SM C, over a 10-day period. This was the first evidence that a pure

SM can restore DNA synthesis and cell proliferation in an organ (lens epithelium) of a living animal.

Schoenle et al.[100] recently demonstrated that pure IGF-1 stimulates gain in body weight, increase in tibial epiphysial width, and thymidine incorporation into costal cartilage when administered continuously for 6 days to hypophysectomized rats.

The following clinical findings support Salmon and Daughaday's somatomedin concept: immunoreactive IGF-1 is decreased in hypopituitarism and increased in acromegaly,[101] treatment of hypopituitary children with GH increases the serum immunoreactive IGF levels.[102] So there is unequivocal direct evidence for the notion that IGF rather than GH itself is the true growth-stimulating hormone. However, direct or indirect effects of GH on various tissues cannot be excluded.

Recent in vivo experiments in hypophysectomized rats indicate that the local administration of hGH to the cartilage growth plate of the proximal tibia results in accelerated longitudinal bone growth.[103] This direct effect of GH does not support the theory that the increased plasma concentration of SMs is a prerequisite to promote longitudinal bone growth, although a local SM production following GH administration cannot be discarded.

Besides somatomedins, several other growth factors have been identified, among them the epidermal growth factor, fibroblast growth factor, nerve growth factor, and platelet growth factor.[104]

VII. BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

The study of the relationship between structure and function in GHs has been attempted following two different approaches. One of them rests on the analysis of the changes in biological activity detected after chemical modification of particular residues in the molecule. We are not going to discuss this work here and the reader is referred to the reviews of Paladini et al.[105] and Wallis.[7]

The other approach is a consequence of early attempts to find an "active core" common to all GHs through controlled enzymatic digestion of the native hormones (see also Section VIII). Initial encouraging results with chymotryptic, peptic, and tryptic digests of bGH led Sonenberg et al.,[106] in 1970, to the isolation of a small fragment of bGH that had some of the properties of the native hormone and, most gratifyingly, was metabolically active in man. Since the whole field of research in GH as well as the clinical studies and therapy in hypopituitary children is held back due to the limited supply of hGH, Sonenberg's results stirred a great deal of interest and several laboratories pursued actively the study of enzymatic digests of various GHs. These research efforts were extended to fragmentation of the molecule by chemical means and by the synthesis of selected sequences in the hGH.[107,108] Table 2 collects all the relevant information accumulated since 1970 on this subject.

Several negative results appear in Table 2 but we tend to favor positive ones for two reasons: first, a reliable, positive result is a fact that needs an explanation and we assume that it has a molecular basis, and secondly, a negative result is more sensitive to artifacts in the experiment like the physical state of the peptide fragments.[109] Also, low or negative responses in an experiment in vivo may be due to increased turnover or accelerated metabolic disposal and not to any intrinsic biological inertness of the peptides.

The information in Table 2 adds further weight to the concept that the structural elements required for body growth-promoting activity, or to produce a variety of acute, short-lived (or even permanent) metabolic effects, and regulatory actions are located in separate, discrete regions of the GH molecule. The hypothesis of a protein hormone with several active centers is thus supported by a reasonable amount of experimental evidence.

This characteristic of GH illustrates a typical property of some active proteins, namely, the coalescence into one chemical species of several independently active molecules. It is interesting that Niall et al.[110] have pointed out internal sequence homologies in hGH which suggest that the hormone may have evolved by genetic reduplication from a smaller ancestral peptide. It is possible that, during evolution (see Section III), this peptide acquired new properties or modified its existing ones, resulting in a molecule whose different fragments have activities in common.

Pure hGH is probably not diabetogenic in the dog,[53] however this property is latent in the molecule since it appears in several fragments of hGH obtained by enzymatic digestion.

When two complementary fragments of GH are recombined under proper conditions, the original biological activities of the hormone are restored in high proportion. This points to the paramount importance of conformation on the proper handling of the hormone by the target cells.

How the many activities packaged in GH are translated into physiological effects is not easy to see. One possibility, besides the obvious spontaneous selection of the different active centers in GH by specific receptors, is connected with the heterogeneity of the hormone obtained from the pituitary gland (see Section IV). The 22K native hormone may be only one of the molecular species released by the pituitary, and many other modified forms with different activities could exist or be generated in the circulation to perform their different duties. In fact, the biological activity recovered from plasma is much higher than the immunological activity measured in it,[44] a result to be expected if active fragments of GH, devoid of immunological reactivity, were present.

Another possible route to the production of active GH-fragments arises from the peculiar nature of the in vivo distribution of ^{14}C-labeled bGH in the rat:[64] the radioactivity distribution curves fall into two different categories; in kidney, liver, and spleen there is an early concentration which reaches a maximum 15 min after the injection of the hormone, and then rapidly declines. In heart, skeletal muscle, pancreas, intestine, bone, and fat, the radioactivity increases gradually and a steady-state is reached after 30 to 60 min. Kidney is the organ where the highest concentration of radioactivity occurs. However, muscle accumulates more than 60% of the initial dose after 2 hr. Very little radioactivity appears in the urine in this period. Kidney, liver, and muscle rapidly produce radioactive fragments soluble in 10% trichloroacetic acid.

The time sequence of GH distribution in the different organs of the rat suggests that the hormone may be transformed, catabolized, or activated in any of the organs that behave as early concentrators: liver is a well-characterized target organ for GH. Very little information is available on the fate of GH in the spleen, but it is well known that in the kidney low molecular weight proteins are extensively filtered, absorbed from the luminal side by renal tubular cells, and released back to the circulation, either as intact molecules or as catabolic products.[111] It is not known if the kidney produces active molecules after its initial massive absorption of native GH, but the possibility merits further investigation. It is interesting to note that chronic renal insufficiency in childhood is often associated with a delay in body growth, in spite of high concentrations of hGH in the circulation.[112]

VIII. IMMUNOCHEMISTRY OF GROWTH HORMONES

A. Early Studies

The first report of the production of antibodies against bGH in rabbits occurred in 1952 when Morrison et al.[113] could support their claim by clear precipitin reactions. Before that date it was even unthinkable that an animal would form antibodies to such an important biologically active substance.[114]

Table 2
BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

Source	Fragment[a]	Biological effect	Relative response[b] (native GH:100%)	Ref.
Enzymatic digestion:				
Trypsin	bGH 96—133	Tibial width increase[c]	5%	1, 2
			±	3
		Weight gain[c]	+	1
		Metabolic[c,d]	+	4
		Lipolysis[e]	3%	5
		Metabolic (humans)[f]	+	6
		Pleiotypic[g]	+	7
			+	8
	oGH 96—133	Tibial width increase[c]	±	3
	(Cam)-bGH 151—191	Tibial width increase[c]	±	3
	bGH 1—95/151—191	Tibial width increase[c]	±	2
		Weight gain[c]	±	1
		Metabolic[c,d]	+	4
		Lypolysis[e]	±	5
		Pleiotypic[g]	+	7
		Somatomedin-like[ch1,h2]	±	8
	hGH 1—134/146—191	Tibial width increase[c]	96.4%	9
		Receptor binding[i1]	111%	9
		Receptor binding[i2]	150%	9
		|^{125}I-hGH|-Radioimmunoassay	73%	9
Pepsin	hGH 31—44	Lipolysis	+	10
Plasmin	hGH 1—134/141—191	Tibial width increase[c]	100%	11
		Lactogenic[j1]	>100%	11
		Weight gain[c]	100%	12
		^{14}C-leucine incorporation[c,k1]	100%	12
		Glucose oxidation[c,l]	100%	12
		α-Amino isobutyric acid and 3-O-methyl glucose uptake[c,k1]	+	12
		Somatomedin-like[c,h3]	100%	12
		Diabetogenic[o1]	+	12
	hGH 1—134/147—191	Tibial width increase[c]	⩾100%	11
		Lactogenic[j1]	⩾100%	11
		|^{125}I-hGH|-Radioimmunoassay	100%	11
	(Cam)-hGH 1—134	Tibial width increase[c]	10—20%	13
		Weight gain[c]	±	12
		^{14}C-leucine incorporation[c,k1]	+	12
		Glucose oxidation[c,l]	+	12
		Orn. decarbox. stimulation[m1]	10%	14
		Lactogenic[j1]	6—10%	13
		Lactogenic[j2]	10%	15
		Somatomedin-like[c,h3]	+	12
		Receptor binding[i3]	3%	16
	(Cam)-hGH 141—191	Tibial width increase[c]	6%	13
		Weight gain[c]	—	12
		^{14}C-leucine incorporation[c,k1]	—	12
		Glucose oxidation[c,l]	—	17
		Orn. decarbox. stimulation[m1]	—	14
		Lactogenic[j1]	<6%	13
		Lactogenic[j2]	10%	15
		Somatomedin-like[c,h3]	—	12
	hGH 20—41	Weight gain[c]	—	17
		Glucose oxidation[c,l]	—	17
		Lactogenic[j3]	—	17
		Somatomedin-like[c,h3]	—	17

Table 2 (continued)
BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

Source	Fragment[a]	Biological effect	Relative response[b] (native GH:100%)	Ref.
	hGH 95—134	Weight gain[c]	2%	17
		Glucose oxidation[c,l]	±	17
		Lactogenic[j3]	3%	17
		Somatomedin-like[c,h3]	–	17
	(Cam)-hGH 15—125[n]	Tibial width increase[c]	+	18
		Orn. decarbox. stimulation[m1]	7%	18
		Lactogenic[j1]	3%	18
		Receptor binding[i3]	2%	16
	(Cam)-hGH 42—134	Weight gain[c]	—	19
		Glucose oxidation[c,l]	—	19
Fibrinolysin	hGH 1—136/148—191	Tibial width increase[c]	⩾100%	20
		Weight gain[c]	⩾100%	20
		Lactogenic[j1]	⩾100%	20
		|^{125}I-hGH|-Radioimmunoassay	100%	20
Subtilisin	hGH 1—139/150—191;	Tibial width increase[c]	>100%	21
	hGH 1—139/147—191	Diabetogenic[o2]	+	21
Thrombin	(Cam)-oGH 1—133	Tibial width increase[c]	—	22
	(Cam)-hGH 1—134	Weight gain[c]	±	23
		Glucose oxidation[c,l]	3%	23
		Diabetogenic[o3]	25—50%	23
	(Cam)-hGH 135—191	Weight gain[c]	±	23
		Glucose oxidation[c,l]	—	23
		Diabetogenic[o3]	—	23
Selective cleavage in:				
Tryptophan	(Cam)-bGH 1—86	Tibial width increase[c]	±	24
	(Cam)-bGH 87—191	Tibial width increase[c]	—	24
Methionine	bGH 6—124/150—179	Weight gain[c]	—	25, 26
		^{14}C-leucine incorporation[c,k1]	2%	25
		Glucose oxidation[c,l]	+	25
		α-Aminoisobutyric acid uptake[c,k1]	—	25
	bGH 125—149;	Weight gain[c]	—	25, 26
	(Cm)-bGH 150—179;	^{14}C-leucine incorporation[k1]	—	25
	bGH 180—191	Glucose oxidation[c,l]	—	25
		α-Aminoisobutyric acid uptake,[c,k1]	—	25
	(Cm)-bGH 6—124	Weight gain[c]	—	25
		^{14}C-leucine incorporation[k1]	—	25
		Glucose oxidation[c,l]	+	25
		α-Aminoisobutyric acid uptake,[c,k1]	—	25
	(Ae)-pGH 5—124	Weight gain[c]	—	27
		^{14}C-leucine incorporation,[c,k1,k2]	+	27
		Somatomedin-like,[c,h3]	—	27
	(Ae)-pGH 125—179	Weight gain[c]	—	27
		^{14}C-leucine incorporation,[c,k1]	—	27
		Somatomedin-like,[c,h3]	—	27
	(Ae)-hGH 15—125	Weight gain[c]	—	27
		^{14}C-leucine incorporation,[c,k1]	±	27
		Glucose oxidation[c,l]	±	27
		α-amino isobutyric acid and 3-O-methyl glucose uptake,[c,k1]	+	27
		Somatomedin-like,[c,h3]	—	27
	hGH 171—191	Diabetogenic[o4]	—	28
	oGH 6—124/150—179	Orn. decarbox.	+	29
		stimulation,[c,m1,c,m2m2]	—	29
	oGH 125—149	Orn. decarbox. stimulation[m2]	±	29

Table 2 (continued)
BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

Source	Fragment[a]	Biological effect	Relative response[b] (native GH:100%)	Ref.
		Somatomedin-like,[c,h1]	—	29
	eGH 5—72/124—178; eGH 73—123	Tibial width increase[c]	—	30
		Weight gain[c]	—	30
Tryptophan and methionine Chemical synthesis:	bGH 87—124	Somatomedin-like,[c,h1,h2]	+	31
	N-α-acetyl hGH 95—136	Tibial width increase[c]	±	32
	hGH 95—134; hGH 77—102 hGH 124—155	Receptor binding[i]	—	33
	hGH 87—123	Weight gain[c]	—	33
		Receptor binding[i]	—	33
	bGH 125—133	Tibial width increase[c]	±	34
		Weight gain[c]	—	34
	hGH 95—124[p]; N-α-Boc-hGH 95—124	Tibial width increase[c]	—	35
	hGH 1—35;	^{14}C-leucine incorporation[c,k]	—	36
	hGH 78—103;	Glucose oxidation,[c,l]	—	36
	hGH 88—124;	Lipolysis[c]	—	36
	hGH 95—134;	Somatomedin-like[h3]	—	36
	hGH 135—172	Receptor binding[i]	—	36
	(Cam)-hGH 140—191	Tibial width increase[c]	±	37
		Lactogenic[j]	±	37
	oGH 95—133	Tibial width increase[c]	—	38
		Weight gain[c]	—	38
	oGH 111—133; hGH 125—156; hGH 88—124	Tibial width increase[c]	±	38
	hGH 83—130;	Tibial width increase[c]	—	38
	hGH 96—134	Weight gain[c]	—	38
		^{14}C-leucine incorporation,[c,k]	±	38
	hGH 1—36; hGH 166—191	Tibial width increase[c]	—	38
	(Cam)-hGH 44—77	Diabetogenic[o3]	+	39
	(Cam)-hGH 52—77	Diabetogenic[o3]	—	39
	hGH 176—180; hGH 172—191; hGH 176—191; hGH 177—191; hGH 178—191	Diabetogenic[o5]	+	40, 41
	hGH 179—191; hGH 180—191	Diabetogenic[o5]	—	40
	hGH 1—13; hGH 3—13; hGH 6—13; hGH 1—15; hGH 1—20	Insulin-like[o5]	+	42
	hGH 1—10; hGH 6—11	Insulin-like[o5]	—	42
	(Cam)-hGH 1—54	Tibial width increase[c]	7%	43
	|Nle^{170}, Ala^{165,182,189}|-hGH 145—191; |Nle^{170}, Ala^{165,182,189}|-hGH 140—191; |Lys^{135,136,138}, Glu^{137,139} Nle^{170}, Ala^{165,182,189}|-hGH 135—191	Tibial width increase[c]	±	44

Table 2 (continued)
BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

	(Cam)-hGH 1—128;	Tibial width increase[e]	—	45
	(Cam)-hGH 19—128;	Weight gain[e]	—	45
	(Cam)-hGH 34—128;	Lactogenic[j1]	—	45
	(Cam)-hGH 44—128;	Receptor binding[i4,i5]	—	45
	hGH 64—128; hGH 88—128; hGH 88—120[p]	Membrane enzymes cooperativity	+	46
	hGH 73—128; hGH 75—120[p]	Weight gain[e]	—	47
Recombination:[q]				
I)	(Cam)-hGH 1—134 and (Cam)-hGH 141—191[r]	Tibial width increase[e]	108%	48
		Orn. decarbox. stimulation[m1]	69%	49
		Lactogenic[j1]	102%	48
		[j2]	97%	49
		Receptor binding[i1]	25%	50
		[i2]	78%	50
		Radioimmunoassay(^{125}I-hGH)	65%	49
II)	bGH 96—133 and bGH 1—95/151—191	Tibial width increase[e]	10%	2
III)	(Cam)-hGH 1—134 and (Cam)-hGH 135—191	A. Weight gain[e]	35%	19
		Glucose oxidation[e,1]	20%	19
		Diabetogenic[o3]	100%	19
		Receptor binding[i5]	20%	19
		|^{125}I-hGH|-Radioimmunoassay	100%	19
		Receptor binding[i1]	22%	51
		[i2]	49%	51
		C. Tibial width increase[e]	51%	52
		Receptor binding[i1]	19%	52
		[i2]	35%	52
		|^{125}I-hGH|-Radioimmunoassay	40%	52
IV)	(Cam)-hGH 1—134 and (Cam)-hPL 141—191	Receptor binding[i1]	19%	53
			11%	54
		Receptor binding[i2]	76%	53
			21%	54
		|^{125}I-hGH|-Radioimmunoassay	100%	53
			46%	54
		|^{125}I-hPL|-Radioimmunoassay	0.1%	53
			0.3%	54
V)	(Cam)-hPL 1—133 and (Cam)-hGH 141—191	Receptor binding[i1]	0.2%	53
			—	54
		Receptor binding[i2]	—	53, 54
		|^{125}I-hGH|-Radioimmunoassay	3%	53
			0.5%	54
		|^{125}I-hPL|-Radioimmunoassay	1.8%	53
			1.4%	54
VI)	hGH1—134/hPL 141—191	Receptor binding[i1]	72%	54
		[i2]	91%	54
		|^{125}I-hGH|-Radioimmunoassay	54%	54
		|^{125}I-hPL|-Radioimmunoassay	0.5%	54
VII)	hPL 1—133/hGH 141—191	Receptor binding[i1]	—	54
		[i2]	93%	54
		|^{125}I-hGH|-Radioimmunoassay	—	54
		|^{125}I-hPL|-Radioimmunoassay	65%	54

Table 2 (continued)
BIOLOGICAL ACTIVITY OF GH FRAGMENTS AND RECOMBINANT FORMS

[a] Residues numbers were derived from table reference 55. The existence of a disulfide bridge between two chains is represented by /. An intra chain disulfide bridge exists when two cysteines are present in the sequence unless specified differently: (Cam) means *S*-carbamidomethyl cysteine; (Cm) *S*-carboxymethyl cysteine; (Ae) *S*-aminoethyl cysteine.

[b] + Means a clear positive response but quantitative comparison is difficult; ± statistically significative, but very low.

[c] Hypophysectomized rats.

[d] |^{14}C|-glucose and |^{14}C|-His uptake; glucose oxidation and incorporation into glyceride-glycerol by adipose tissue.

[e] Glycerol release in isolated rat fat cells.

[f] Changes in blood urea nitrogen, calciuria, urinary creatine excretion, insulin resistance, and plasma fibrinogen level in hypopituitary children.

[g] Stimulation of protein and nucleic acid synthesis in human fibroblasts.

[h$_1$] In vitro |^{35}S| sulfate and |^{3}H| thymidine uptake by costal cartilage.

[h$_2$] Competition for insulin binding sites in human placental membrane preparations.

[h$_3$] |^{3}H| thymidine uptake by costal cartilage from injected rats.

[i$_1$] In liver cell membranes from pregnant rabbits.

[i$_2$] In mammary gland membranes from pregnant rabbits.

[i$_3$] In IM-9 lymphocytes.

[i$_4$] In hepatocytes.

[i$_5$] In liver cell membranes from female rats.

[j$_1$] In pigeon crop sac.

[u$_2$] |^{3}H|aminoacids incorporation into casein-like protein of mid-pregnant mouse mammary gland.

[j$_3$] Induction of *N*-acetyl lactosamine synthetase in mammary tissue explants from pregnant mice.

[k$_1$] Isolated diaphragm.

[k$_2$] In vivo pulse labeling of protein in diaphragm and liver from rats.

[l] By isolated epididymal adipose tissue.

[m$_1$] In rat liver.

[m$_2$] In rat kidney.

[n] Obtained by additional cleavage at Met^{14} of hGH 1—134.

[o$_1$] Glucosuria production in partially pancreatectomized, dexamethasone-treated rats.

[o$_2$] Glucose intolerance production and hyperinsulinemia in fasted dogs. Intact hGH did not produce these effects.

[o$_3$] Glucose intolerance production in ob/ob mouse.

[o$_4$] Inhibition of glucose uptake by rat soleus muscle and of the activity "in vitro" of glyceraldehyde 3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase.

[o$_5$] Changes in: glucose uptake by rat diaphragm, glyceraldehyde 3-phosphate dehydrogenase activity, glycemia, and plasma insulin level in rats or rabbits.

[p] Peptide amide.

[q] Noncovalent association of the two fragments was achieved by mixture under different experimental conditions (I-V). In recombinant IIIC, a covalent coupling of both fragments was generated with thrombin. In VI and VII the covalent linkage was obtained through reformation of the disulfide bond between Cys^{53} and Cys^{165}

[r] Similar results were obtained by the authors using the natural or synthetic C-terminal peptide.

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Rapid advances made in antibody production technology soon permitted that concentrations as low as 0.2 μg/mℓ of hGH could be detected.[115]

The specificity of the antiserum against GH was proven by the then newly described techniques of double diffusion in agar gels[116-118] and by microimmunoelectrophoresis.[117]

A remarkable result was obtained by Li et al.[118] who were able to measure the GH content of the human pituitary gland by immunoassay and by the tibia test. Quite comparable values were obtained: 5.7 mg and 3.8 mg per gland, respectively, strongly supporting the validity of the quantitative precipitin method applied.

Attempts to assay the levels of GH in blood were made by hemagglutination-inhibition and complement fixation techniques, but these methods were not sufficiently sensitive.[119] Utiger et al.,[120] employing a radioimmunoassay procedure, succeeded in measuring GH in extracted plasmas and the minimum detectable amount of hGH was of the order of 3 to 6 ng.

A major breakthrough was achieved when Hunter and Greenwood[121] developed a procedure for the preparation of ^{131}I-labeled hGH of high specific activity. This tracer allowed them to measure the GH levels in unextracted plasmas of normal human subjects and acromegalics[122] utilizing Yalow and Berson's technique[123] for plasma insulin. It followed very soon[124,125] the detection of the acute changes in plasma levels of hGH that occur under various physiological conditions or experimental stimulus.

B. Antigenicity of GHs

In the decade following 1960, it was shown by a number of investigators that it was possible to produce antibodies against all available purified GH preparations, regardless of the species of origin. (See Hayashida[8] for a complete review.)

The rabbit and the guinea pig have been the two most commonly employed species for the production of antiserum against GH. It was soon found that the rat posed special problems: the hypophysectomized rat would respond with continued growth if injected daily, for a month or more, with either bovine or whale GH; whereas injections of human or monkey GH would result in a failure of growth reponse after approximately 10 days of treatment.[126] These findings were interpreted as being due to an immune response of the rat against primate GHs but not against GHs from the other species, and it suggested that the antigenic structure of primate GHs must be quite different from that of the rat. These observations correlate very well with the evolutionary "jump" that primate GHs have made respective GHs from the other mammals (see Figure 1).

Rat GH may then be considered a relatively poor antigen in the rabbit but a good one in the monkey.[8] Human GH should be a better antigen than bGH or eGH in the rabbit and this was found to be the case in our laboratory where anti-hGH titers higher than anti-bGH or anti-eGH titers were systematically obtained in this species.[127]

The experimental and therapeutic use of hGH in man since 1957[128-130] has led to the discovery of the production of homologous antibodies. In 1964, Roth et al.[131] and Parker et al.[132] first described the binding capacity and association constant of antibodies against hGH in human sera.

The following possibilities, all based on the existence of some structural difference between the administered hormone and the endogenous circulating hormone, have been suggested to explain the formation of homologous antibodies:[131]

1. During extractions and purification a small fraction of hGH is altered and thereby endowed with an antigenic potency not present in the native molecule.
2. Changes may occur in hGH during storage alone or in the intramuscular injection site as a result of proteolytic action due to enzymes present as contaminants.
3. The hGH injected is heterogeneous due to individual genetic differences in the donors of the glands used.

The demonstration that hGH-binding antibodies occur in humans after the administration of hGH may explain some of the resistance to therapy.[133] Roth et al.,[131] however, make the additional comment that a high concentration of antibodies that bind hGH with a weak energy might actually have a beneficial effect by protecting the hormone from rapid destruction in the body and allowing its release slowly between injections.

The groups of Illig[38,39] and Kaplan et al.[134] have studied in detail the relationship between different hGH preparations used in therapy and the incidence of antibodies formation. Illig et al.[38,39] described the increased tendency of GH antibodies formation in a group of children with familial isolated growth hormone deficiency type A which is effective already before birth and which causes a lack of immunotolerance to homologous hGH. In these patients, Phillips et al.[37] investigated the molecular basis of the defective production of GH, as already mentioned.

C. GH Activity in Plasma

Hayashida[8] expressed his doubts about the identity of the substance being measured with a RIA for GH since he found in hypophysectomized rats significant levels of presumably immunoreactive pituitary GH. He concluded, from this and other studies, that what is currently being measured in the usual RIAs for plasma GH in the rat, and perhaps in other animals, may not be identical to the circulating form of pituitary GH.

Tibial bioassays and RIAs with human and rat plasmas have confirmed a wide divergence between the detected levels of GH by both methods.[44] Pooled human plasmas yielded a 200-fold disparity between the concentration of GH activity and the immunoassayable GH.

Antiserum to rGH inhibited the response of rats (tibia test and body weight assay) to pure rGH, but not to bioassayable plasma growth active material which does not seem to be SM C.[44]

The parallelism between the dose response curves of bioassayable plasma hormone and authentic pituitary GH suggests that the plasma hormone may be a derivative of the latter generated in the circulation as well as in the pituitary.[44]

In contrast to the wide discrepancies between bioassay and RIAs, the results of radioreceptor assays (RRA) are in close agreement with those of the RIA (see Sonksen and West[22] for review).

One recent example that RIA and RRA detect the same substances is the receptor modulation assay developed by Rosenfeld and Hinz using IM-9 lymphocytes.[135] When this assay is applied to serum samples from acromegalic patients, a very close correlation between RIA and RRA results is apparent. Nevertheless, further investigations are required to fully clarify the many intriguing questions posed by the bioassayable GH-like activity of plasma studied by Ellis, Vodian, and Grindeland.[44]

D. Comparative Studies

Hayashida[8] has summarized, up to 1974, the immunochemical cross-reactions of GHs, or pituitary extracts of mammalian and submammalian species with a variety of antisera to mammalian GHs. This survey has suggested that all vertebrate GHs tested share some common immunochemical determinants.

Complement fixation, as originally applied,[119] was not sufficiently sensitive. It has since been improved and the microcomplement fixation procedure of Wasserman and Levine[136] could be used to detect either small differences in structure between very closely related antigens or small similarities between only remotely related antigens.[137] Thus it was possible to show significant reactions between p, b, and oGHs with anti-hGH serum.[138,139] This was also our experience[140] with h, b, o, and eGHs which gave indications of cross-reactivity using antisera obtained in rabbits and a hemagglutination method. With the microcomplement fixation method, eGH clearly reacted with a rabbit anti-hGH serum[141] and analogous evidence of interaction was obtained using a rabbit anti-bGH serum and both h or eGHs.[142]

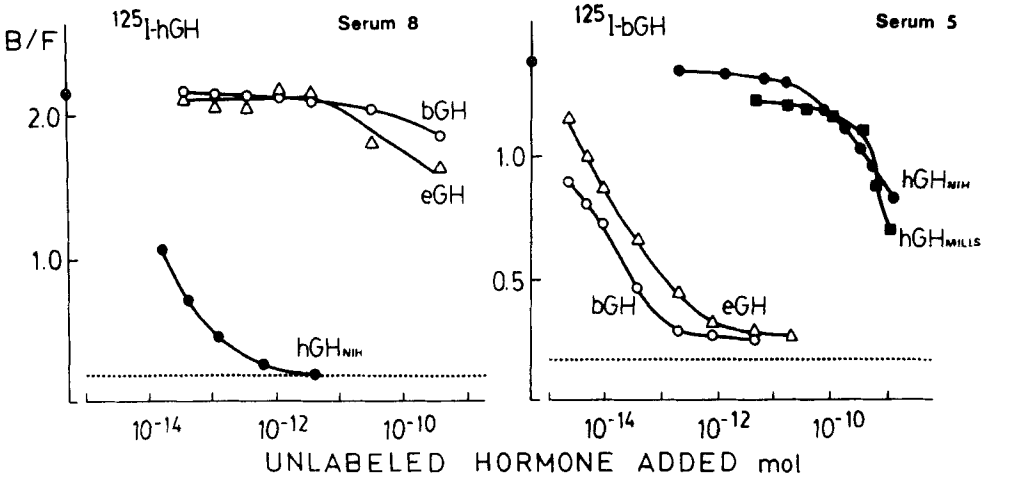

FIGURE 2. Displacement curves for hGH_{NIH} (standard reference preparation), hGH_{Mills} (prepared as indicated in Reference 166), bGH and eGH. Serum 8 (1:1500) is a human serum specific for hGH; serum 5 (1:800) is a human serum containing only antibodies against bGH or eGH. ^{125}I-hGH: 1.5 ng/mℓ; ^{125}I-bGH: 0.59 ng/mℓ. ---, nonspecific binding. B/F: bound to free ratio. (Adapted from Reference 146.)

RIA was utilized by Parker et al.[143] to detect cross-reactions between pGH and rGH in homologous and also in heterologous assays.[144]

An interesting experimental situation arises when human patients are therapeutically injected with hGH for extended periods. In these circumstances, it is not uncommon to find antibodies against hGH in their plasma, as already discussed, and also the rather frequent presence of "heterologous" antibodies exhibiting anti-porcine,[145] anti-bovine, and anti-equine GH[146] activities. No cross-reaction with hGH could be demonstrated in the patients showing antibodies to pGH,[145] and displacement experiments[146] indicated that hGH was an effective competitor of ^{125}I-hGH whereas b and eGHs were quite inefficient. Conversely, when the tracer was ^{125}I-bGH, both bovine and equine growth hormones were good displacers while the human hormone was poor (Figure 2). Recent experiments[147] with the same human sera have shown that other animal hormones like porcine, rabbit, and rat GHs are also good displacers of ^{125}I-hGH.

The affinity constants of the various antibodies found in human sera by Poskus et al.[146] had all similar values and were of the same magnitude as those usually found in rabbits immunized with these antigens. These results suggest that the antibodies against b, e, p, r, and rabbit GHs belong to a separate population distinct from that directed against the human hormone.

Perhaps the closest experiment to those just described that can be found in the literature was performed by Hayashida in 1969[148] when the immunochemical relatedness of several mammalian GHs was examined by a RIA procedure using monkey antiserum against rGH and ^{125}I-rGH as tracer. hGH did not react in such system (see Table VIII in reference 8), but p, rabbit, and whale GHs were quite effective displacers and b and oGHs rather good ones. One may speculate that in these experiments only the antibodies directed against the common immunochemical determinants in nonprimate hormones were acting.

The heterologous immunity obtained with hGH has only been found in human beings therapeutically treated with hGH for relatively long periods.[145,146] In laboratory animals immunized against various GHs, including hGH, aside from weak cross-reactions and the previously mentioned experiments by Hayashida,[148] no significant "heterologous activity"

of the type described by Murthy and Mc Garry[145] and by Poskus et al.[146] was detected up to now.

The mechanism by which hGH can express determinants of other animal growth hormones is obscure although the high degree of conservation of structure in these molecules during evolution must be involved.

E. Enzymatically and Chemically Modified GHs

An idea that was put to test very early in the GH field was that of disclosing a common "active core" in all mammalian GHs through controlled enzymatic digestion of the native hormones. A "beef α-core" obtained by partial hydrolysis of bGH with chymotrypsin[149] was active in promoting growth in rats, but in spite of some initial indications of being also active in man this claim could not be substantiated. Various other attempts in the same direction followed and the reader is referred to a brief review by Sonenberg et al.[150] for further details.

Immunological studies employing precipitin tests with rabbit antiserum to intact bGH suggested that the immunoreactivity of the "α-core preparation" was significantly reduced.[151]

Laron et al.[152] obtained some evidence of cross-reactivity between bGH and hGH with an impure bGH fragment isolated from a pepsin digest of this hormone, and Glick[153] reported that one of the patients treated with a tryptic digest of bGH developed an antiserum which was capable of reacting significantly with hGH in a RIA. Furthermore, he observed that this human antiserum cross-reacted with monkey GH and a variety of nonprimate GHs including b, p, and rGH as well as a bovine α-core preparation.

The study of the immunological reactivity of GH by the use of enzymatically or chemically obtained derivatives which contain the amino acid backbone mostly unchanged, but, nevertheless, with the secondary or tertiary structures probably altered, are useful for exploring the dynamic role of molecular conformation in these processes.

Experiments related with the modification of the disulfide bonds in hGH provide a good example to demonstrate that the results obtained usually depend on: (1) the methodology applied to prepare the derivative, (2) the specificity of the acting antibodies, and (3) the nature of the procedure used to detect the antigen-antibody interaction.

Plasmin-digested hGH was found[154] to be composed of the N-terminal residues 1 to 134 and the C-terminal residues 141 to 191, connected by a disulfide bridge. Agar gel diffusion studies with guinea pig antiserum to hGH showed a reaction of qualitative immunochemical identity between this derivative, hGH, and totally reduced and alkylated hGH. When both fragments were separated by reduction of the disulfide bridge and carbamidomethylation, it was found that the N-terminal fragment had greater immunological activity than the C-terminal fragment. In accord with these studies, it was previously reported that the N-terminal fragment possesses greater biological activity than the C-terminal fragment.[155]

Totally reduced and alkylated hGH and plasmin digested hGH are as potent in man as unmodified hGH but the enzymatic derivative is less immunoreactive.[156,157] As discussed by Hayashida,[8] the disulfide bridges are not essential for the biological activity of hGH but their intactness appears to play a significant role in determining its immunoreactivity when one is employing an antiserum to the native hormone. Aubert et al.[158] have partially confirmed this hypothesis with reduced and carboxy or carbamidomethylated hGH and a hGH antiserum purified by affinity chromatography: the derivative exhibited only 70% to 80% of the reactivity of native hGH. Performic acid oxidized hGH, which retains very little of the original conformation, had low immunoreactivity in the same test.

Zakin et al.[140] found that special precautions had to be taken to discriminate the changes in immunological properties due to the specific chemical reactions applied apart from those caused by the intervening manipulations of the proteins. Total reduction of the disulfide bridges and carboxymethylation of the resulting thiols did not substantially affect the reac-

tivity of hGH in a hemagglutination assay, but the same treatment applied to b and eGHs made both proteins almost inactive.

Performic acid oxidation, a well-known procedure to modify tryptophan, methionine, and cystine residues in proteins, differentially affected the immunoreactivity of h, b, and eGHs.

The oxidized human hormone retained all its reactivity in hemagglutination,[140] but in RIA had a low affinity for the antibodies together with an important residual reactivity,[105] while the majority of the determinants in bGH were permanently inactivated as measured by hemagglutination. The oxidized equine hormone could regain most of its original reactivity in the same test by a short exposure to high pH, or to 8 *M* urea. These treatments, although immunologically effective, did not restore the native conformation in oxidized eGH as proved by circular dichroism measurements. These results supported the suggestion that the determinants in hGH, and possibly in other mammalian GHs, as well, must be located in a small area of the molecule.[140]

Another approach to the detection of structural determinants in common with nonprimate GHs has used limited sulfitolysis of the disulfide bonds to "open up" the hormone molecule.[159] When bGH was partially reduced by cleavage of one disulfide bond, it was able to fix more complement than native bGH, reacting with an antiserum to hGH. It was felt that sulfitolysis had exposed an area of the molecule that was similar or identical to a portion of the hGH molecule. When this procedure was applied to hGH, it was again demonstrated that the partially reduced hormone fixed more complement, or cross-reacted better than the untreated hGH preparation, utilizing rabbit antiserum to bGH.[139]

From these studies, and others that will be mentioned in connection with the mapping of the antigenic sites in GHs, it can be concluded that the disulfide bridges are located outside these sites. However, it should be remembered that when the bridges are modified, the tertiary structure may reflect the change and influence the reactivity of the epitopes.[8]

The immunoreactivity of various fragments of h and bGH has been explored: hGH 20—41 and 95—134[160] obtained by plasmin digestion (of reduced and Cam-hGH) have very low reactivity in RIA carried out with the antibodies against the native hormones; hGH 1—134[161] obtained by thrombin digestion is a good displacer of ^{125}I-hGH in a homologous RIA. The remaining fragment, hGH 135—191 instead, reacts very weakly.

It is difficult to assess the validity of marginal reactions in these experiments since the presence of traces of the intact hormone cannot usually be excluded.

With bGH, Ferrara et al.[142] were able to localize the majority of the determinants reactive in a complement fixation assay, within peptide 6—124, obtained by cyanogen bromide treatment of the native hormone.

Another interesting approach is the study of the mixed recombinants obtained by incubating the N-terminal fragment of hGH (or hPL) with the C-terminal fragment of hPL (or hGH).[162] The resulting noncovalent complexes recover full radioimmuno and radioreceptor activities in a way which demonstrates that the antigenic determinants for both a monospecific antiserum to hGH and a monospecific antiserum to hPL are in the N-terminal fragments of their respective antigens (see Table 2).

In our laboratory, the efforts to map the antigenic determinants in GHs started when eGH was being sequenced.[163] Peptide fragments obtained by cyanogen bromide cleavage were isolated and identified. Their immunological reactivity was tested by hemagglutination and complement fixation methods using rabbit antisera against the native hormone. Antigenic determinants were detected in the fragments comprising amino acid sequences 5—72 and 73—123 of eGH, this last one being predominant. Fragment 124—178 had very low reactivity.

Nitration of peptide 73—123 did not modify its immunological properties, but oxidation diminished them. Comparison of the antigenicity of this fragment with that of the homologous peptide oGH 86—123 (differing in only 4 positions),[164] lent support to the hypothesis that at least one antigenic determinant area in eGH is dependent on sequence 86—123. The

successful synthesis of the homologous peptide hGH 73-128 made possible a comparative immunological study[141] in an attempt to explain the cross-reactivity existing between the native e and hGHs.[140] Such cross-reactivity was partially explained by hemagglutination and complement fixation experiments using rabbit anti-hGH serum and both the eGH 73-123 and hGH 73-128 fragments. Antibodies raised in rabbits by both fragments were also tested.

Antiserum against eGH 73-123, although reacting strongly against the immunogen, were practically ineffective against the 2 native hormones and against hGH 73-128, whereas antisera against hGH 73-128 recognized a similar antigenic structure in the fragment eGH 73-123 and in the native equine and human hormones. A similar predictive approach was used recently by Sakata and Atassi[165] to map epitopes by homology in human and bovine serum albumins.

As mentioned before, the principal determinant in bGH was also located in the same region of the molecule as in e and hGHs, namely, in cyanogen bromide fragment 6-124.[142] By oxidation of this peptide with N-bromosuccinimide, the short peptide 87-124 could be obtained and it was possible to demonstrate that it contained most of the antigenicity found in the original fragment.

Based on all these experiments, we assumed that human, bovine, equine (and probably ovine) GHs share a common antigenic area which can be detected within the boundaries of amino acids 73 and 128 in their molecules.

A more detailed investigation of this area in hGH has been attempted using a series of synthetic overlapping peptides, whose antigenicity was measured by RIA.[166] A preliminary survey of a series of peptides, covering different lengths of hGH antigenic region 73-128, was made using a rabbit antiserum and ^{125}I-hGH as tracer. It was found that peptide 98-128 was responsible for the total antigenicity of hGH at least as detected in the RIA, strongly indicating that a relevant epitope(s) is located in this region of the molecule. All other tested peptides, except 121—128, had a low reactivity which was detectable when their concentration was 2 or more orders of magnitude higher than that of 98-128. The diminished affinity of 98-128 for the antibody as compared with hGH is the cause of the right shift (approximately 3 orders of magnitude) shown by the inhibition curve of the peptide in the RIA (Figure 3).

Antibodies to native hGH bind weakly to 98-128 because they are directed to native-like conformations which are present at extremely low concentration. Todd et al.[167] have pointed out that the binding constant for antibodies to such a peptide is very small, the normal binding constant being attenuated by a factor of 10^{-4} to 10^{-5}.

Since it was found that a high enough concentration of 98-128 completely displaces a tracer amount of ^{125}I-hGH from the complex (Figure 3), we assume that this is a case of true cross-reactivity, as described by Berzofsky and Schechter.[168]

The analysis of the parallelism of the binding curves is difficult to make and a decision on the value of the association constant for 98-128 is not warranted with present data. One can still say qualitatively that the average affinity of the peptide is approximately three orders of magnitude lower than that of hGH. To discard the possibility that the results described might be the exclusive property of a particular rabbit serum, other sera against hGH were also assayed. In fact, RIAs performed with antisera from two other rabbits, guinea pigs, and from two patients with isolated growth hormone deficiency, type A, submitted to replacement therapy, gave results consistent with those obtained with the first rabbit serum.[166] The patients provided a unique opportunity to assay human antibodies against hGH (see also Section VIII.E.).

F. Studies with Monoclonal Antibodies

All the efforts already described to localize antigenic determinants in the GH family of proteins have been made with polyclonal antisera. These antibodies were invaluable to set up quite sensitive immunoassays and obtain a wealth of information, but their use for the

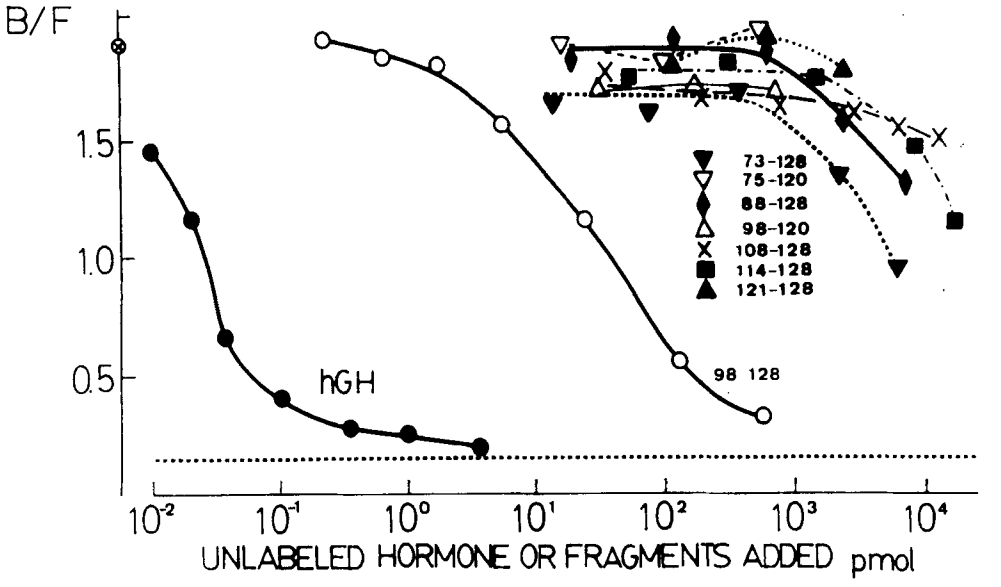

FIGURE 3. Radioimmunoassay for hGH and several synthetic peptides using a rabbit anti-hGH serum (1:500.000). Tracer: ^{125}I-hGH (0.4 ng/mℓ). ----, nonspecific binding. B/F: bound to free ratio. (Adapted from Reference 166.)

analysis of the antigenic structure of these hormones is limited by the heterogeneity of their specificities. Hence the subject is being re-explored using murine monoclonal antibodies (MAbs) secreted by hybridoma cell lines.

A series of monoclonal antibodies directed to hGH have been reported and partly characterized by three laboratories.[169-174] One of them [170-172] was also able to obtain four MAbs against hPL and one against hPRL. These antibodies showed a wide spectrum of specificities ranging from strict specific binding to hGH (QA68), hPL (EB4), or hPRL (QB1) to equal binding to hGH and hPL for NA39 (hGH immune spleen), and EB1, EB2, and EB3 (hPL immune spleen). In addition, 2 anti-hGH MAbs (NA71 and NA27) showed a weak cross-reactivity with hPL. None of the anti-hGH or anti-hPL antibodies were bound by hPRL. However, Retegui et al.[174] have found a significant cross-reaction with hPRL and some of their MAbs against hGH. The 10 anti-hGH MAbs obtained by Bundesen et al.[173] showed a cross-reactivity with hPL covering a 4000-fold range of specificity.

Distinct antigenic determinants were identified by Ivanyi[172] in hGH and hPL on the basis of competitive inhibition assays with the already-mentioned murine MAbs. Effective competition for antigen binding within a pair of antibodies was interpreted as overlapping combining site specificities, whereas a lack of competition suggested binding to sterically distinct structural moieties. The results obtained point to the existence of one specific determinant each in hGH and hPL, two mutually exclusive determinants in a region common to both hormones and another determinant in hGH, only weakly cross-reactive.

The immunoreactivity of some of the various modified forms of hGH known to occur in the pituitary gland and in plasma has also been explored using MAbs. Since the 20K variant of hGH (see Section III.) was only about 1/3 as active as a standard preparation of hGH in displacing ^{125}I-hGH from binding to rabbit antibodies,[53] Ivanyi[171] investigated whether the effect could be selective for any of the epitopes detected by his MAbs. The results of RIAs suggested that 20K displaced to a very similar extent the binding of all 4 tested antibodies to ^{125}I-hGH and that the antigenic activity of the 20K variant was not significantly different from the control preparation of hGH. Ivanyi concluded that quite probably neither of the

epitopes under study had been altered in their expression as a result of the structural deletion of amino acids 32-46 in 20K-hGH.

Chrambach et al.[175] were able to fractionate hGH by preparative electrophoresis. Fractions D and E were 2 chain forms of hGH, cleaved at residues 135-146 to 150 (quite similar to forms α_2 and α_3, isolated by Lewis et al.[53] from the pituitary gland), which show enhanced bioactivity. Bauman[176] proved that both forms give virtually identical displacement curves as native hGH in RIAs with the four anti-hGH MAbs of Ivanyi[171] As stated before for the 20K-hGH, this result shows that neither of the epitopes being detected by the MAbs was affected by the gap (amino acid 135 through 150) in these molecules.

Aggregated, trimeric, dimeric, and monomeric hGH were isolated from a clinical grade of hormone by Wallis et al.[177] Using a conventional rabbit antiserum for RIA, the monomeric and oligomeric fractions were equally active whereas the aggregated fraction had only about 10% of displacing potency. Three of the four MAbs of Ivanyi reacted in a similar fashion to the rabbit antibody but the fourth MAb was much less reactive with dimeric and trimeric hGH indicating that the site recognized by this antibody could have been masked selectively.

Some experiments have also been performed with synthetic fragments of hGH known to be immunologically active (see Section VIII.E.). Paladini et al.[178] tested by RIA peptide 44-128 and they found that it had a similar displacing activity with all 4 anti-hGH MAbs of Ivanyi.[171] A similar result was obtained by Retegui et al.[174] using a different set of anti-hGH MAbs, and 3 synthetic peptides extending from residues 19 to 128, 73 to 128, and 98 to 128 of hGH. In both groups of experiments, the inhibitory concentrations of the peptides were three to four orders of magnitude higher than those needed for native hGH (see Section VIII.E. for discussion of the possible reasons for the low affinity of the peptides).

These results suggest that the sequences corresponding to the synthetic peptides participate in the structure of a major antigenic site of which various portions are recognized by the monoclonal antibodies.

The effects of MAbs on hormone-receptor binding were also explored. Cadman et al.[179] investigated if Ivanyi's anti-hGH MAbs could interfere with the interaction of labeled hGH and receptors from rabbit liver or mammary gland cells. Their results showed that only two of the four MAbs tested did block almost completely binding in the two types of receptors. The authors concluded that either a unique site in hGH was responsible for the binding to both receptors or, if two sites were involved, they should be so close together that the antibodies affect them identically. It was also postulated that the hGH-binding site must be be larger than an antigen determinant since at least two MAbs of nonoverlapping specificity almost completely blocked receptor binding.

Retegui et al.,[180] using the smaller Fab fragments of five anti-hGH MAbs, found that three of them inhibited in similar fashion the binding of the hormone to the receptors in the human lymphoid cell line IM-9 and in the liver membranes from pregnant rabbits. Hence they suggest that the receptors in both preparations react with the same region of the hormone which is tentatively localized near peptide 98-128, a known[166] antigenic site of hGH, as already discussed.

The results obtained by Cadman et al.[179] and Retegui et al.[180] are basically in accord with each other and by making clear that different MAbs have different effects on receptor-binding interactions they strengthen the notion that MAbs may help to determine the detailed nature of such interactions.

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