could potentially account for the differential effects on craniofacial growth. Other pathologic processes appear to be at work in the calcified tissues of patients with Turner’s syndrome, as is apparent from the contradictory effects on dental crown (simpler than control) and root (greater number and complexity) morphologies noted by Midtbo and Halse.18

These differences indicate potential differences between the response of patients with Turner’s syndrome and those with GH deficiency to systemic GH. Indeed, this was observed in Turner’s original article describing this then new syndrome.3 In our study of the craniofacial growth of patients with Turner’s syndrome who received systemic GH over a 1-year period, mandibular growth was affected more by the systemic GH treatment than was maxillary growth. This discordant growth response could be beneficial in a particular retrognathic patient if the amount and direction of the mandibular growth compensated for the presenting discrepancies of that patient. It is imprudent to draw too many conclusions from this short-term study, however, and the importance of continual monitoring of craniofacial growth should not be underestimated, especially in patients expected to undergo long-term therapies.

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REFERENCES

9. Jones R, Simmons K. Cephalometric analysis of children with Turner syndrome. Table clinic presented to students and faculty of Indiana University School of Dentistry; 1993; Indianapolis, IN
14. Demirjian A. Biological Age. Sonart Inc; 1993

Methods in Determining Growth Hormone Concentrations: An Immunofunctional Assay

Christian J. Strasburger, MD

ABSTRACT. Actual measurement of growth hormone (GH) levels in children suspected of having GH deficiency can confirm this diagnosis. However, there currently is no standard for the test. Although there is general agreement that in a short-statured child, a value of <10 μg/L in response to an appropriate provocative test indicates the possible need for GH replacement therapy, there is no agreement on the best testing method. Different immunoassay kits use different methods to measure serum levels and, therefore, produce different results. In this article, the evolution of assays and the reasons for disparities in results are reviewed and discussed. An immunofunctional assay using monoclonal antibodies as a new standard is described. Pediatrics 1999;104:1024–1028; growth hormone immunoassay, growth hormone binding protein, immunofunctional assay, monoclonal antibody.

ABBREVIATIONS. GHD, growth hormone deficiency; GH, growth hormone; RIA, radioimmunoassay; MOAB, monoclonal antibody; GHB, growth hormone-binding protein; IFA, immunofunctional assay.
Although the diagnosis of growth hormone deficiency (GHD) in a child is based primarily on auxologic criteria, and in an adult on evidence of pituitary disease, the diagnosis is almost always confirmed biochemically by measuring GH levels after provocative testing. The results obtained from these measurements have profound effects on establishing the diagnosis and are the key criteria for reimbursement for the costs of GH replacement therapy. Recommendations exist concerning the provocative testing procedures for the diagnosis of GHD, and cutoff levels for diagnosis have been proposed; however, no consensus has been established for the biochemical procedure of measuring GH levels itself.

In the United States, as well as in Europe, it is widely accepted that peak GH levels in response to provocative tests should be <10 μg/L as a prerequisite for GH replacement therapy in children. Although most commercial immunoassay kits for GH determination are calibrated against international reference preparations of the hormone, the comparative measurement of serum samples by different immunoassay kits produces vastly disparate results. In view of the heterogeneity of the results obtained with different GH immunoassays, the establishment of cutoff levels for confirming the diagnosis of GHD appears arbitrary.

The first GH immunoassays were based on the competitive radioimmunoassay (RIA) principle described by Yalow and Berson. Later, Miles and Hales proposed an immunometric approach to the quantification of GH levels in serum. The advent of monoclonal antibody (MOAB) technology has led to the availability of a wide variety of commercial GH assays, all of which are suitable for the determination of GH levels in a large series of serum samples. In contrast to most bioassays for GH, which until recently have been suitable only for investigation of GH reference preparations or purified standard materials, the relatively straightforward immunoassay technology requires only small volumes of serum or plasma for GH quantification. Rather than measuring the biologic activity of GH, however, these immunoassays detect GH-like molecules in biologic samples that interact with the antibodies used in the particular assay.

Various factors have been shown to account for the heterogeneity in GH determinations by different immunoassays. For example, it is known that the molecular isoforms of GH in serum are not homogeneous, but consist of monomers, dimers, and oligomers. Monomeric GH molecules of the major 22-kd isoform, consisting of 191 amino acids, account for only approximately half of the GH molecules in the circulation. Approximately 10% are the 20-kd splice variant, in which amino acids 32 through 46 are absent. Chemically modified isoforms and fragments of various lengths also exist. Furthermore, approximately half of all the GH molecules in the circulation are bound to the high-affinity GH-binding protein (GHBP), which represents the extracellular part of the GH receptor. The binding of GH to GHBP may interfere with GH quantification by immunoassays.

Because the different antibodies used in immunoassays bind to a different spectrum of GH isoforms, GH concentrations measured by immunoassay are likely to depend on the particular antibody used. Moreover, because the distribution of the different GH isoforms varies among individuals, the results from different immunoassays cannot be interrelated easily by using a single conversion factor. Other confounding factors also contribute to the heterogeneity of the results from GH immunoassays, including the choice of assay calibrator or reference preparation, differences in immunoassay design (eg, competitive vs two-site assays), and matrix differences among the standards used for calibration and among the samples themselves.

When serum samples are measured for their GH concentration, the clinical question that has to be answered is the amount of biologically active, functional GH in the circulation at the time of the blood sampling. The answer commonly provided by the analytical results, in contrast, is a quantitation of immunoreactive GH molecular forms in the sample, to which variants, fragments, and aggregates of GH contribute to different extents depending on the epitope specificity of the antibodies used in the assay. The optimum assay for GH would be a bioassay suitable for quantification of GH activity in small volumes of serum by a method that facilitates the processing of a large number of samples. In defining such a bioassay, the GH molecules to be analyzed should promote an easily quantifiable biologic signal that is transmitted by human GH receptors. In reality, the suitability of most bioassays is restricted by their high detection limits. Moreover, the majority of bioassays are too cumbersome to perform on a routine basis.

GH has been shown to induce dimerization of its receptor as the initial step of signal transduction in target cells, which leads to phosphorylation of the intracellular domain of the human GH receptor and receptor-associated proteins. This process initiates the signal transduction cascade involving JAK2 kinase and STAT (signal transduction and activation of transcription) proteins. The radiograph crystallography structure of a complex formed by one molecule of GH and two GH receptor ecto domains was resolved, and the epitopes of the ghGH molecule that interact with either receptor molecule have been characterized. The GH molecule sequentially binds to a first receptor molecule with a large contact surface area involving a total of 31 amino acid side chains of the C-terminal part of the fourth helix bundle, parts of the random coil sequence between helix 1 and helix 2, and parts of helix 1. After a 1:1 complex has been formed between GH and the first receptor molecule, a second receptor attaches to binding site 2 of GH with a considerably smaller contact surface area and lower affinity.

The availability of panels of MOABs to human GH has led to the recognition of the importance of epitope specificity in GH immunoassays. Therefore attempts have been made to identify MOABs with binding properties for GH that are comparable with those of the GH receptor itself but with a greater
affinity than that of the natural GH receptor. The latter is on the order of 0.3 nmol/L and limits the use of cells that express the GH receptor in competitive binding assays to GH levels $>5$ $\mu$g/L. We have sought to identify MOABs specific for both binding domains on the human GH molecule that interact with the receptor molecules and have attempted to design an immunoadsorption procedure that reflects more closely in its results the biologically active proportion of GH in a given serum sample. However, only the interaction of GH with the second receptor molecule involves interaction at a limited number of amino acid side chains, which can be identified by the epitope of one MOAB. In contrast, the 31 amino acids involved in interaction on binding site 1 with the receptor cannot be identified by one MOAB.

We therefore designed an immunoadsorption assay as follows: an MOAB (code 7B11) that targets binding site 2 for the GH receptor on the GH molecule, and recombinant GHBP, representing the ectodomain of the GH receptor. The antibody 7B11 is immobilized on microtiter plates and allowed to bind over a 3-hour incubation period those GH isoforms with an intact binding site 2. The receptor-binding site of the GH molecule cannot be targeted by a single MOAB because the epitope recognized by MOABs spans a maximum of 10 amino acid side chains. Therefore, after a wash step to remove all other sample constituents, an excess of biotin-labeled recombinant GHBP is added and the samples are incubated overnight. The labeled GHBP interacts with GH molecules bound by antibody 7B11, which also contain an intact binding site 1 (Fig 1). After an additional wash step to remove excess unbound biotinyl-GHBP, microtiter plates are incubated for 30 minutes with streptavidin labeled with a detectable probe, such as europium, to give a time-resolved fluorescence endpoint, enzymes to give a colorimetric endpoint, luminophores to give a chemoluminescent endpoint, or iodine 125 for $\gamma$ counting.

With its use of GHBP as the labeled component in this two-site assay for GH, the method was called an immunofunctional assay (IFA). The IFA recognizes only those molecular forms of GH that have both receptor interaction sites and can thus initiate a biologic response in target cells. In the IFA, recombinant 22-kd human GH (IRP 88/624) was found on a weight basis to be almost twice as potent as a pituitary-derived GH preparation such as IRP 80/505.

Recent experiments with recombinant 20-kd human GH showed a relative potency of 10% $\times$ 3% for this isoform, compared with the 22-kd GH isoform in the IFA. Growth hormone dimers, on a molar basis, had a slightly higher reactivity than monomers, as would be predicted by the design of the IFA. Placental lactogen, which may reach concentrations that are 1000-fold those of GH in serum samples taken during pregnancy, was found not to cross-react in the IFA. The only serum samples in which higher concentrations were detected by the IFA than by a polyclonal RIA were pregnancy sera. This indicates that the placental GH variant is recognized by the IFA to a greater extent than by the polyclonal antibody raised against pituitary-derived GH and used in the competitive RIA.

When GH was measured by the IFA in sera taken from nonpregnant subjects and these measurements were compared with the results obtained in the same samples by a polyclonal competitive RIA using identical recombinant 22-kd GH calibrators (IRP 88/624), the overall agreement was good, with the IFA giving results that were on average 27% lower. This result implies that 73% of the molecules detected by polyclonal RIA retain the integrity, as detected by the IFA. This proportion on an individual basis, however, varies between 50% and 95% of immunoreactive forms. Polyclonal RIAs still provide the best agreement between GH immunoassay results using different methods in different laboratories and, for this reason, the recommendations for diagnostic cutoff values are based on polyclonal RIAs.

The finding that variable proportions of GH molecules detected by RIA retain functional integrity compromises further the diagnostic value of GH determinations by traditional immunoadsorbs. For example, a peak GH level of 12 $\mu$g/L (determined by RIA) in response to a provocative test may reflect a sample containing 11 $\mu$g/L of functionally intact GH (as determined by IFA) and may lead to the conclusion that GH secretion is normal. On the other hand, the sample may contain only 6 $\mu$g/L of functionally intact GH, supporting the diagnosis of at least partial GHD in childhood.

High levels of circulating GHBP lead to diminished recovery of GH in the IFA. In this situation, the majority of GH in the sample are bound to GHBP, and not fully available to receptors on target cells. This is in keeping with the recent description of a GH receptor mutation leading to a truncated protein and to increased production of GHBP in a child with short stature.

The IFA has been reviewed previously, along with an eluted stain assay and a 22-kd exclusion assay. A limited comparison between the IFA and an RIA was performed in 19 serum samples covering a GH concentration range of 1.0 to 25.8 $\mu$g/L, as measured by
the RIA. Comparison of the two assays yielded an $R^2$ of only .88. A comparison between the IFA and a bioassay using NB2 rat lymphoma cells yielded an $R^2$ of .97.\textsuperscript{21}

The practical handling of the IFA is fully comparable with that of microtiter plate-based sandwich immunoassays. The hands-on time needed for determination of 100 samples is between 90 and 120 minutes, and results are obtained within 24 hours.

Assays for the quantification of GH have to be judged critically for their methodologic approach; GH levels cannot be interpreted or even compared among laboratories without detailed knowledge of the methodologic processes used to generate the results.

Bioassays generally provide the type of information that is most useful to the clinician, although animal models or cell assays make use of receptors that differ in association constants and other properties from those of the human GH receptor. Most conventional bioassays, however, are not suited for the analysis of human serum or plasma samples and are too cumbersome to perform on a routine basis in endocrine centers.

Immunoassays generally have major advantages over bioassays in terms of performance, sensitivity, and convenience, but their results are highly variable and reflect the immunoreactivity of an undefined proportion of the GH molecular isoforms in the sample with the antibodies used in the assay. Other sources of the variability among different immunoassay methods reflect their design, choice of calibrator, and matrix effects.

The IFA for GH uses a site-specific MOAB, in combination with a recombinant GHBP, and recognizes GH isoforms that have intact receptor interaction sites. Only these isoforms retain the potential to initiate a biologic signal in target cells by inducing receptor dimerization as the initial step of signal transduction.\textsuperscript{21} The IFA shows better agreement with the NB2 bioassay than with RIAs. It is as convenient to use as immunoassays and can be used routinely for GH determinations in endocrine laboratories.

The heterogeneity of GH immunoassay results poses a major problem in defining standards for the diagnosis of GHD in pediatric patients. Recommendations as to cutoff values for GH provocative tests are meaningless unless the assay methodology is defined. Because of the wide variety of GH assay methods available, consensus statements that include cutoff recommendations have referred to the competitive polyclonal antibody-based RIA, because this approach shows the highest comparability of results obtained in different laboratories. Radioimmunoassay, however, is now used infrequently in routine practice. Therefore, the present recommendation is that every clinician should be aware of how his or her GH results are derived and that every laboratory should be aware of how its preferred method compares with the polyclonal RIA.

After the advent of a recombinant international reference preparation (IRP 88/624) as the first chemically and physically homogeneously defined calibrator, it makes sense to use this preparation in GH assays. Additionally, the results should be stated in mass units such as micrograms per liter.

Now that the IFA is available commercially, it will have to prove its advantage over other existing methods for GH quantification in terms of clinical decision-making. Because the IFA recognizes only intact and biologically active GH serum samples, it provides more meaningful information as to whether a given patient is GH-deficient. Theoretically, this should make the IFA a standard methodologic approach. In view of the enormous cost and burden for the patient that is implied by the diagnosis of GHD and its subsequent treatment, vigorous standardization of all components involved in establishing the diagnosis is mandatory.

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REFERENCES

Growth Hormone Stimulation Test Results as Predictors of Recombinant Human Growth Hormone Treatment Outcomes: Preliminary Analysis of the National Cooperative Growth Study Database

George M. Bright, MD*; Joanne R. Julius, MS‡; John Lima, PharmD*; and Sandra L. Blethen, MD, PhD‡

ABSTRACT. Growth hormone (GH) stimulation tests are considered a prerequisite to clinical trials of recombinant human GH (rhGH) therapy, but the test results may not be predictive of the treatment outcomes with rhGH. We examined the GH stimulation test results as a predictor of the treatment outcome in a cohort of prepubertal subjects in the National Cooperative Growth Study. A standard is proposed in which a diagnosis of GH deficiency is considered appropriate when a patient has significant first-year catch-up growth and that a positive stimulation test result predicts this outcome. With this construct, a traditional interpretation of GH stimulation test results correctly identifies 64% of the rhGH treatment outcomes. The analysis shows an upper limit of diagnostic sensitivity of 82% and a lower limit of specificity of 25% in our study population. The results of our recent studies suggest that the sensitivity and specificity of the current GH stimulation tests are attributable in part to broad intersubject variation in GH clearance, rates of GH elimination, and GH volume of distribution. The combined studies suggest that the use of subject-specific pharmacokinetic parameters will improve the diagnostic interpretation of GH stimulation test results and improve rhGH treatment outcomes. Pediatrics 1999; 104:1028–1031; growth hormone stimulation tests, recombinant human growth hormone, pharmacokinetic parameters, maximal stimulated growth hormone concentration.

ABBREVIATIONS. GH, growth hormone; hGH, human GH; rhGH, recombinant human GH; NCGS, National Cooperative Growth Study; ∆Ht SDS, change in height standard deviation score; GHmax, maximal stimulated GH concentration.

In the early years of growth hormone (GH) therapy, human GH (hGH) was extracted from the pituitary glands of organ donors and distributed through the National Pituitary Association. The demand for GH frequently exceeded the supply, and criteria were developed for determining prospectively which GH-deficient patients would most likely respond favorably to hGH treatment. Early efforts were aimed at determining whether the patient had deficient production of GH, but we have since realized that states of functional GH deficiency may arise either from a defect in GH production and secretion or from insensitivity of the GH response mechanisms. The pulsatile nature of GH secretion mitigated against casual blood sampling to determine the adequacy of GH production and gave rise to numerous varieties of stimulation tests of GH release. Such stimulation tests have become standard not only in endocrine practice but also for the approval of GH treatment by most health care insurers. It is not surprising then that renewed attention is being given to the diagnostic accuracy of these tests.

The literature is replete with comparisons of one stimulation test with another, and no single test has achieved the status of a “gold standard.” The limitations of GH stimulation tests have been reviewed, with a consensus being that the “available methods for measuring GH secretion are neither convenient nor reliable.” The results of GH stimulation tests in models that have attempted to explain the outcomes of hGH treatment have not always been reliable predictive variables. Are GH stimulation tests useful or not for determining which patients will benefit
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