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LH Measurements by *in Vitro* Bioassay and a Highly Sensitive Immunofluorometric Assay Improve the Distinction between Boys with Constitutional Delay of Puberty and Hypogonadotropic Hypogonadism

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ABSTRACT. The basal and gonadotropin-releasing hormone (GnRH) stimulated levels of LH were measured in 21 boys with delayed puberty using conventional RIA, mouse interstitial cell *in vitro* bioassay (B-LH), and a highly sensitive immunofluorometric method (F-LH). On the basis of subsequent clinical follow-up, the subjects were diagnosed as idiopathic constitutional delay of puberty (CD, $n = 13$) or hypogonadotropic hypogonadism (HH, $n = 8$). The basal RIA LH levels were similar in the two diagnostic groups (HH, 2.92 ± 0.76, CD 3.53 ± 1.37 IU/L). In contrast, the mean basal B-LH was significantly lower in boys with HH than with CD (1.10 ± 0.45 *versus* 2.91 ± 1.23 IU/L; $p < 0.01$). A similar finding was made by F-LH measurements which were clearly lower in the HH than the CD group (0.073 ± 0.04 *versus* 1.71 ± 0.97 IU/L, $p < 0.01$). Also upon GnRH stimulation (3.5 μg/kg i.v.), the distinction between the CD and HH groups was better with the B-LH and F-LH measurements. The basal B/I ratio of the CD group (0.90 ± 0.43) was more than that of the HH group (0.42 ± 0.25, $p < 0.01$) and this ratio increased significantly (more than 2-fold, $p < 0.01$) during GnRH stimulation in the CD group, but not in the HH patients. Such differences were not found between the B/F ratios of the CD and HH groups. Measurements of basal and GnRH stimulated B- and F-LH levels clearly improved the distinction between CD and HH in comparison to the conventional RIA method, due to the low sensitivity and likely cross-reactions with some non-LH constituents of serum in the latter assay. This problem is, to a great extent, eliminated by better sensitivity and specificity of B-LH and F-LH measurements. For the same reasons, the difference in B/I ratios between the CD and HH samples, and the increased B/I ratio after GnRH stimulation in the CD group, were not observed in B/F ratios. In conclusion, the measurements of basal and GnRH-stimulated concentrations of serum B-LH and F-LH clearly improve the differential diagnostics between CD and HH. The discrepancies measured between the B/I and B/F ratios in these samples call for reevaluation of the bio/immuno ratios of circulating LH. (*Pediatr Res* 27: 211–214, 1990)

Abbreviations

B/F ratio, ratio of serum B-LH/F-LH concentrations
B/I ratio, ratio of serum B-LH/I-LH concentrations
B-LH, bioactive LH
CD, constitutional delay of puberty
F-LH, immunoreactive LH measured by immunofluorometric assay
GnRH, gonadotropin-releasing hormone
hCG, human chorionic gonadotropin
HH, hypogonadotropic hypogonadism
I-LH, immunoreactive LH measured by radioimmunoassay
CV, coefficient of variation

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CD and HH pose a diagnostic problem in pediatric endocrinology. This differentiation is important for prognostic reasons and to avoid too long androgen treatment of children with CD. No hormonal measurement has thus far proven to be absolutely reliable in discriminating the two conditions (1, 2). Basal levels of immunoreactive LH and FSH show no clear differences (3–5), and even after stimulation tests with GnRH and hCG some overlap remains. In many cases the final diagnosis must await outcome of the clinical follow-up (3–5). Some information is available on discordant activation of the pituitary secretion of immunoreactive and bioactive LH during normal puberty, with a trend toward increase in the B/I ratio of LH as puberty advances (6–12). These findings are corroborated by findings in adults with hypogonadotropic hypogonadism, in which the B/I ratio is suppressed (13, 14). However, the recently developed highly sensitive immunometric measurements of gonadotropins (15, 16) have disclosed quite different data on the quantitative changes of serum gonadotropins during puberty, and their value has not yet been tested in the diagnostics of pubertal disorders. We therefore decided to compare the measurements of LH by conventional RIA, *in vitro* bioassay, and a highly sensitive immunofluorometric assay in boys with CD and HH. The results demonstrate that both of the latter techniques improve discrimination between the two diagnostic groups.

MATERIALS AND METHODS

Patients. A total of 21 prepubertal boys, referred to the hospital because of delayed pubertal development or suspicion of gonadotropin deficiency, was studied. Data on the stage of maturation of the boys are presented in Table 1. On subsequent clinical follow-up for at least 5 y, 13 of the boys developed normal spontaneous puberty, and were classified as CD. The diagnostic criteria of this group were spontaneous puberty appearing more than 2 y later than average, but progressing normally, and testis

size appropriate for bone age (17, 18). The boys were followed up until at least full genital stage 4 (including normal testis growth) (19) for clinical confirmation of diagnosis. Eight of the boys were diagnosed as HH. The diagnostic criterion was a prepubertal testis size at bone age more than 13.0 y or testis size less than mean −2 SD of the normal boys at later bone age (17). Of the HH patients, four were subsequently diagnosed as Kallmann's syndrome, one as Prader-Willi syndrome, and three remained as idiopathic. The clinical diagnostics of these patients are described in more detail previously (5), where also some of the I-LH data were presented. None of the boys had received any androgen therapy before the study. At the time of the GnRH challenge tests, the stage of pubertal development, in particular that of bone age, of the boys was similar in the two groups (Table 1).

Methods. The GnRH challenge test was performed by injecting 3.5 μg/kg body wt of GnRH (Relefact, Hoechst, Frankfurt am Main, F.R.G.) as a 1-min i.v. injection at 0850–0900 h. Venous blood samples were obtained at −20, 0, 20, 30, 60, and 120 min, and stored at −70°C until analyzed.

The I-LH was measured by a double antibody RIA (reagents donated by NIADDK, NIH, Bethesda, MD) and the results were expressed in IU/L in terms of the 1st International Reference Preparation of human pituitary gonadotropins (68/40). The intra- and interassay CV of the method were 9 and 13%, respectively, and the sensitivity of the assay was 1.9 IU/L.

F-LH was measured using the time-resolved immunofluorometric assay technique (Delfia, Pharmacia-Wallac Oy, Turku, Finland) essentially as described recently by Apter *et al.* (16). The intra- and interassay CV of the method were less than 5%, and the sensitivity was 0.02 IU/L.

B-LH was measured by the mouse interstitial cell *in vitro* bioassay, described by van Damme *et al.* (20), with some modifications (21). Each assay tube contained about 50 000 interstitial cells in a volume of 100 μL medium 199 (supplemented with 20 mmol/L *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 0.1 mmol/L 1-methyl-3-isobutylxanthine, 10^5 IU/L heparin, and 0.1% BSA, pH 7.4) and of a 100 μL aliquot of a standard or unknown serum dilution. The serum samples were diluted, on the basis of the expected LH levels, either 1:10, 1:30, 1:100, or 1:300 with the supplemented medium 199, and at least two dilutions per sample were analyzed in triplicate. The standards were diluted in the same medium, and LH-free serum was added to them and the higher serum dilutions to adjust the serum content of each assay tube to a constant 5%. The LH-free serum was prepared from male peripheral serum by immunoadsorption to microtiter wells (anti-hLH Microtitration Strips from a Delfia hLH Kit, Pharmacia-Wallac Oy) coated with a MAb to the β-subunit of LH. This treatment removed more than 98% of the LH immunoreactivity present in the serum samples (21). By this procedure it was possible to obtain parallel dose response curves of the standards and serum dilutions in the bioassay. The sensitivity of the assay was 0.3–0.5 IU/L, and the intra- and interassay CV less than 5 and 10%, respectively. The end-point of the bioassay, testosterone production of the mouse interstitial cells, was measured in the incubation media by a direct RIA as described (22).

Table 1. *Chronologic and bone age, testis size, and pubertal stage of the patient groups with CD (n = 13) and HH (n = 8) (mean ± SD)*

		CD	HH
Chronologic (y)		15.8 ± 1.3	16.6 ± 7.2
Bone age (y)		13.2 ± 1.0	13.1 ± 2.2
Testis size (mL)		5.3 ± 3.0	1.7 ± 0.7*
Genital stage† (no. of patients)	G1	1	5
	G2	10	3
	G3	2	0

* $p < 0.01$ *versus* the CD group.

† Boys at G1 had prepubertal testicular volume (<2.2 mL).

Student's *t* test was used for comparisons of two means, and one-way analysis of variance followed by Duncan's new multiple range test, for multiple comparisons. The testis volumes were compared using the Mann-Whitney U test. A *p* value of less than 0.05 was chosen as the limit of statistical significance. The study was approved by the Ethical Committee of the Children's Hospital, University of Helsinki.

RESULTS

The basal levels of LH (means of the −20- and 0-min samples) differed between the three assay methods used (Fig. 1*A*). No difference was found in basal I-LH levels between the CD (n = 13) and HH (n = 8) groups (3.53 ± 1.37 and 2.92 ± 0.76 IU/L, respectively; mean ± SD). In contrast, B-LH of the CD group (2.91 ± 1.23 IU/L) was significantly higher than that of the HH group (1.10 ± 0.45 IU/L) ($p < 0.01$). The difference between the two groups was even more (more than 20-fold) in the F-LH measurements (CD 1.71 ± 0.97 IU/L; HH 0.073 ± 0.04 IU/L, $p < 0.01$).

The maximal GnRH-stimulated levels of I-LH, B-LH and F-LH remained low, less than 10 IU/L, in seven of the eight subjects of the HH group (in all eight when measured by F-LH), but increased clearly in all subjects of the CD group (B-LH more than 15 IU/L, I-LH and F-LH more than 10 IU/L) (Fig. 1*B*). The difference between low and high responses was greatest in the bioactivities, and only one of the HH subjects had a response reaching the range of the CD group. The F-LH assay was the only method where no overlap existed between stimulated levels of the two groups. The I-LH measurements were clearly worse in this respect, because both the low CD and the high HH

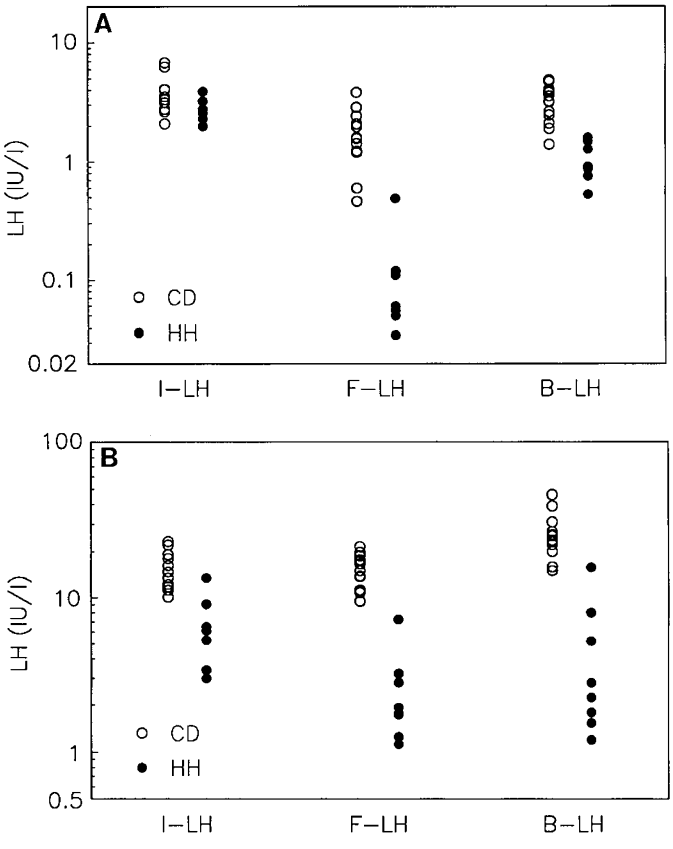

Fig. 1. Basal (*A*) and maximally GnRH-stimulated (*B*) levels of LH measured by I-LH, highly sensitive F-LH, and *in vitro* B-LH in 13 patients with CD (*open symbols*) and eight patients with HH (*closed symbols*).

responses to GnRH overlapped at a concentration of about 10 IU/L.

Time courses of the compiled LH responses to GnRH stimulation are shown in Figure 2. The increases in B-LH and F-LH were higher than those of I-LH, and the former levels stayed up longer. Clearly higher responses to the releasing hormone stimulation were seen in the CD than HH group in all three measurements. No differences in LH responses were found in either group according to bone age or stage of puberty or within the HH group between the different diagnoses (results not shown).

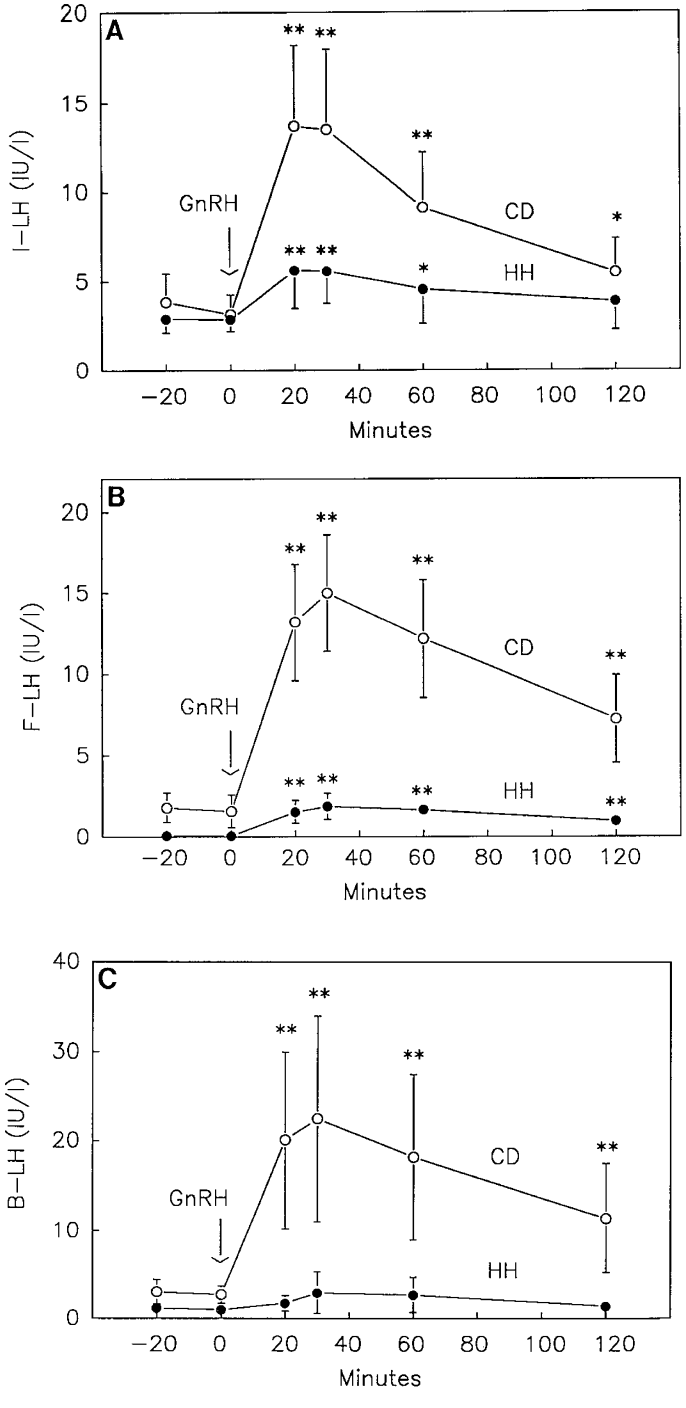

Fig. 2. Compiled LH responses (mean ± SD) of the patients with CD (*open symbols*) and HH (*closed symbols*) to GnRH stimulation. The I-LH levels are *A*, the F-LH levels in *B*, and those measured by *in vitro* B-LH in the *C*. If no error bars are shown, they are within the symbols. The *asterisks* indicate significant changes from the −20 and 0 min levels (* $p < 0.05$; ** $p < 0.01$).

The B/I and B/F ratios of the basal and GnRH-stimulated CD and HH samples are shown in Figure 3. The basal B/I ratio was about 2-fold higher in the CD than HH group (0.90 ± 0.43 *versus* 0.43 ± 0.27, $p < 0.01$), and increased in the former group more than 2-fold after GnRH stimulation ($p < 0.01$). No change was observed after the releasing hormone in the HH group. The B/F ratios were very different. This ratio was basally 1.99 ± 0.65 in the CD group and it was unaffected by the releasing hormone. In the HH group, a very high B/F ratio (about 15) was measured in the basal HH samples. After GnRH, it decreased to a level of about 1.7, and was thereafter indistinguishable for the CD group.

DISCUSSION

Our results clearly demonstrate the benefits of improved assay sensitivity and specificity, as offered by F-LH and B-LH measurements, in discriminating CD and HH. Admittedly, the patient groups studied were somewhat heterogenous in terms of their chronologic age, testis size, and stage of puberty. Especially the larger testis volume in boys with CD suggests that the gonadotropin priming may have been different between the groups already before the time of testing. However, the main point of ours is clear, *i.e.* although the CD and HH groups could not be discriminated by conventional measurements of I-LH, the measurements of B-LH and F-LH brought about clear distinction between them.

The difference between the low and high post-GnRH levels was greater in the B-LH and F-LH than the I-LH measurements. Only one of the eight HH cases showed a B-LH response (and none, when assessed by F-LH) within the CD range. In contrast,

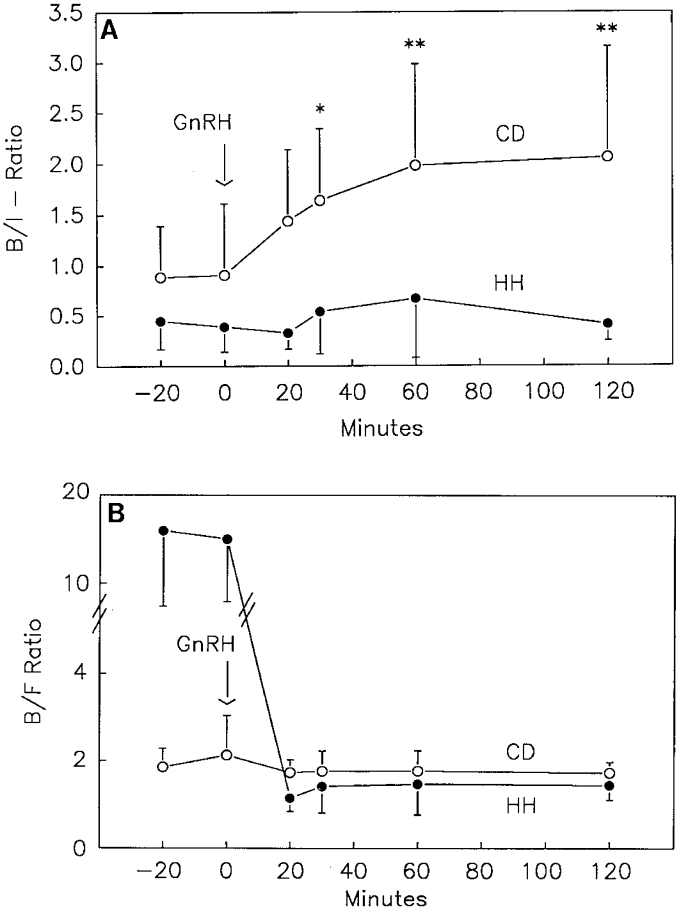

Fig. 3. The B/I (*A*) and B/F (*B*) ratios of the LH levels measured in the CD (*open symbols*) and HH (*closed symbols*) groups during GnRH stimulation (mean ± SD). The *asterisks* in the B/I panel indicate differences from the −20 and 0 min levels (* $p < 0.05$; ** $p < 0.01$).

the I-LH responses of the two groups were much closer. Because the final diagnosis was in each case based on clinical signs and follow-up, the aberrant response found in one subject of the HH group may best highlight the heterogenous nature of this clinical condition. It is of clinical importance that none of the CD patients would have been falsely diagnosed as HH with the present tests, and subjected to unnecessarily long hormone treatment. Such a distinction could not have been done with the I-LH measurements.

The B/I ratio of LH was higher in the CD than the HH group, and it increased during GnRH stimulation only in the former group. The B/I ratio of LH increases also during progression of normal puberty (6–12). The response of the CD group is in agreement with previous findings on increased B/I ratio during stimulation of the adult pituitary by endogenous and exogenous GnRH (14, 23–25). The etiology of HH is heterogenous (26, 27), but a common factor may nevertheless be the lack of sufficient hypothalamic GnRH secretion. In adults, such a condition (*e.g.* after long term treatment with GnRH agonist) is related to decreased B/I ratio of circulating LH (27–29). Inasmuch as the acute GnRH stimulation was able to increase the B/I ratio only in CD, this response may require priming of the pituitary by previous GnRH pulses. Such priming was evidently missing in the HH group.

However, when the F-LH measurements are taken into account, the meaning of the pubertal and post-GnRH increases in the B/I ratios may have to be reevaluated. When the concentrations measured were in the optimal range of B-LH measurements (>1 IU/L), the B/F ratios neither differed between the CD and HH groups nor increased in the CD group after GnRH stimulation. An explanation to the discrepancies between the B/I and B/F ratios is that RIA overestimates low LH levels, because of its low sensitivity and of cross-reactions with free LH subunits and/or other, as yet unidentified, constituents of serum. The F-LH measurements in contrast detect only intact LH molecules with high specificity and sensitivity. Hence, the correlation of B-LH levels is better with F-LH than with I-LH. When the LH levels increase (with advancing puberty or after GnRH), the real increase in immunoreactive LH is greater than that measured by I-LH, hence resulting in erroneous increase of the B/I ratio. In contrast, also the low levels of F-LH are correct and no change in the B/F ratio is seen during GnRH stimulation. In the HH group, the basal B-LH levels are overestimated because of insufficient sensitivity of this assay, and now the B/F ratio decreases when the B-LH levels reach the optimal range of the bioassay after GnRH. Our present data suggest therefore that the meaning of the B/I ratios reported in the past may have to be reevaluated using the specific and sensitive methods of detection of LH immunoreactivity.

In conclusion, our present data demonstrate the potential advantage of B-LH and F-LH measurements in the differential diagnostics of delayed puberty in boys. In addition, our results point out a possible problem in the calculations of the bio/immuno ratios using conventional immunoassay methods.

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