Binding of $^{125}$I-Human Growth Hormone to Specific Receptors in Human Cultured Lymphocytes

CHARACTERIZATION OF THE INTERACTION AND A SENSITIVE RADIORECEPTOR ASSAY

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SUMMARY

The interaction of human growth hormone with human lymphocytes from an established culture (IM-9) was studied using $^{125}$I-human growth hormone. The binding of $^{125}$I-human growth hormone was rapid; with human growth hormone at 0.1 nm a steady state was observed in 90 min at 30$^\circ$. Bound labeled human growth hormone was dissociated rapidly by addition of excess unlabeled human growth hormone. Binding of $^{125}$I-human growth hormone to cultured lymphocytes was relatively insensitive to alterations in the pH and in the concentrations of Ca$^{2+}$, Mg$^{2+}$, or EDTA. At 30$^\circ$ there was very little degradation of labeled human growth hormone or of the specific receptor sites. Tryptic digestion destroyed the capacity of cells to bind human growth hormone. The IM-9 cells bound all human growth hormone preparations but not unrelated hormones or nonprimate growth hormones. The binding of $^{125}$I-human growth hormone was inhibited 10 to 14% with 1 to 2 ng per ml of unlabeled human growth hormone and 50% with 30 to 40 ng per ml, well within the range of hormone concentrations in vivo. Analysis of steady state data revealed a single order of binding sites with an affinity constant of 1.3 X 10$^5$ M$^{-1}$ and about 4000 binding sites per cell. Numerous human growth hormone preparations were assayed by use of this receptor system as well as by immunoassay and by bioassay in vivo. The potencies of the human growth hormone preparations determined by the radioimmunoassay correlated more closely with the bioassay than with the radioimmunoassay.

The first step in the action of a polypeptide hormone is binding to specific receptors on the target cell. The methods that were devised for the direct study of adrenocorticotropic binding to its specific receptors in the adrenal (1-5) and of angiotensin binding to its receptors in blood vessels and in other cells (6-8) have been applied widely by us and by others to the direct study of many polypeptide hormones in their interaction with their specific receptors (9).

We recently described (10-13) specific receptor sites for human growth hormone (hGH) in a line of cultured human lymphocytes (4265), which had been initiated in 1965 from peripheral blood cells of a patient with chronic myelogenous leukemia. The binding of $^{125}$I-hGH to these cells is rapid, saturable, and reversible (10, 11). Hormones that have growth promoting effects in man compete with $^{125}$I-hGH for binding (10); nonprimate growth hormones (which do not stimulate growth in man) and unrelated hormones (insulin, thyrotropin) have no effect on binding (10).

The capacity of human growth hormone preparations to compete for the binding of $^{125}$I-hGH to receptors correlates much better with their growth promoting potencies than with their immunological potencies. Human placental lactogen which has a weak growth stimulating effect in man also has a definite but weak affinity for the growth hormone receptor (10).

Recently we have found that another lymphocyte line (IM-9), initiated in 1967 from bone marrow cells of a patient with chronic myelogenous leukemia (14) demonstrated greater binding per cell and greater sensitivity to low physiological concentrations of hormone. In the present study, using the IM-9 cell line, we present detailed studies of the interaction of hGH with its receptors as well as an extensive comparison of the reactivity of many hGH preparations in the radioreceptor assay, the in vivo bioassay, and the radioimmunoassay.

MATERIALS AND METHODS

All human growth hormone preparations were generous gifts from Dr. A. E. Wilhelmi, Emory University. Bovine thyrotropin and bovine, porcine, and ovine growth hormones were generous gifts of Dr. R. W. Bates, National Institutes of Health and the National Pituitary Agency. Porcine insulin was a gift of Eli Lilly. Sodium $^{125}$I (carrier-free) was purchased from Union Carbide, tetrabutylammonium chloride from Gold Leaf Pharmacia, bovine serum albumin (BSA) (Fraction V), human serum albumin (HSA) (crystalline), and rabbit y globulin (Fraction II) from Pentex, and trypsin and soybean trypsin inhibitor from Worthington Biochemical Corporation. All other chemicals were of reagent grade.

The cultured human lymphocytes, IM-9, which were generously provided by Dr. D. N. Buell of the National Cancer Institute, were maintained at 37$^\circ$ in Eagle's minimum essential medium
supplemented with 10% fetal calf serum (Grand Island Biological Associates). The cells were used in the binding studies when they were in stationary phase of growth (14). Viability of the cells was determined by their capacity to exclude trypan blue dye (15). 

\[ \text{125I-human growth hormone was prepared at specific activities of 90 to 100 \mu Ci per \mu g (average of 90 to 1.0 atom of iodine per molecule) by special modifications (9, 10) of the chloramine-T method (16). Preparation hGH 1394, with an in vivo bioactivity of 2 i.u. per mg, was the labeled as well as the unlabeled hormone, except as noted otherwise.} \]

For the binding studies the cells and \text{125I-hGH} were incubated with or without unlabeled hormone preparations (10); to determine binding of \text{125I-hGH} to receptors, aliquots of the incubation mixture were transferred to chilled buffer in microfuge tubes and centrifuged for 1 min. The supernatants from tubes with \text{125I-hGH} without unlabeled hGH were saved to test integrity of the \text{125I-hGH} (see Fig. 4); the other supernatants were discarded. The pellet of cells at the tip of each tube was excised. The radioactivity bound to the cells, expressed as the percent of the total radioactivity in the system, is plotted as a function of the duration of the incubation. The radioactivity bound to the cells in the presence of \text{125I-hGH} alone is referred to as "total binding." Radioactivity bound to cells in the presence of \text{125I-hGH} plus an excess of unlabeled hGH (10 to 50 \mu g per ml) is referred to as "nonspecific binding." "Specific binding" is the difference between total binding and nonspecific binding (for example, see Fig. 5).

\[ \text{of tale were added to adsorb the \text{125I-hGH}.} \]

Generally, 90% of the labeled hormone was adsorbed.

\[ \text{RESULTS} \]

Effects of Duration of Incubation, Temperature, and Cell Concentration on Binding—Binding of labeled human growth hormone to cultured lymphocytes was time and temperature dependent. When cells were incubated with \text{125I-hGH} at 30° a steady state was approached by 90 min (Fig. 1). In the presence of a large excess (10 to 50 \mu g per ml) of unlabeled hGH only a small percentage of the \text{125I-hGH} was bound. This has been referred to as "nonspecific" binding. Notice that nonspecific binding increased continuously with time (Fig. 1). Cell viability (trypan blue exclusion), even after 240 min of incubation at 30°, was unaltered. At 15° binding of the labeled hormone was much slower and a steady state was not approached even after 6 hours of incubation, while at 37° specific binding of the labeled hGH was decreased, the cells were clumped and cell viability was markedly reduced. Therefore, subsequent studies of binding were done at 30° for 90 min.

At 30° the binding of \text{125I-hGH}, in the absence and presence of unlabeled hGH was directly proportional to the cell concentration over the range 1 to 40 \times 10^6 cells per ml (Fig. 2). For most experiments we used 10 to 30 \times 10^6 cells per ml.

We have previously reported that the addition of an excess of

\[ \text{2 P. Gorden, and C. M. Hendricks, personal communication.} \]
unlabeled hormone at 5 to 30 min after the addition of labeled hormone to cells is followed by prompt dissociation of a significant fraction of the bound radioactivity (11). In the present study after 90 min of incubation of labeled hormone with cells at 30\(^\circ\), the addition of unlabeled hGH was followed by a prompt dissociation of the bound labeled hormone (Fig. 3). By 240 min, 75% of the labeled hormone bound to specific receptor sites was dissociated (the nonspecific binding increased continuously with time) (Fig. 3). Thus, the interaction of hGH with its specific receptors, like the interaction of some other peptide hormones with their receptors, appeared to be a rapid reversible process (9).

Effects of pH, Ca\(^{2+}\), Mg\(^{2+}\), and EDTA—The binding of \(^{125}\)I-labeled hGH to the cultured lymphocytes occurred over a broad range of pH. Little difference in the binding was noted over a pH range of 6.4 to 8.8. All subsequent experiments were performed at pH 7.0, since at this pH the binding of \(^{125}\)I-labeled hGH and the competition of unlabeled hormone were highly reproducible, and cell viability remained unchanged during the incubation period.

Divalent cations and chelating agents have been shown to have important effects in some receptor hormone interactions (4, 7, 17, 19–24). Since the buffer that was used for these studies (which is the same as that used for studies of the insulin receptor in these cells) contained Ca\(^{2+}\), Mg\(^{2+}\), and EDTA, we studied the effects of these agents on the binding. Individually none of the three had any effect on \(^{125}\)I-labeled hGH binding to cultured lymphocytes over a 10-fold range (1.0 to 10 mM). Similarly, combinations of Ca\(^{2+}\) and Mg\(^{2+}\) up to 10 mM were without effect.

Degradation of Labeled hGH and of hGH Receptor—In general, biological preparations that contain specific binding sites for polypeptide hormones also contain systems for degradation of the hormone and for degradation of the receptor (9, 17, 25–29). All three systems appear to be functionally independent processes even though they may be located in the same subcellular structures, e.g. the plasma membrane. The degradation processes must be evaluated carefully before quantitative studies of binding to specific receptors can be interpreted properly (27). Using three methods for determining degradation of \(^{125}\)I-labeled hGH, we found trivial degradation of the labeled hormone in 90 min (Fig. 4). Even with the most sensitive method of detection (25), namely the capacity for rebinding of labeled hormone to receptors on fresh lymphocytes, only 10% of the \(^{125}\)I-labeled hGH was degraded in 4 hours of incubation at 30\(^\circ\).

To determine whether receptors were degraded under our assay conditions, cells were preincubated at 30\(^\circ\) for 0 to 180 min prior to the addition of the \(^{125}\)I-labeled hGH for the standard 90-min incubation. Regardless of the duration of the preincubation, binding of \(^{125}\)I-labeled hGH to the cells, in the absence and presence of unlabeled hGH, was unchanged, i.e. both specific and nonspecific binding were unaltered. The \(^{125}\)I-labeled hGH that remained in the supernatant after the 90 min incubation was indistinguishable from radioactive hGH that had never been exposed to cells in its reaction with trichloroacetic acid and with tacle and its binding to fresh cells (Fig. 5). This excluded the release of soluble receptors or of hormone-degrading enzymes by the cells during several hours of incubation and suggested that the slight degradation of labeled hormone observed in the previous experiment occurred on the cells and not in the medium.

Effect of Trypsin on Binding—Exposure of cells or cell membranes to trypsin has been reported as being able to destroy some hormone receptors without a decrease in cell viability (23, 26, 30–33). The insulin receptor on cultured lymphocytes (4265) was destroyed by trypsin (26). In the present study we found that the hGH receptors in these cells (IM-9) were more susceptible to inactivation than were the insulin receptors (Fig. 6). Differential sensitivity of receptors on the same cell to trypsin has been reported (30).

Specificity and Sensitivity of Binding—As with cell line 4265 (10), the IM-9 cells bound human growth hormone preparations but not unrelated hormones (insulin and thyrotropin) or nonprimate growth hormones. Porcine, bovine, and ovine growth hormone (1 \(\mu\)g per ml) did not displace labeled human growth hormone. We have previously indicated that with cell line 4265 a minimum of 5 to 10 ng per ml of unlabeled hGH was necessary to observe inhibition of labeled hGH binding to the cultured lymphocytes (10). With cell line IM-9 greater sensitivity was achieved; 1 to 2 ng per ml of unlabeled hGH inhibited 10 to 14% of the binding of labeled hormone, 30 to 40 ng per ml inhibited 50% and 10 \(\mu\)g per ml inhibited 90% of the binding of labeled hormone (Fig. 7).

The data in Fig. 7, when replotted by the method of Scatchard (34) and subjected to linear regression analysis showed 4000 binding sites per cell of a single order with an affinity constant of 1.3 \(\times\) 10\(^5\) M\(^{-1}\) (Fig. 8). There is some suggestion that a second site of higher order may be present (see Fig. 8) as has been reported for some other peptide hormone interactions with their receptors (3, 5, 35–40).

In our previous study with cell line 4265 and four hGH preparations that varied 100-fold in biological potency we found an excellent correlation between growth promoting activity in vivo (41) (bioassay potency) and capacity to inhibit binding of \(^{125}\)I-labeled hGH to receptors on lymphocytes (radioreceptor potency); however, with the two preparations of intermediate potencies, the relative immunological potency clearly exceeded the biological and radioreceptor potencies (10). In the present study, using IM-9 cells, we have extended these observations.

Twelve hGH preparations were compared with hGH 1394 in our radioreceptor assay and with estimates (see legend to Table I) of biological activity in vivo (41). For 11 of the 12 preparations, the bioassay/radioreceptor potencies were in the range of 0.2 to 2.0 (Fig. 9 and Table I). In general it appeared that with purification of the hormone the radioreceptor potency increased relatively more than did the bioassay potency. In other words, with
The binding of \[^{125}\text{I}}\text{-hGH}\) to both lines of cultured lymphocytes appears to be representative of growth hormone binding to its biologically important receptors in man; presumably binding of hGH to such structures in target cells is the first step in the biological action of hGH. The specificity of the binding is exactly what one would predict from the enormous body of data on the biological specificity of growth hormone in man; human growth hormones are active while unrelated hormones and nonprimate growth hormones are not \(^{42}\). Further, for each human growth hormone preparation, the potency in binding to the receptor parallels remarkably its biological potency in vivo. Even human placental lactogen, or somatomammotropin, a hormone with definite but weak growth stimulating effect in man \(^{43}\), has reactivity in the binding assay that parallels its growth promoting efficacy \(^{10}\). Finally, the range of hGH concentrations over which most of the receptor binding occurs in vivo coincides exactly with the full physiological range of hGH concentrations in vivo \(^{44, 45}\).

The correlation between the radioreceptor assay and the radioimmunoassay is not as close as the correlation between the radioreceptor assay and in vivo bioassays. During early stages of purification, the immunological potency of the hGH preparations increases more rapidly than the receptor potency, whereas at further stages of purification the radioreceptor potency increases more rapidly. These differences may well be explained by differences in what these assays measure and by the presence of multiple components in hGH. All pituitary hGH preparations including hGH 1394, as well as circulating hGH from all patients, are composed of at least two components which differ in molecular weight by about 2-fold \(^{46-49}\). The higher molecular weight component is always less abundant, and its relative proportion varies severalfold from preparation to preparation \(^{50-52}\); it has radioreceptor potency/immunological potency that is roughly one-tenth that of the lower molecular weight species \(^{50-52}\).

In a bGH preparation at an early stage of purification the higher molecular weight component is in greater relative abundance than in the more highly purified preparations.\(^{2}\) During later stages of purification the immunologically-rich, receptor-poor component(s) are removed. These findings appear to account for the immunoassay/radioreceptor assay data; a similar process may account for the bioassay/radioreceptor assay data. With increasing purification of the hormone, the bioassay potency appears to increase a little less rapidly than radioreceptor potency (Fig. 9). Biological potency in vivo is a composite result of many factors including binding affinity, intrinsic activity, and degradation rate, whereas the radioreceptor assay as applied to hGH is almost entirely a measure of binding affinity. Even if we assume that the two forms of hGH do not differ in binding affinity or in intrinsic activity, it does seem most reasonable to assume that the high molecular weight component has a longer lifetime in the circulation, and therefore that the ratio of in vivo bioassay potency to radioreceptor potency for the higher molecular weight component exceeds that of the lower weight component. With impure hGH preparations, which are relatively rich in the higher molecular weight component, bioassay/radioreceptor potencies are higher than with purer hGH preparations, which are relatively enriched in lower molecule weight component.

An analogous situation exists with insulin and proinsulin. The higher molecular weight precursor of insulin has an affinity for binding to the insulin receptor that is about 5% that of in-
insulin, its intrinsic activity is equal to that of insulin, and its lifetime in the circulation is several times greater than that of insulin (53–55). With both the in vitro bioassay (glucose oxidation in isolated fat cells) and the radioreceptor assay, proinsulin is 50% as active as insulin. With the in vitro bioassay, proinsulin is about 20% as active as insulin due largely to the prolonged lifetime of proinsulin in vivo (50). For mixtures of the two components the in vitro bioassay/radioreceptor assay will fall as the proportion of proinsulin to insulin is reduced. A similar situation also exists with the immunoassay/receptor assay. Proinsulin is about half as potent as insulin in the insulin immunoassay (56, 57). For a mixture, immunoassay potency/receptor assay potency will depend on the relative proportions of the two components. The existence of multiple molecular forms intermediate between proinsulin and insulin (56, 57) makes the actual situation even more complex; a similar complexity for hGH components seems likely (58).

The interaction of hGH with its receptors is similar in most respects to the interaction of ACTH, angiotensin, and other peptide hormones with their receptors (1–9). At 30°C binding is rapid and reaches a steady state in 2 hours or less. The addition of unlabeled hormone produces prompt dissociation, and most of the bound labeled hormone is disassociated in a few hours. The equilibrium constant, which is equal to what one would have predicted from the known in vitro concentrations of the hormone (44), is in the range reported for other systems as are the number of sites per cell (9). Somewhat remarkable are the lack of sensitivity of the binding to changes in pH, the narrow range of useful temperatures for the assay, extreme sensitivity of the binding to changes in pH, the narrow range of equilibrium constant, which is equal to what one would have predicted from the slope of the line with the abscissa. The association constant, derived from the slope of the line, is 1.3 X 10^5 M^-1. There are 4000 binding sites per cell, derived from the intercept of the line with the abscissa.

Table I

Comparison of assays of human growth hormone preparations

| Preparation | Bioactivity estimated | Radioreceptor activity | Radiimmunoactivity |
|-------------|----------------------|------------------------|--------------------|
| 1394        | 100                  | 100                    | 100                |
| 1395        | 100                  | 50                     | 80                 |
| 1563C       | 90                   | 230                    | 165                |
| 1544C       | 85                   | 230                    | 165                |
| 1490B       | 50                   | 170                    | 145                |
| 1208        | 50                   | 100                    | 100                |
| 1519B       | 25                   | 30                     | 100                |
| 1563A       | 20                   | 25                     | 20                 |
| 1563D       | 20                   | 40                     | 100                |
| 1018B       | 12.5                 | 12                     | 40                 |
| Human Ca    | 10                   | 20                     | 100                |
| Human Ba    | 10                   | 15                     | 45                 |
| 1519A       | 2                    | 0.6                    | 0.7                |
| 1518A<sup>a</sup> | <1.5                 | 0.12                   | <1                 |
| 1679A<sup>b</sup> | 3                    | 31                     |                    |
| B           | 11                   | 75                     |                    |
| C           | 27                   | 85                     |                    |
| D           | 50                   | 85                     |                    |
| 1679A<sup>b</sup> | 2                    | 16                     |                    |
| B           | 10                   | 75                     |                    |
| C           | 21                   | 150                    |                    |
| D           | 90                   | 150                    |                    |

<sup>a</sup> This preparation is not included in Fig. 9 or Fig. 10.
<sup>b</sup> Included in Fig. 10 only.

Lack of degradation of receptor or of hormone even after many hours at 30°C.

This very low level of degradative activity is but one of the characteristics of the cultured lymphocytes that makes them almost ideal reagents for receptor studies and assays (26). The specific hormone binding by the cells is exceedingly reproducible
Fig. 9. Comparison of in vivo bioassay with radioreceptor assay. Twelve hGH preparations were compared with hGH 1394 for their ability to inhibit the binding of $^{125}$I-hGH to lymphocytes. Since the displacement curves were all parallel, we expressed the relative receptor potency as the concentration of hGH 1394 that inhibited 50% of the labeled hormone binding divided by the concentration of the test hGH that inhibited 50% of the binding. The bioassay potency, supplied by Dr. A. E. Wilhelmi, is the potency of the test hGH (i.u. per mg) divided by the potency of hGH 1394 (2 i.u. per mg). Lines have been drawn where the ratios of bioassay potency to radioreceptor assay potency are 2, 1, 0.5, and 0.2.

Fig. 10. Comparison of radioreceptor assay with radioimmunoassay. Twenty-one hGH preparations were assayed at multiple concentrations in the radioreceptor assay and in a standard hGH radioimmunoassay and compared in both assays to hGH 1394. The potency in each assay is the concentration of hGH 1394 that inhibited 50% of the $^{125}$I-hGH binding divided by the concentration of test hGH that inhibited 50% of the binding.

from batch to batch, and, in this form, bound and free labeled hormone are separated rapidly by simple centrifugation. The sensitivity and specificity of the radioreceptor assay make it ideal as a supplement or replacement for the in vitro bioassays for hGH which are insensitive and imprecise and therefore very costly in material. The multiple immunologically reactive hGH components in plasma, which cannot possibly be characterized by conventional bioassays, have been studied in normal adults and in acromegalics by use of the hGH radioreceptor assay (50, 52). One disadvantage of receptors on live cells, that the range of experimental conditions is narrow, has been overcome by solubilization of the hGH receptor in aqueous media without detergents, analogous to the solubilization of the insulin receptors of these cells (59).

In addition to 4265 and IM-9 we have detected hGH receptors in lymphocyte lines derived from patients with mononucleosis but not in cells derived from patients with Burkitt's lymphoma (60). In all cell lines in which we detected hGH receptors we also found insulin receptors* but not vice versa; when both are present, the insulin receptors are more numerous and often are of a higher affinity, making their detection easier. We (61) and others (62) have also detected hGH receptors in liver.

Contrary to earlier expectations, recent studies with insulin show that receptor concentrations per cell are not fixed but in fact, fluctuate widely, both in man and in experimental animals (23, 61, 63-66). In several insulin-resistant states (e.g. obesity or glucocorticoid excess) hormone binding to receptors is decreased and in some insulin-sensitive states (e.g. with fasting or hypophysectomy) hormone binding is elevated (63-66). The altered binding accounts in large part for the altered sensitivity, and cells with altered insulin binding have been shown to have a concomitant alteration in response to insulin in vitro. We expect that similar approaches will be extended to GH and permit direct studies, in man and experimental animals, of disorders of skeletal growth such as growth-hormone resistant (Laron) dwarfism, primordial dwarfism, and growth failure associated with glucocorticoid administration.

Note Added in Proof—The following new publications also contain pertinent data on hGH interactions with its receptors: "Radioreceptor Assay for Growth Hormone" (67), "Induction of a Polypeptide Hormone Receptor in Rat Liver" (68), and "Insulin Interactions with its Receptors: Experimental Evidence for Negative Cooperativity" (69).

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