Heterogeneity of Growth Hormone in the Nocturnal Serum of Children¹

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ABSTRACT. The nocturnal serum of 13 nongrowth hormone deficient, hyposomatomedinemic short children and of 12 normal children of average height was analyzed by both polyclonal and biconal radioimmunoassays. The biconal/polyclonal ratio for immunochemical grade human growth hormone was 1.0, but for the nocturnal sera in both groups, this ratio was significantly less than 1.0 (range 0.5–1.2, average 0.7–0.8). The ratio did not differ significantly between the two groups of children. (*Pediatr Res* 19: 981–985, 1985)

Abbreviations

RIA, radioimmunoassay
GH, growth hormone
hPL, human placental lactogen
hPRL, human prolactin
hTSH, human thyroid-stimulating hormone
hFSH, human follicle-stimulating hormone
hLH, human luteinizing hormone
hGH, human growth hormone
RRA, radioreceptor assay

The purpose of this study was to compare the RIA profile of the endogenous GH between normal children of average height and hyposomatomedinemic non-GHD, short children identified in a preceding investigation (1) by means of newly available monoclonal RIA techniques (2) together with the conventional polyclonal assay (3). The gel-filtration behavior of the serum GH also was compared between the two groups of children.

METHODS

SUBJECTS

The normal group comprised six boys and six girls, 12 to 18 yr old, between the 5th and 95th percentiles in height. History and physical examination revealed no evidence of acute or chronic illness.

The hyposomatomedinemic, non-GHD, short (less than third percentile) children consisted of 10 boys and three girls, 7 to 10 yr of age, who had been identified during phase 2 of a preceding study (1). Their clinical characteristics are summarized in Table 2 of the preceding report (1). Mean serum immunoreactive GH²

response to L-dopa or glucagon was >12 ng/ml, showing normal immunoreactive GH status.

STUDY DESIGN

The children were hospitalized for 5 days. They received no medications. The short children had not received hGH injections during the previous month. Their diet was 2300 calories, 100 g protein, 250 g carbohydrate, and 100 g fat. Via an inwelling heparin lock inserted at 2000–2100, 5 ml heparinized blood were removed on retiring, and at 30, 60, 90, and 120 min after sleep began. The blood was kept at 4° C until 800, when it was spun; the serum was stored at –20° C for up to 2 wk before analysis by RIA (4). Some sera also were examined by gel filtration.

MATERIALS

The kits were obtained from Kallestad Laboratories (New York, NY) ("polyclonal kit") and from Hybritech Laboratories (Los Angeles, CA) ("biconal kit"). Two mouse monoclonal antibodies (33.2 and 101.3) to hGH were purchased from Hybritech. According to the manufacturers, the antibodies were directed at different epitopes of the hGH molecule. The precipitating antibody (rabbit antiserum to mouse IgG) was obtained from Calbiochem-Behring (Los Angeles, CA). The labeled hGH used in the monoclonal assays was obtained from Kallestad Laboratories. Immunochemical grade hGH, used as the cold hormone in these assays, was from National Institutes of Health, Bethesda, MD, as were the other hormones—hPL, hPRL, hTSH, hFSH, hLH, and β -lipotropin. For gel filtration, Ultrogel AcA 44 was purchased from LKB (New York, NY), and disposable columns (1 $\times$ 50 cm) from BioRad, Richmond, CA. All other reagents and chemicals were obtained from Sigma Chemicals Co., St. Louis, MO.

ANALYTICAL METHODS

The Polyclonal RIA. The polyclonal RIA kit was obtained from Kallestad Laboratories, Inc. It contained rabbit antiserum to hGH, labeled ¹²⁵I-hGH, goat antiserum to rabbit gamma globulin, and 0.01 M phosphate buffer containing bovine serum albumin. The assay was a double antibody sequential saturation technique. The samples were incubated with hGH antiserum and ¹²⁵I-hGH to form hGH-antibody complexes, which were then precipitated with the second antibody. The bound fractions were counted in a gamma counter.

In each assay, GH-free human serum containing varying amounts (1–30 ng/ml) of National Institutes of Health immunochemical grade hGH provided the standard curve.

The Biconal RIA. This comprised plastic beads coated with monoclonal mouse antibody to hGH, ¹²⁵I-labeled monoclonal mouse antibody to hGH (different from the one coating the

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² The conventional, commercially available RIA with polyclonal rabbit antiserum was used to measure immunoreactive GH in the provocative test. Our technique for carrying out this RIA is described in "Methods."

beads), and a buffer wash. The assay was a solid phase two-site assay. Samples bound to the antibody on the bead and to the different ^{125}I -antibody. The bead was then washed to remove all unbound labeled antibody and counted in a gamma counter. As in the polyclonal RIA the standard curve was determined in each assay with immunochemical grade hGH dissolved in GH-free serum from GHD children.

Two Monoclonal RIA's. Two RIA's using monoclonal mouse antibodies were developed. These assays were of the sequential saturation type; details are described below.

Optimal dilution of the monoclonal antibody. A stock solution of 1 mg/ml monoclonal antibody was serially diluted from 1 in 100 to 1 in 300,000 in assay buffer, a 0.01 M phosphate solution of pH 7.4 containing 0.5% bovine serum albumin. An aliquot (500 μl) of the assay buffer was mixed with 100 μl of each antibody dilution and 100 μl of ^{125}I -hGH. The tubes were vortexed and incubated for 4 h at 37° C. Thereafter, 200 μl of the precipitating antibody solution was mixed into each tube of the assay. Precipitating antibody was rabbit antiserum to mouse IgG supplied by Calbiochem-Behring. This was diluted one in five parts in assay buffer, and then equally with 12% polyethylene glycol. The total assay mixture was then incubated for 16 h at 4° C, after which the tubes were centrifuged for 30 min at 3000 $\times$ g. The supernatant was decanted and the residue counted in a gamma counter. The percent absolute binding was plotted against the dilution to determine the dilution of monoclonal antibody which bound about 30% of the labeled hGH.

Displacement Curve. After the optimal antibody dilution was determined, a displacement curve of the antibody using varying amounts of immunochemical hGH was examined for sensitivity and specificity. The assay was based on sequential saturation technique where 500 μl of assay buffer, 100 μl diluted antibody, and 100 μl of sample were incubated for 4 h at 37° C. The standards (3 to 100 ng/ml immunochemical grade hGH) were prepared in GH-free human serum. Nonspecific binding was determined by spiking duplicate tubes in the assay with 10 μg hGH in place of sample or standard. After the initial 4 h incubation, 100 μl of ^{125}I -hGH were mixed into all tubes and incubated for 2 h at 37° C. The precipitating antibody solution (200 μl) was then added. The entire assay mixture was incubated for 16 h at 4° C and then spun, drained and counted in order to calculate the percent of bound ^{125}I -hGH in each tube. A log-logit plot was established to correlate percent binding and the amount of hormone in the standard samples. As in the polyclonal and biconal assays, control sera containing known amounts of the immunochemical grade hGH were included in each assay of unknown sera. In addition to tests of sensitivity, and inter- and intraassay variability, the antibody was tested for specificity to hGH. Cross-reactivity was measured by testing the ability of adrenocorticotrophic hormone, hPRL, hTSH, hLH, hPL, hFSH and β -lipotropin, to displace the labeled hGH.

Gel Filtration Analyses. The chromatography was carried out on a 1 $\times$ 50 cm Ultrogel AcA 44 column. The eluting buffer was 0.023 M ammonium carbonate, pH 8.6, with 0.1% bovine albumin and 0.02% sodium azide.

The column and buffer were stored at 4° C and were run at 25° C. One ml aliquot of a child's nocturnal serum was loaded on the column. The flow rate was 1 ml/1.5 min and 1 ml fractions were collected. The fractions were separately lyophilized, reconstituted in 250 μl of water, and assayed in the polyclonal and biconal RIA's.

RESULTS

DATA ON THE FOUR RIA SYSTEMS

Polyclonal (Kallestad) Assay. The intraassay and interassay coefficients of variation were 4 and 6%, respectively. No detectable cross-reactivity (<0.01%) was observed with hFHS, hLH, hTSH, or hPL. Human prolactin cross-reacted with 2.5% of the

activity of hGH. The sensitivity of the assay was 1.0 ng/ml and the working range was 1–12 ng/ml.

Biconal (Hybritech) Assay. Intra- and interassay coefficients of variation were 3 and 6%. Cross-reactivity of hLH, hFSH, or hPL was <0.01%, while cross-reactivity of human prolactin was 0.5%. The sensitivity of the assay was 0.2 ng/ml and the working range was 1.0–25.0 ng/ml.

Monoclonal RIA. Determination of optimal dilution of antibody. A maximal binding of 50% for 33.2, and of 42% for 101.3, was obtained at a dilution of 1 in 1000. The binding became undetectable (<4%) at a dilution of 1 in 100,000 for 33.2, and of 1 in 1,000,000 for 101.3. At a dilution of 1 in 30,000, the absolute binding ranged from 30 to 35% for 101.3. At a dilution of 1 in 3000, the absolute binding ranged from 30 to 35% for 33.2. These latter dilutions of antibody were used for all subsequent assays.

The intra- and interassay coefficients of variation for degree of binding were 3 and 6%. Stock solutions of antibody 33.2 and 101.3 (diluted 1 in 3000) were kept frozen in 1 ml aliquots, and diluted for use in each assay as required. No change in binding was noticed in frozen aliquots over a period of 12 wk.

Displacement curves for each antibody. Figure 1 shows dis-

Fig. 1. Upper: Displacement curve for monoclonal antibody 101.3 using immunochemical grade hGH standards of 3, 6, 12, 25, and 50 ng/ml and ^{125}I -hGH. Lower: Displacement curve for monoclonal antibody 33.2 using immunochemical grade hGH standards as defined in Figure 1A.

placement curves for each monoclonal antibody. The intra- and interassay coefficients of variation averaged 5 and 9% for both antibodies. The assay showed no significant cross-reactivity ($<0.05\%$) with adrenocorticotrophic hormone, hTSH, hFSH, hPL, or β -lipotropin. Human prolactin cross-reacted about 0.5%. For 33.2, 15 ng/ml hGH was required for 50% displacement. For 101.3, only 12 ng/ml was required for similar displacement.

The useable range for 33.2 was 1–32 ng/ml and for 101.3 was 8–100 ng/ml. The sensitivities of the two monoclonal RIA's were 16 ng/ml and 8 ng/ml respectively.

SLEEP TESTS

Normal Children and Adolescents, 12 to 18 Yr Old. A typical sleep test is shown in Figure 2, and the group is summarized in Table 1. On retiring, the polyclonal serum GH value averaged less than 1 ng/ml. At 30 and 60 min, it averaged 14 ng/ml, then declined progressively during the next 90 min. Because the biclonal and polyclonal assays had similar thresholds, about 1

Fig. 2. Typical sleep tests comparing nocturnal serum hGH levels of a normal (A) and a non-GHD, hyposomatomedinemic, short child (B) as measured by four types of RIA.

Table 1. Nocturnal immunoreactive serum GH in normal and non-GHD hyposomatomedinemic short-children as measured by four types of RIA (mean $\pm$ SD)

	Normal	Non-GHD*
Peak polyclonal (ng/ml)	20.0 $\pm$ 2.2 (n = 12)	17.7 $\pm$ 2.4 (n = 13)
Peak biclonal/polyclonal	0.84 $\pm$ 0.07†	0.81 $\pm$ 0.07†
Peak 33.2 monoclonal/polyclonal	1.28 $\pm$ 0.35 (n = 5)	1.18 $\pm$ 0.13 (n = 6)
Peak 101.3 monoclonal/polyclonal	0.90 $\pm$ 0.41 (n = 8)	0.98 $\pm$ 0.24 (n = 9)
33.2 monoclonal (% ND‡)	58 (7/12)	45 (5/11)
101.3 monoclonal (% ND§)	27 (3/11)	10 (1/10)
30 min polyclonal (ng/ml)	13.9 $\pm$ 2.9 (n = 12)	15.1 $\pm$ 2.9 (n = 13)
30 min biclonal/polyclonal	0.83 $\pm$ 0.09†	0.79 $\pm$ 0.07†
30 min 33.2 monoclonal/polyclonal	1.14 $\pm$ 0.11 (n = 3)	1.19 $\pm$ 0.57 (n = 5)
30 min 101.3 monoclonal/polyclonal	1.00 $\pm$ 0.10 (n = 7)	1.06 $\pm$ 0.58 (n = 7)
33.2 monoclonal (% ND)	75 (9/12)	55 (6/11)
101.3 monoclonal (% ND)	36 (4/11)	30 (3/10)
60 min polyclonal (ng/ml)	14.4 $\pm$ 2.5 (n = 11)	12.6 $\pm$ 2.4 (n = 13)
60 min biclonal/polyclonal	0.81 $\pm$ 0.06†	0.69 $\pm$ 0.04†
60 min 33.2 monoclonal/polyclonal	1.16 $\pm$ 0.28 (n = 4)	0.98 $\pm$ 0.47 (n = 3)
60 min 101.3 monoclonal/polyclonal	1.46 $\pm$ 1.25 (n = 8)	1.72 $\pm$ 1.95 (n = 4)
33.2 monoclonal (% ND)	67 (8/12)	73 (8/11)
101.3 monoclonal (% ND)	27 (3/11)	60 (6/10)

* $p > .05$ for comparison of all variables between normal and non-GHD groups.

† Significantly less than 1 ($p < 0.05$).

‡ Not detectable: <16 ng/ml.

§ Not detectable: <8 ng/ml.

ng/ml, the biclonal to polyclonal ratio was calculated for all of the sera which contained >1 ng/ml or more of polyclonal immunoreactivity. At 30 and 60 min, the average ratio was significantly less than one by Student's t test, being 0.83 and 0.81, respectively ($p < 0.05$).

At 30 and 60 min, the 33.2 monoclonal reactivity was below its threshold of 16 ng/ml in 75 and 67% of the samples, respectively; and the 101.3 assay was below its sensitivity of 8 ng/ml in 36 and 27% of the samples. For samples in which the 33.2 monoclonal assay was above its threshold sensitivity of 16 ng/ml, the 33.2 to polyclonal ratio averaged 1.14 (30 min) and 1.16 (60 min) (not significantly different from 1.0 by Student's t test). For samples in which the 101.3 monoclonal assay was above its threshold of 8 ng/ml, the 101.3 to polyclonal ratio averaged 1.00 (30 min) and 1.46 (60 min) (not significantly different from one).

Biclonal to polyclonal ratios for individual normal children varied between 0.54 and 1.20. The 101.3 and 33.2 to peak polyclonal ratios for individual normal children varied between 0.52 and 1.64 for 101.3 and between 1.06 and 1.27 for 33.2. By single factor analysis of variance techniques, we found generally significant F ratios ($p < 0.05$), indicating that some subjects had statistically different ratios.³

³ These data are filed with the National Auxiliary Publication Service as document 04319.

Hyposomatomedinemic, Non-GHD, Short Children, 7 to 10 Yr Old. A representative sleep test is shown in Figure 2B and the results for the entire group are summarized in Table 1. The polyclonal GH immunoreactivity usually peaked at 30 min, occasionally at 60 min. The peak polyclonal value averaged 17.7 ng/ml.

At 30 and 60 min, the biclonal to polyclonal ratio was significantly less than one, being 0.79 and 0.69 respectively. As shown in Table 2 of the preceding report, the average biclonal to polyclonal ratio in the non-GHD, hyposomatomedinemic short children ranged from 0.52 to 1.18.

At 30 and 60 min, 55 and 73% of the samples had undetectable monoclonal 33.2 level (<16 ng/ml), while 30 and 60% were undetectable in the monoclonal 101.3 assay (<8 ng/ml). For those samples above the thresholds in the 33.2 and 101.3 monoclonal assays, the 33.2/polyclonal and 101.3/polyclonal ratios averaged 1.19 and 1.06 at 30 min and 0.98 and 1.72 at 60 min, respectively (not significantly different from one by Student's *t* test, $p > 0.05$).

Statistical Comparisons between Normal (Age 12 to 18) and Hyposomatomedinemic, Non-GHD Short Children (Age 7 to 10). With Student's *t* test or Fisher's exact test for comparing percentages, no significant differences in any of the serum GH immunochemical levels or ratios shown in Table 1 were found between the groups.

INCUBATION EXPERIMENTS

After the differing results in the four RIA's were recognized, the possibility was considered that the native hGH molecule was degraded in blood, with differential effects of the degradation process on immunoreactivity in the four assays. To investigate this question, we incubated either clinical grade or immunochemical grade hGH (20 ng/ml) in heparinized blood from a GHD child (<1.0 ng/ml of endogenous serum GH by the polyclonal RIA), and in the corresponding plasma at 25° C or at 37° C, under either air or 95% O₂ + 5% CO₂. In none of the four assays did immunoreactive levels change significantly during 5 h of incubation.

GEL-FILTRATION ANALYSES

Twenty-six nocturnal sera containing >10 ng/ml GH in the polyclonal RIA (12 normal subjects and 14 hyposomatomedinemic non-GHD short children) were examined by gel-filtration chromatography. For comparison, 20 ng/ml of immunochemical grade hGH were added to the serum of a GHD child, and this sample also was analyzed. Typical results are shown in Figure 3. After chromatography of the serum containing added hGH, the polyclonal immunoreactivity eluted between fractions 36 and

Fig. 3. Gel-filtration profile of GH-deficient serum spiked with immunochemical grade hGH on Ultrogel ACA44. Solid line indicates absorption at 280 nm. Broken line is immunoreactive GH determined by the polyclonal assay.

42, with a peak at tube 40. The overall recovery was about 80%. No other polyclonal immunoreactive peak was observed. The same type of chromatogram was obtained in all 26 nocturnal serum samples. The overall recovery of the serum polyclonal immunoreactivity ranged from 65–90% and was in fractions 36 to 42. The biclonal to polyclonal ratio of the recovered GH immunoreactivity did not differ significantly from that in the serum applied to the column.

Similar results were obtained when the GHD serum containing 20 ng/ml added immunochemical hGH was stored at 5° C for 8 h before gel filtration.

DISCUSSION

The average biclonal/polyclonal immunoreactivity ratio of nocturnal serum was significantly below 1.0 in both normal and hyposomatomedinemic, non-GHD, short children. If the endogenous GH in these sera was identical to the native hGH molecule which was used to standardize the four RIAs, this ratio would have been 1.0. Because the average ratio was 0.7 to 0.8, at least 20–30% of the endogenous GH probably differs in structure from the native hormone molecule. One possibility would be a mixture of about 75 parts of the native molecule with biclonal/polyclonal ratio = 1.0, and 25 parts of an altered molecule with a ratio = 0. Alternatively, all the endogenous GH could be an altered form of hGH with a biclonal/polyclonal ratio of 0.75. Intermediate hypotheses are possible. The extensive intra- and interindividual differences in the biclonal/polyclonal and monoclonal/polyclonal ratio (range, 0.5 to 1.5)³ suggest that endogenous serum GH probably is a complex mixture of altered forms. However, the possibility cannot be excluded that the standard hGH molecule was not itself native, but was altered by its purification. Because the antigenic specificities of the five antisera used in the four RIAs are not defined, we cannot speculate about where in the hGH molecule the alterations occurred.

The structural alteration or heterogeneity responsible for the low biclonal/polyclonal ratio could arise in the pituitary gland, where two different GH genes are present, where the primary gene transcript is clipped and spliced, where the pre-GH product of the ribosomes is proteolytically shortened, and where structurally different forms of 22K GH have been recognized (5). Alternatively, the low biclonal/polyclonal ratio of the serum GH could reflect alteration of native GH by peripheral tissues, with reentry of modified GH molecules into the blood. The incubation experiments (see "Results") gave no evidence that exposure of hGH to plasma or blood constituents altered the hormone's immunoreactivity.

The absence of a statistically significant difference in any feature of the RIA profile, or in the gel-filtration chromatogram, between normal and non-GHD, hyposomatomedinemic short children, argues against the bioinactive GH hypothesis for the latter group as a whole. However, the considerable interindividual differences in the various RIA ratios within each group³ makes it impossible to exclude this possibility in every case. Furthermore, the gel-filtration technique used was probably not capable of distinguishing between 22K and 20K GH.

Besides the RIA profile and gel chromatogram, other ways of searching for bioinactive GH are by RRA and bioassay. Recently we compared serum GH by the pregnant rabbit liver RRA, by the human "IM9" lymphocyte RRA, by the NB2 mouse lymphoma bioassay, and by the polyclonal RIA in 12 control and in 4 hyposomatomedinemic, non-GHD, short children (6). Ratios of RRA/RIA and of bioassay/RIA ranged from as low as 0.36 to as high as 1.65 for nocturnal serum GH in both groups of children. Furthermore, RRA/RIA ratios averaging only 0.6 were observed by Sneed *et al.* (7) on serum GH during pharmacologic provocative tests in short normal children. These findings with RRA and bioassay methods are consistent with the present conclusion that serum GH is heterogeneous in children of both average and below average height.

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