Development of a Bioassay System for Human Growth Hormone Determination with Close Correlation to Immunoassay

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Serum growth hormone (GH) level is measured largely through immunoassays in clinical practice. However, a few cases with bioinactive and immunoreactive GH have also been reported. We describe here a new bioassay system for GH determination using the BaF/GM cell line, which proliferates in a dose-dependent manner on hGH addition; cell proliferation was blocked by anti-hGH antibody. This bioassay had the lowest detection limit (~ 0.02 ng/ml) reported thus far and the highest specificity for GH. The bioassay

results were compared with those of an immunoradiometric assay across 163 patient samples in various endocrine states. A close correlation (the ratio of bioactivity/immunoreactivity was 1.04 ± 0.33 , mean $\pm$ SD) was observed between bioactivity and immunoreactivity in these samples. The newly developed system is a specific, sensitive, easy, and fast bioassay system for GH determination; we consider it useful for evaluating GH bioactivity in various endocrine states. *J. Clin. Lab. Anal.* 26:328–335, 2012. © 2012 Wiley Periodicals, Inc.

Key words: growth hormone; bioassay; BaF/GM cell line; high sensitivity; IRMA

INTRODUCTION

Growth hormone (GH) plays an important physiological role in a variety of clinical settings by exerting either endocrine or metabolic effects. Currently, immunoassay methods based on an antibody's ability to recognize specific isoforms of GH are exclusively used to determine serum human GH (hGH) levels in both physiological and pathological states (1, 2). Because human GH consists of more than 1 isoform (the major 22-kDa form along with 20-kDa and 17-kDa isoforms), measured hGH concentrations can differ between hGH assay kits (3, 4). Furthermore, it is clinically useful to measure the bioactivity of serum GH to determine whether the GH is biologically active or inactive, since Kowarski and Schneider suggested a discrepancy between immunoreactivity and bioactivity for serum GH (5). Insensitivity to intrinsic GH despite the presence of normal GH receptor could be caused by bioinactive GH, although the incidence of short stature caused by bioinactive GH is very low. Hence, it is desirable to develop and use serum GH assay by both bioactivity and immunoreactivity to diagnose abnormality in GH secretion. Previous studies have reported the diagnosis and

treatment of various pediatric endocrine diseases such as precocious puberty, small for gestation age (SGA), obese children, and tall stature children using methods that test the immunoreactivity of GH (6–10). However, only a few studies using bioactivity-based methods have been reported (9, 11).

In this paper, we describe a new bioassay method for assessing GH bioactivity. We evaluated the suitability of this method through an intensive GH bioactivity assay of 163 serum samples of patients in various endocrine states, including patients with idiopathic short stature (ISS), SGA, precocious puberty, obesity, and tall stature, and compared their values against those of normal stature

[†]This paper is dedicated to the memory of Professor Kenji Fujieda, who passed away on March 19, 2010.

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controls along with immunoradiometric assay (IRMA) values.

MATERIALS AND METHODS

Subjects

Serum samples were collected from 23 control children of normal stature (aged 8 months–17 years), 32 patients with Japanese ISS (aged 3 years–14 years 6 months), 36 patients with Xin Jiang ISS (aged 2 years 6 months–14 years 5 months), 9 children with tall stature (aged 5 years 2 months–10 years 8 months), 10 patients with precocious puberty (aged 1 years 2 months–6 years), 11 patients with SGA (aged 3–7 years), and 10 children with obesity (aged 7 years 4 months–9 years 10 months). All samples were collected after obtaining informed consent from the parents or guardians. This study was approved by the Ethical Committee of Asahikawa Medical University, Japan.

The clinical characteristics of the subjects are summarized in Table 1. The standard deviation (SD) of height and weight for Japanese children was calculated from the mean height and weight for that age referred from the national statistics for Japanese children aged 0–15 years in 2000, while SD for Xin Jiang children was calculated from the mean height and weight for that age referred from the national statistics for Chinese children aged 0–15 years in 1995. All the 108 patients chosen for this study were determined to have no chromosomal aberrations or complications that might affect natural growth (e.g., congenital heart defects, gastrointestinal malformations, thyroid dysfunction, leukemia, or epilepsy).

Normal control samples in our study were selected at random from the population of children with normal stature among outpatients' serum samples. Chromoso-

mal abnormalities, dimorphic syndrome, endocrine diseases, and metabolic diseases were also ruled out in these controls.

Basal and peak samples were obtained from GH stimulation tests using arginine and insulin stimulation tests in 32 Japanese ISS patients.

Basal blood samples were obtained after overnight fasting before any physical activity. Further samples were collected every 15 min until 3 h, and the GH concentration was then determined. The samples containing the highest GH concentrations were chosen as the "peak samples." Although individual variability was noted, the majority of such peak samples were collected at 30–60 min, except some peak samples obtained at 2 h.

Reagents and Antibodies

Standard hGH was obtained from the JCR Pharmaceuticals Co., Ltd. (Kobe, Japan). Reagents and hormones were purchased as follows: human thyroid stimulating hormone (hTSH), human luteinizing hormone (hLH), and human follicle stimulating hormone (hFSH) from Aspen Bio Pharma, Inc. (Colorado, USA); prolactin (PRL) and fibroblast growth factors from Acris Antibodies GmbH (Herford, Germany); human epidermal growth factor (EGF) and human insulin-like growth factor-1 (IGF-1) from Assay Designs/Stressgen (NY, USA); hydrocortisone from LKT Laboratories, Inc. (Minnesota, USA); insulin (Novorin R 40 IU) from Novo Nordisk (Japan); and L-thyroxine sodium from Toyobo Co., Ltd. (Osaka, Japan). Monoclonal anti-human growth hormones antibody was purchased from Oy Medix Biochemica Ab (Kaunianinen, Finland). The growth hormone receptor (GHR) antagonist pegvisomant (SOMAVERT) was obtained from Pfizer Pharmaceuticals Co., Ltd. (Tokyo, Japan).

TABLE 1. Clinical Characteristics of the Study Subjects

	Clinical criteria	No (M:F)	Age (range in years)
Control	Height within $\pm 2SD$	23 (13:10)	0.6–17
Japanese idiopathic short stature (ISS)	Height below $-2SD$	32 (18:14)	3–14.5
Xin Jiang idiopathic short stature (ISS)	Height below $-2SD$	36 (22:14)	2.5–14.4
Tall stature	Height over $+2SD$	9 (7:2)	5.1–10.6
Precocious puberty	Secondary sexual characteristics developed before 7 years and 6 months	10 (0:10)	1.1–6
Small for gestation age (SGA)	Height and weight below $2SD$ at birth	11 (7:4)	3–7
Obese	Body weight exceeds 120% of the standard	10 (8:2)	7.3–9.7

In Vitro Bioassay with BaF/GM Cell Line

To improve upon the disadvantages and limitations of the previous bioassays, we used the BaF/GM cell line (1). To establish a GH-sensitive cell line, the cDNA of the chimera of the extracellular domain of hGHR (cDNA 44–829, *HindIII-MluI* site) and of the transmembrane and cytoplasmic domain of human thrombopoietin receptor (cDNA 1474–1908, *MluI-XhoI* site) was constructed in pCR3-Uni (Invitrogen Corp., NV Leek, the Netherlands) and transfected in Ba/F3 cells. Stable transfectants (BaF/GM cells) were selected using G418. Ba/F3-GM cells proliferated according to the concentration of hGH (1). We developed a newly modified method using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] as the endpoint, which was easier, more convenient, faster, and had greater

safety advantages than the previous one that used MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium].

In accordance with routine practice, 16 h before the start of the bioassays, the cells were washed three times by centrifugation (5 min at 1,000 rpm) with the assay medium (RPMI 1640), transferred to the assay medium supplemented with 10% horse serum (GIBCO Japan, Tokyo, Japan) without hGH, and incubated for 16 h to decelerate the rate of cell replication. After incubation, the cells were collected by centrifugation (5 min at 1,000 rpm) and resuspended in the assay medium with 10% horse serum at a concentration of 3×10^5 cells/ml; then, 100- μ l aliquots were distributed in each well of a 96-well microplate (CELLSTAR, Greiner Bio-One, Tokyo, Japan). Standard hGH was diluted with assay medium supplemented with 10% horse serum at a concentration of 0.015–240 ng/ml.

Samples were incubated at 56°C for 30 min to inactivate the serum, and each sample was diluted to give three different concentrations. Then, 10 μ l of diluted standards or serum samples were pipetted in triplicate into the 96-well microplate. For all dilutions, we used sealed test tubes (Nalge Nuck Int., Yokohama, Japan) to prevent protein adhesion to the walls. The cultures were incubated in 5% CO₂ at 37°C for 22 h. At the end of the incubation, the colorimetric endpoint was determined using Cell Titer 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Wisconsin, USA) according to the manufacturer's protocol with a slight modification. Here, 22 μ l of One Solution Reagent containing the tetrazolium compound MTS and an electron-coupling reagent (phenazine ethosulfate; PES) was added to each well and incubated at 37°C for 3 h under the same conditions. During this time, viable cells changed the yellow MTS salt to purple formazan. Bioactive responses were determined with a THERMO max microplate reader (Molecular Devices, California, USA), which assessed optical densities at the test wavelength of 490 nm and a reference wavelength of 650 nm to correct for differential scattering. A range of hGH standards were run on each plate, and all hGH concentrations were expressed as the final concentration obtained in the assay wells. The same serum ("control serum") with a known hGH concentration (as determined by IRMA and by our bioassay with good correlation) was used at every assay for quality control.

Several hormones and growth factors at different concentrations, such as PRL (0.1–1,000 μ g/l), human IGF-1 (0.1–1,000 ng/ml), human insulin (0.1–1.0 μ IU/ml), hydrocortisone (10–1,000 ng/ml), (25–1,000 IU/l), FSH (100–100,000 IU/l), TSH (1–100 mU/l), L-thyroxin Na (80.4–643.5 nmol/l), EGF (10–1,000 ng/ml), fibroblast growth factor (10–1,000 ng/ml), and growth hormone releasing factor (GRF; 0.1–1,000 ng/ml), were assayed by

adding 10 μ l of each to a well to estimate the influence on the BaF/GM cell line.

In the blocking study, 10 μ l of hGH antibody (diluted 350 or 3,100 times with 0.01 M PBS containing 0.1% BSA) were added to the wells of the standard or sample.

Finally, 10 μ l of the GHR antagonist SOMAVERT (diluted 350 times) was pipetted into wells instead of the hGH antibody together with the standard or sample.

Intra-assay variation was estimated by assaying the same serum samples (three different concentrations within a readable range on the standard curve) ten times. Inter-assay variation was determined from ten consecutive assays of three serum samples within the readable range on a standard curve; three samples were assayed on each of 10 days.

Immunoassay

The immunoreactivity of serum hGH was determined using IRMA (Daiichi Radioisotope Laboratories, Tokyo, Japan).

Statistical Analysis

Student's paired *t*-test was used to compare basal and peak values in the GH stimulation test. Mann–Whitney *U* test (nonparametric) was used to compare between two groups in the bioassay/IRMA ratio (B/I ratio) distribution. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Specificity and Accuracy for GH Bioassay in the BaF/GM Cell Line

Purified hGH (after appropriate dilution) stimulated BaF/GM cell proliferation in a dose-dependent manner (Fig. 1A), and was used to obtain a standard curve for the subsequent GH bioassay. The quantitation limit was set at approximately 0.020 ng/ml.

Human serum also stimulated cell growth in a dose-dependent manner, giving a curve similar to that produced by the standard purified hGH (Fig. 1B). The dilution range of human serum was 0.69–11% at the final concentration. Cell proliferation by human serum was inhibited by adding anti-hGH antibody to the serum.

To examine the specificity of hGH for the BaF/GM cell line, other growth-related hormones and growth factors were applied to the cell line. None of these, except IGF-1, influenced cell growth proliferation at the following levels: PRL 1,000 μ g/l, human insulin 1.0 μ IU/ml, hydrocortisone 1,000 ng/ml, FSH 100,000 IU/l, TSH

Fig. 1. (A) Stimulation of the proliferation of BaF/GM cells by hGH and IGF-1. Points represent the mean values of triplicate wells. GH standard (○), IGF-1 (●). (B) Effect of human serum on the proliferation of BaF/GM cell cultures after 22-h incubation without (○) and with (▲) hGH antibody. The hGH standard curve is shown by (●).

100 mU/l, L-thyroxin Na 643.5 nmol/l, EGF 1,000 ng/ml, fibroblast growth factor 1,000 ng/ml, and GRF 1,000 ng/ml. IGF-1 increased cell proliferation at concentrations higher than 30 ng/ml and stimulated cell proliferation in a dose-dependent manner (Fig. 1A).

Cell proliferation induced by hGH was completely blocked by the anti-hGH antibody (Fig. 2). The GHR antagonist SOMAVERT also completely inhibited cell proliferation.

The intra-assay coefficients of variation (CV) were lower than 10% at three different concentrations with values of 3.4, 5.8, and 9.8% (at 20, ten, and five times dilutions of standard sample, respectively). The inter-assay coefficients of variation were 5.6, 7.9, and 9.3%, respectively.

Fig. 2. Effect of GH antibody on cell proliferation induced by hGH. BaF/GM cell cultures were incubated for 22 h with hGH in the presence or absence of anti-GH antibody (monoclonal) or GHR antagonist. The dilution of the antibody was $\times 100$. GH standard (○), GH standard + anti-GH antibody (monoclonal) $\times 100$ (●), and GH standard + GHR antagonist (SOMAVERT) (▲)

Bioactivity of hGH in Human Serum

We assayed hGH levels in serum samples using the GH stimulation test in Japanese ISS patients. The mean values of bioactivity and immunoreactivity were 1.26 ± 0.19 and 1.21 ± 0.20 for basal levels, and 8.33 ± 1.04 and 8.31 ± 0.87 for peak levels (mean $\pm$ SE) ($n = 64$), respectively; no significant differences were noted (Fig. 3A). The mean ratios of bioactivity/IRMA (B/I ratio) at basal and peak values in the GH stimulation tests were 1.09 ± 0.36 and 0.98 ± 0.27 (mean $\pm$ SD) ($n = 64$), respectively (Fig. 3B), showing no significant difference.

We assayed hGH levels in serum samples from patients with various growth-related issues using both bioassay and immunoassay (Fig. 4). The mean values for bioactivity and immunoreactivity in serum samples from tall stature patients were 2.41 ± 0.60 and 1.92 ± 0.97 (mean $\pm$ SE) ($n = 9$); precocious puberty patients, 2.19 ± 0.57 and 2.41 ± 0.78 ($n = 10$); SGA patients, 0.97 ± 0.24 and 0.86 ± 0.24 ($n = 11$); and obese children, 0.56 ± 0.20 and 0.74 ± 0.26 ($n = 10$), respectively. The mean values for bioactivity and immunoreactivity ($n = 23$) for the normal control children were 2.18 ± 0.77 and 2.07 ± 0.66 , respectively. Compared to the control group, no significant differences were noted for either the bioactivity or immunoreactivity values in the pathological states. There were also no statistically significant differences between the bioactivity and immunoreactivity values for each of the disease groups or between the control group and disease groups.

We also evaluated the B/I ratio in a variety of clinical states (Fig. 5). The mean B/I ratio in the serum samples from the normal controls was 1.02 ± 0.21 (mean $\pm$ SD) ($n = 23$); the B/I ratio for overall ISS (basal value for

Fig. 3. (A) Bioactivity and immunoreactivity of GH in Japanese ISS children showing basal and peak values. GH peak values (○) with GH stimulation test, GH basal values (●) (mean $\pm$ SE) ($n = 64$). (B) Correlation ratio of B/I at basal and peak values, with B/I ratio representing the bioactivity/immunoreactivity ratio. Basal (●) and peak (○) were plotted in the graph ($n = 32$).

Japanese ISS patients and for Xin Jiang ISS patients) was 1.02 ± 0.29 ($n = 68$), for tall stature patients was 1.45 ± 0.44 ($n = 9$), for precocious puberty patients was 0.91 ± 0.61 ($n = 10$), for SGA patients was 1.22 ± 0.27 ($n = 11$), and for obese patients was 0.82 ± 0.16 ($n = 10$). Significant differences ($P < 0.05$) were noted in the B/I ratios for tall stature and obese patients compared to the normal controls. However, no differences were noted for ISS, precocious puberty, and SGA patients compared to the controls.

The correlation between the bioactivity and immunoreactivity in the same sample was determined by comparing the results of the BaF/GM bioassay and immunoassay (IRMA) in various serum samples ($n = 163$), including

Fig. 4. Bioactivity and immunoreactivity of hGH in normal controls (mean $\pm$ SE) ($n = 23$), ISS (GH basal values, $n = 68$), tall stature ($n = 9$), precocious puberty ($n = 10$), SGA ($n = 11$), and obese ($n = 10$) children. GH bioactivity values (●) and GH immunoactivity values (○).

Fig. 5. The bioassay/IRMA ratio (B/I ratio) distribution in a variety of clinical states. Normal control ($n = 23$) shown by open circles; ISS ($n = 68$), tall stature ($n = 9$), precocious puberty ($n = 10$), SGA ($n = 11$), and obese ($n = 10$) children are plotted as closed circles in the graph. *represents significant difference vs. control ($P < 0.05$).

ISS ($n = 67$), precocious puberty ($n = 10$), obesity ($n = 10$), tall stature ($n = 10$), SGA ($n = 11$), and normal controls ($n = 23$) (Fig. 6). There was a close positive correlation between bioactivity and immunoreactivity, and the mean overall B/I ratio was 1.04 ± 0.33 (mean $\pm$ SD) ($n = 163$).

DISCUSSION

The use of GH assays for diagnosing GH-related diseases has grown tremendously, and clinical laboratories now handle an increasing number of requests for GH determination. The presently available GH assays are based on various immunological detection methods. Although these immunoassays are widely used in routine clinical diagnosis and research, their major limitation is that they may not reflect the level of hormonal bioactivity in the

Fig. 6. Correlation between hGH bioactivity and immunoreactivity in 163 samples related to idiopathic short stature (Japanese ISS basal and peak values, Xin Jiang basal values), precocious puberty, obese, tall stature, small for gestation age (SGA), and normal control. The bold diagonal line in the graph represents points where the ratio of the bioassay value to the IRMA value equaled to 1.

sample, but rather reflect the combined immunoreactivity of the compounds structurally related to the immunogen (12). To overcome the shortcomings of immunoassays, bioassays have been developed, based on the binding of GH to GHRs, with GHR preparations being in the form of plasma membrane preparations (13–17) or as cell lines expressing GHR, such as the human lymphoblastoid line IM-9 (18). However, these methods require tissue culture facilities or preparation of plasma membrane fractions; moreover, they were relatively not sensitive. Therefore, after the Nb2 bioassay (19) modification, research efforts have been primarily directed toward the development of more convenient cell proliferation ESTA bioassay systems (20). This assay in the optimized protocol includes an anti-prolactin antibody to eliminate interference by prolactin, and it is very sensitive to standard hGH (~ 0.02 ng/ml). However, when applied to serum samples, its sensitivity is limited to approximately 2 ng/ml (21). Recently, more sensitive detection tools—such as the Ba/F3 cell proliferation bioassay (22, 23)—have been reported, with more than twofold sensitivity, i.e., 1 ng/ml for human serum samples. However, for detection limits lower than 1 ng/ml, they have poor sensitivity, leading to concerns regarding the quantification of the actual somatogenic activity of human serum samples (12). Moreover, a recent paper suggested that the Ba/F3 bioassay would not be a useful tool for identifying patients with altered forms of GH (24). The assay described here using the BaF-GM

cell line was demonstrated to have the lowest detection limit thus far, with a detection limit of approximately 0.02 ng/ml for human serum samples, i.e., over 50 to 100-fold more sensitive than the abovementioned recent cell proliferation bioassay. We consider that this greater sensitivity was because of the inherently greater sensitivity of the cell line.

The present assay needs 22 h of cell culture for hGH measurement; thus, it is less time consuming as compared to the Ba/F3-hGHR assay that requires 48 h. An additional advantage is that the bioassay could be adapted for the MTS endpoint detection system (25), which has much higher sensitivity than the MTT system used in previous assays. The MTS assay is a single step, single solution assay, and requires no washing or cell harvesting, thus being less labor- and time-intensive; further, it eliminates the solubilization steps normally required for MTT assays, which is less sensitive and has a lower color response range (25).

The BaF/GM cell line proliferated in a dose-dependent manner when hGH was added to the cell culture, while cell proliferation was completely blocked by the hGH-antibody (Fig. 2). The GHR antagonist inhibited all bioactivity of GH by binding to GHR and preventing GH activation of the receptor. These results suggest that this assay for GH bioactivity depends on GH-GHR signal transduction and intracellular signaling. The GH specificity of the cell line is supported by the result that the bioactivities of the other hormones and growth factors tested herein, except free IGF-1 (26), were almost absent, which correlated well with a recent report (23). Free IGF-1 concentrations over 30 ng/ml induced cell proliferation (Fig. 1; however, this concentration would be beyond the physiological human state because most circulating IGF-1 (97–99%) is bound to IGF-binding proteins (IGFBPs) with only 2–3% IGF-1 being in the free form; moreover, the levels of free IGF-1 even in the tallest children are under 25.28 ng/ml (23, 27, 28). The observed cell proliferation by high concentration of free IGF-1 cannot be achieved when human serum sample is applied in physiological state.

Since very few reports describe the bioactivity of hGH in clinical states related to SGA, precocious puberty, obesity, or tall stature, we evaluated the bioactivity and immunoreactivity of hGH and calculated the B/I ratio in these pathological states. Interestingly, we found that the B/I ratio was significantly higher in tall stature patients and significantly lower in obese children than in the controls ($P < 0.05$) (Fig. 5). Serum samples in tall stature children thus had greater amounts of bioactive GH; we speculate the following reasons for the same: tall children might have GH isoforms with a biological profile that cannot be identified by immunoassay methods [2]; alternatively, certain unknown growth factors in the serum

might influence bioactivity but not immunoreactivity. In contrast, in obese children, the B/I ratio was decreased. In a previous report (11), not only immunoreactive but also bioactive GH concentrations were low, but the B/I ratio was not estimated. In the present study, the decrease in the B/I ratio allows us to speculate that an unknown factor in serum (such as adipocytokine) might inhibit GH-GHR receptor binding and/or signal transduction for cell proliferation in addition to decrease in immunoreactive GH. These findings suggest the importance of evaluating bioactive GH levels in different endocrine states, although more patient samples need to be examined for confirming those hypotheses.

We demonstrated that serum hGH bioactivity and immunoreactivity were correlated well (Fig. 6), even at the secretory peaks of GH stimulation tests (Fig. 4). This close correlation has also been reported for the Ba/F3/GHR cell proliferation assay (23) although a recent study (24) reported that the correlation was not so good for the same assay; however, several reports concerning the ESTA bioassay showed that the bioactivity of GH at the secretory peak as obtained by the GH provocation test tended to be greater than the immunoreactivity (24, 29–31). The discrepancy between the bioactivity and immunoreactivity values in the ESTA assay may be due to transient changes in the distribution of different GH isoforms present in the circulation. Alternatively, it may be caused by other growth-stimulating factors in the serum, such as interleukins, colony stimulating factors, and IGF-1, but not GH. The close correlation noted in our assay, unlike in the ESTA bioassay, suggests its accuracy and reliability as a bioassay method.

This assay had a very low detection limit of 0.02 ng/ml in human sera, and 50 to 100-fold more sensitivity than the detection limit of the currently used ESTA bioassay. Further, in pediatric endocrinology, it is sometimes necessary to evaluate GH bioactivity without conducting GH stimulation tests; here, this assay would be highly advantageous.

Most importantly, the assay is sensitive enough to estimate even low levels of GH present in serum samples under various pathological states. Thus, this assay provides a practical method for analyzing serum samples for GH bioactivity. Its key advantage is that it is capable of evaluating the total GH bioactivity, and may thus contribute to an improved understanding of patients with bioinactive GH, where assessing the levels of bioactive GH is critical. Moreover, this assay could be applied for estimating bioactive levels of GH in aging studies. We expect this novel bioassay to open up novel avenues for determining the physiological and pathological hGH status in various GH-related diseases.

We thus consider the newly developed system to be a specific, sensitive, easy, and a fast bioassay for GH deter-

mination that would be useful for evaluating GH bioactivity in a variety of endocrine states.

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