Monoclonal Antibody-Based Discrepancies between Two-Site Immunometric Tests for Lutropin

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We compared five two-site immunometric assays (including three commercially available kits) for measuring lutropin (LH) in serum. Four of the assays involved monoclonal antibodies directed against the alpha–beta dimer, intact LH; these assays measured significantly lower concentrations of LH in 19 (out of 83) samples than did a commercial method not involving such antibodies. In five serum samples, two of the intact LH assays failed to detect any significant immunoreactivity above the detection limit. Findings of normal in vitro LH bioactivity in these samples did not confirm the low immunoreactivity of the intact LH assays. The inability of these assays to detect bioactive LH creates confusion in daily, routine testing as well as in research monitoring of bio/immuno ratios. The data presented here confirm our previous findings (Clin Chem 1991;37:333–40) and emphasize the need to avoid the use of monoclonal antibodies specific for the intact LH dimer.

The use of monoclonal antibodies in diagnostic applications increased dramatically during the 1980s. Monoclonal antibodies are particularly well suited for implementing the potentials inherent in a two-site immunometric assay design (e.g., sensitivity, specificity, range, and kinetics). Doubts have been raised, however, about whether a monoclonal antibody may be "too specific" and thus miss some subtype(s) of an antigen present in multiple forms (1, 2). In a recent paper (3), we showed that different two-site immunometric assays involving one or both of two anti-lutropin (lutenizing hormone, LH) monoclonal antibodies underestimated the LH content in about 25% of samples from healthy individuals, compared with results from assays not involving these two antibodies.3 These antibodies have low cross-reactivity with human chorionic gonadotropin (hCG), and recognize only the intact LH dimer, not the free subunits. Furthermore, the assays based on one of the "intact specific" antibodies could detect no LH in one individual, a 31-year-old healthy mother of two children.

Today, several immunometric LH assays, claimed to be highly specific for LH and unaffected by hCG, have become commercially available. Some of these reportedly make use of monoclonal antibodies that recognize only the intact LH dimer. In this study, we report the results of comparing two such LH tests with a commercially available immunofluorometric assay (IFMA) of LH (4) and two inhouse IFMAs involving two intact-specific monoclonal antibodies as described earlier (3). We also measured the in vitro biological activity of LH in selected samples.

Materials and Methods

All assays are calibrated against the 1st International Reference Preparation (IRP) 88/40, human pituitary LH for immuonassay. All determinations were performed in duplicate, according to the manufacturers’ instructions.

Commercially Available LH Immunoassays

Amerlite LH-30 (Amersham International plc, Amersham, Bucks., U.K.): This assay makes use of an antibody-Whole-LH monoclonal antibody on a solid phase and a peroxidase (EC 1.11.1.7) labeled anti-beta-LH monoclonal antibody as tracer antibody. A 50-μL sample is incubated in one step for 30 min at 37 °C. The detection limit (2 SD above the mean for the zero standard) is <0.12 IU/L and the standard range extends from 2

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3 Nonstandard abbreviations: LH, lutropin; hCG, human chorionic gonadotropin; IFMA, immunofluorometric assay; IRP, International Reference Preparation; NR, normal ratio results; LR, low ratio; and 0R, zero ratio.
to 200 int. units/L. The percentage of cross-reaction with hCG (int. unit/int. unit x 100) is <0.0003. The detection system is based on enhanced chemiluminescence (5).

Enzymun-Test® LH (Boehringer Mannheim, Mannheim, F.R.G.): This kit involves a monoclonal capture antibody directed against a conformational epitope present only in whole LH. As tracer antibody, a Fab'-fragmented monoclonal antibody against an epitope on the beta subunit is used. The tracer antibody is labeled with horseradish peroxidase. A 100-μL sample is incubated in a single incubation for 2 h at 25 °C. The detection limit (3 SD) is <0.5 int. unit/L and the standard range extends from 2 to 157 int. units/L. The addition of hCG at 10 000 int. units/L (1st IRP 75/537) is claimed to produce no detectable response. Detection is by photometry.

Delfia® LHspec (Pharmacia Wallac, Turku, Finland): This kit, recently described (4), makes use of an anti-beta-LH monoclonal capture antibody and a Eu-labeled anti-beta-LH monoclonal antibody as tracer antibody. A 25-μL sample is incubated for 45 min with the solid-phase antibody at room temperature. After a wash step, the tracer antibody is added and the sample is incubated for 15 min at room temperature. The detection limit (2 SD) is <0.05 int. unit/L and the range of standards extends from 0.6 to 250 int. units/L. Cross-reactivity with hCG is <1% (by wt.). Detection is based on the principle of time-resolved fluorescence.

Noncommercial LH Immunocasys

We also used two IFMAs described recently (3). For capture antibodies, both assays involve use of monoclonal antibodies (I1 and I3, respectively) directed against epitopes present only in the intact LH and not in the free subunits. As tracer antibody, an alpha-subunit-specific monoclonal antibody (A2) was used. Both assays are based on time-resolved fluorescence, and the practical performance is identical to that of Delfia LHspec, except that incubation times are 90 + 30 min. Detection limits (2 SD) were <0.05 int. unit/L. Cross-reactivity (by wt.) with hCG was 1.7% (I1/A2) and 0.1% (I3/A2), respectively.

Bioassay

An in vitro bioassay based on the testosterone response of mouse interstitial cells to LH, as described by Van Damme et al. (6) and modified by Ding and Huhtaniemi (7), was used to measure the concentrations of bioactive LH in serum samples. The determinations were performed in triplicate against a standard dose–response curve (1st IRP 68/40). The detection limit of the assay was <0.5 int. unit/L. Each determination was performed in triplicate at two dilutions (five-, 10-, or 30-fold).

Serum Specimens

We obtained 83 samples from normal healthy individuals, 56 women (ages 22 to 76 years) and 28 men (ages 22 to 56). The endocrinological status of most of the subjects was not studied. Included in the comparison were samples from the premenopausal (31 years old) woman referred to above, whose LH was not detected by the I3/A2 IFMA, and a sample from her mother. All samples were tested for the presence of hCG by using the Delfia hCG (Pharmacia Wallac) assay to exclude pregnancy and other causes of increased concentrations of hCG. Because the detection limit of the Enzymun-Test LH was 0.5 int. unit/L, only samples with LH concentrations >1 int. unit/L as measured by Delfia LHspec were included in the comparison. Because of shortages of serum samples, only 23 samples were assayed with Enzymun-Test LH.

Results

The concentrations of LH measured by the five immunometric LH assays are presented in Figure 1. The results are given as the ratios between results by each method to those by the Delfia LHspec plotted against the results by the Delfia assay. On the basis of the clear separation of three groups of ratios by the I3/A2 IFMA in panel A, we divided the samples into 64 normal-ratio (NR) samples, 14 low-ratio (LR) samples, and five zero-ratio (OR) samples. With I1/A2 (panel B), a similar discrimination was seen, but with the five OR samples in panel A now being clearly measurable, although at only 20-38% of the Delfia LHspec value. In the comparison with Enzymun-Test LH (eight NR, 10 LR, and five OR samples), the LH concentrations in the LR samples were significantly lower than in the NR samples (P<0.05, and significantly higher than in the OR samples (P<0.001). Although the Amerlite LH-30 assay (panel D)
did not produce a separation of the three categories as shown in panels A and B, the differences between the Amerlite NR and LR samples on the one hand, and LR and 0R samples on the other, were highly significant (P < 0.001).

Table 1 presents the LH concentrations measured in the five 0R subjects, all women, by all five immunometric assays and the bioassay. The concentrations of LH measured by the I3/A2 IFMA and Enzymun-Test LH are below or very close to the analytical detection limit of the respective tests. LH concentrations measured by the I1/A2 IFMA were 20–38% of the Delfia LH_{spec} concentrations. With the Amerlite LH-30 assay, the corresponding percentage was 5.2–22%. The in vitro bioactivity was measured in four of the samples, with bio/Delfia LH_{spec} ratios ranging from 1.6 to 5.6.

**Discussion**

The results reported in this comparison of LH assays clearly demonstrate that the four two-site IFMAs involving antibodies specific for the intact LH grossly underestimate the LH content of certain individuals. The results are in agreement with the expectations from our previous work (3), in which we distinguished two populations with the I1/A2 and I3/A2 IFMAs by comparison with IFMAs involving only beta- and alpha-specific monoclonal antibodies (beta–beta or beta–alpha combinations). We discriminated between the two populations (NR and LR, identified by the ratio of the I3/A2 IFMA to Delfia LH_{spec}) by using the ratio of the Enzymun-Test LH results and Amerlite LH-30 results, respectively, to those of Delfia LH_{spec}. The separation, although statistically significant (P < 0.05 to <0.001), was not as marked as for the I3/A2 IFMA. This, however, could be a consequence of the respective beta-specific monoclonal antibodies used in combination with the intact specific antibodies.

Five individuals, all women, had concentrations of LH that either were not detectable or were close to the detection limit when assayed with the I3/A2 IFMA. The results obtained with Delfia LH_{spec} were within the reference values (the exact endocrinological status was not known for all cases). By the I1/A2 IFMA, these samples were clearly measurable, but low compared with the results obtained by Delfia LH_{spec}. Measurement with Enzymun-Test LH gave results similar to those by the I3/A2 IFMA, i.e., close to or below the detection limit (0.5 int. unit/L) of the assay. The result obtained with the Amerlite LH-30 assay, on the other hand, resembled more the I1/A2 IFMA because the five samples were well above the detection limit but low in relation to Delfia LH_{spec}. Previously we compared the Delfia LH_{spec} with another commercially available immunoradiometric assay (Miaclone; Serono Diagnostics, Coisinsa, Switzerland), which codetects free beta subunits (4). As could be predicted from our results presented here and earlier (3), the correlation between these two assays was excellent, with no evidence for the separation into NR and LR populations documented in the present study for use of immunometric assays involving monoclonal antibodies recognizing only the intact LH molecule.

The in vitro biological activity was measured in four 0R samples. The bio/immuno ratios calculated from the results in Table 1 for Delfia LH_{spec} ranged from 1.6 to 5.6 (mean 3.1), ratios close to bio/immuno ratios published earlier (8) for sera from the follicular phase measured with the same methods. The apparent bio/immuno ratios for the Amerlite LH-30 test ranged from 7.3 to 38 (mean 18.7), and for the I1/A2 IFMA from 4.2 to 12.5 (mean 8.4). The I3/A2 IFMA and Enzymun-Test LH, with immunoassay values near or below the detection limits, gave apparent bio/immuno ratios of >400 and >23, respectively. Based on these measurements, highly confusing results can be expected in healthy individuals because of artifactually low measurements of the LH immunoreactivity. The apparently high proportion of LR individuals, —25% (3), would also result in unduly wide reference limits for LH as well as bio/immuno ratios.

In a recent study (9), a more detailed characterization of the LH from the 0R premenopausal woman, subject 1 in Table 1, was undertaken. Nothing in her endocrinological status indicated anything unusual, and her fertility was proven by two pregnancies. Interestingly, her mother (subject 2 in Table 1) was also a 0R individual. The father and three siblings (two sisters and one brother) of subject 1, as well as her two children, were all LR individuals, clearly indicating an autosomal

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Table 1. Serum LH Concentrations of Five Zero-Ratio* Women

<table>
<thead>
<tr>
<th>Subject</th>
<th>I3/A2</th>
<th>I1/A2</th>
<th>Delfia LH_{spec}</th>
<th>Enzymun-Test LH</th>
<th>Amerlite LH-30</th>
<th>In vitro LH bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;0.05</td>
<td>1.28</td>
<td>4.3</td>
<td>0.7</td>
<td>0.42</td>
<td>24.0</td>
</tr>
<tr>
<td>2b</td>
<td>0.07</td>
<td>7.95</td>
<td>25.6</td>
<td>&lt;0.5</td>
<td>5.2</td>
<td>56.3</td>
</tr>
<tr>
<td>3</td>
<td>0.07</td>
<td>2.91</td>
<td>8.9</td>
<td>&lt;0.5</td>
<td>1.5</td>
<td>28.0</td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.05</td>
<td>16.2</td>
<td>42.4</td>
<td>0.7</td>
<td>9.4</td>
<td>68.8</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.05</td>
<td>0.53</td>
<td>2.7</td>
<td>&lt;0.5</td>
<td>0.14</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Ratio of I3/A2 IFMA to Delfia LH_{spec} <0.01.

b Postmenopausal mother of subject 1; all other subjects were premenopausal.

N.D., not determined.
pattern of inheritance of this immunological LH variant.

The Delfia LH\textsuperscript{spec} used in this work as the comparison method recognizes intact LH as well as free beta subunits. The discrepancies seen between this method and the four intact LH IFMAs cannot be explained by this difference in specificity, as has been shown previously (3, 4) through comparisons with immunometric assays based on beta-specific capture antibodies with alpha-specific tracer antibodies. Determination of the LH concentrations of the 83 samples in this study with an IFMA involving a beta subunit-specific capture antibody and an alpha subunit-specific tracer antibody (B1/A2 in reference 3) also gave results highly similar to those presented in Figure 1 with the Delfia LH\textsuperscript{spec} (data not shown). In addition, free beta subunits would not produce a response in the in vitro bioassay.

The reason for the inability of certain anti-LH monoclonal antibodies to detect the LH of some individuals is not known. In the previous study (3), we suggested a conformational change covering a larger area of the alpha-beta interface. The consistency of the phenomenon in samples obtained from the same individuals during the menstrual cycle, and the fact that two separate populations can be distinguished, suggest a genetic origin. This conclusion is further strengthened by the result reported here, where a mother and her daughter were both found to be OR individuals. We have recently found (unpublished) that of 32 commercially available anti-LH monoclonal antibodies tested, all monoclonal antibodies classified as intact specific (n = 12) showed restricted reactivity to LH in LR and OR individuals, albeit to different degrees.

The results reported here, together with previous results (3), strongly emphasize the pitfalls in the use of monoclonal antibodies that recognize the intact LH and not the free subunits, however specific these may be in discriminating between LH and hCG. Because these antibodies poorly detect LH variants possessing normal bioactivity, their use is certain to cause confusing situations both in routine endocrinological investigations and in research studies where bio/immuno ratios are monitored.

References