NOTE

A New Radioimmunoassay for Human Chorionic Gonadotropin Using Monoclonal Antibody

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Abstract

A new radioimmunoassay (RIA) for human Chorionic Gonadotropin (hCG) was developed using murine monoclonal antibody to the β-subunit of hCG (β-hCG). The IgG fraction of the monoclonal antibody which did not react with 125I-β-hCG was purified from hybridoma ascites, and covalently coupled to Sepharose 4B. This solid-phase antibody was incubated with standard hCG or serum sampled for 48 hours. The reaction medium was then removed by centrifugation and 125I-β-hCG and anti-β-hCG rabbit polyclonal antibody were added to the precipitate. The alcohol precipitation method was used for separating "bound" and "free" forms in the second reaction.

The sensitivity for hCG in this assay system was 0.5 mIU/ml serum and the cross-reactivity with human Luteinizing Hormone (hLH) was 0.4%. This assay system was shown to be clinically applicable. Serial serum samples from two patients with trophoblastic disease were assayed and minute amounts of hCG, which could not be determined by conventional assay methods, could be assayed by this new RIA.

Monitoring of human chorionic gonadotropin (hCG) is an important element in the successful management of patients with trophoblastic diseases and a highly sensitive assay is mandatory for precise documentation of the status of the disease, of the need to change therapy, the timing of cessation of therapy and follow-up of remission (Tomoda Y. et al., 1977). The utility of RIA or hemagglutination reaction (HAR) techniques, widely used for hCG assay up until now, has been limited because of immunological cross reaction between hCG and human luteinizing hormone (hLH) (Tomoda Y. and Hreshchyshyn M. M., 1968; Wilde C. E. et al., 1965).

In the meanwhile, Vaitukaitis et al. developed a RIA method using specific antisera to β-hCG which has been widely used clinically. Although this method exhibits substantially reduced hLH cross-reactivity, there is still 10% cross-reactivity and the sensitivity of the assay is limited to about 5 mIU/ml (Tomoda Y. et al., 1977; Vaitukaitis et al., 1972; Vaitukaitis et al., 1971). Since, in patients with trophoblastic diseases,
there is a close correlation between the level of hCG and the status of the disease, a highly sensitive and specific hCG assay, with minimum cross-reactivity between hCG and hLH, would have greater utility in detecting the true negative level of hCG on which one could base the very important judgement of remission and the critical decision on timing of cessation of treatment (Tomoda Y. et al., 1977). Recently a highly sensitive and specific immunoassay for hCG has been introduced in which antiserum to the synthetic peptide analogue corresponding to the C-terminal region of the β-hCG has been shown to exhibit considerably reduced cross-reactivity between hCG and hLH (Furuhashi Y. et al., 1982; Matsuura S. et al., 1978; Morgan F. J. et al., 1975). Difficulties in preparation of high titer antiserum have limited the clinical application of this method.

In 1975, Milstein and Koehler (1975) reported the method of monoclonal antibody preparation by cell fusion. Monoclonal antibodies are characterized by their homogeneity and reproducibility (Gametshu B. and Harnson R. W., 1984; Kennet R. H. et al., 1980; McMichael A. J. and Fabre J. W. 1982; Mitchell M. S. and Oettgen H. F. 1982; Moyle W. R. et al., 1982). Taking into consideration the fact that high affinity monospecific antibodies can be applied for a highly sensitive immunoassay of hCG (Ridgway E. C. et al., 1982; Stuart M. C. et al., 1982; White A. et al., 1982; Armstrong E. G. et al., 1984), we prepared monoclonal antibody to β-hCG and we developed a new and substantial specific assay method.

Materials and Methods

1. Preparation of solid-phase monoclonal antibody

Murine monoclonal antibody (4G2) to β-hCG prepared by Furuhashi et al., was used. The cross-reactivity between this monoclonal antibody and hLH was approximately 3% when determined by the sandwich-type enzyme immunoassay method using anti-hCG rabbit IgG-beta galactosidase complex and the solid phase of this monoclonal antibody (Furuhashi Y et al., 1983). Moreover, this monoclonal antibody, obtained by chance, was so unique that it did not combine with [125I] β-hCG labeled by the chloramin-T method (Fig. 1).

The monoclonal antibody was purified by the method described by Ey et al., using protein-A affinity chromatography: Hybridoma ascites was dialyzed against 0.14M phosphate buffer (pH 8) and the dialysate was charged on a protein-A sepharose 4B column (Pharmacia) which was then eluted with 0.1 M citrate buffer (pH 5.8) and the IgG fraction was collected (Ey PL et al., 1978). The IgG fraction, 1 mg, was applied to 1 ml of CNBr-Sepharose
4B gel forming covalently bound solid-phase monoclonal antibody (Porath J et al., 1967).

2. Standard hormone preparations

The second International Standard hCG, provided by the National Institute of Medical Research (Mill Hill, London), was used as standard hCG. For standard hLH, AFP-4345-β (immunoactivity, 12583 IU/mg) was kindly supplied by NIH. Pooled sera obtained from numerous healthy male subjects was used for the preparation of a standard calibration curve.

3. Adsorption rate

For the purpose of examining the rate of absorption of standard hormone preparation, hCG and hLH were diluted with PBS (0.01 M phosphate buffer, pH 6.4, containing 0.14 M NaCl) containing 0.5% bovine serum albumin (BSA) to make solutions of 25, 50 and 100 mIU/l, respectively.

Two milliliters of each solution were reacted with 0.02 ml of the abovementioned solid-phase monoclonal antibody at 4°C for 48 hours, then centrifuged at 2000 rpm for 5 minutes. The hCG content of the supernatant was assayed with a standard calibration curve which had been prepared by stepwise dilution of hCG in an hCG-RIA system using anti-hCG antibody. Similarly, the hLH content of the supernatant was assayed with a standard calibration curve prepared by stepwise dilution of hLH in the same assay system as above (Tomoda Y and Hreshchyshyn MM, 1968).

4. Anti β-hCG serum

The anti β-hCG rabbit polyclonal antibody (prepared by immunizing rabbits with highly purified β-hCG) was supplied by Teikoku Zoki Company Ltd., Tokyo (Lot No. 1762-2). The cross-reactivity between this antibody and hLH was 10% by the homologous β-hCG RIA (Fig. 2).

5. Preparation of [125I] β-hCG

5 μg of β-hCG (NIH-CR 115 B) was mixed with 1 mCi of Na[125I] and iodination was performed according to the modified method described by Greenwood et al. (1962) using chloramin-T. The free 125I was removed on Sephadex G-25 gel. The specific activity of [125I] β-hCG prepared in this manner was 50 μCl/μg.

6. Assay procedure

0.02 ml/tube of the solid-phase monoclonal antibody was placed into a series of plastic test tubes, and then 2 ml of standard hCG dissolved in the sera obtained from numerous healthy male subjects, or serum samples were added to the tubes. The mixtures were incubated at 4°C for 48 hours with shaking. After centrifugation (2000 rpm for 5 min), the precipitate was washed twice with 2 ml of PBS (0.01 M phosphate buffer, pH 6.4, containing 0.14 M NaCl), after which 0.2 ml of the anti β-hCG antibody and 0.2 ml of [125I] β-hCG were added and the mixture was incubated for competitive reaction at 4°C.

After 72 hrs of incubation, 1.6ml of 100% ethanol containing 10% ammonium acetate (Tomoda Y. and Hreshchyshyn M. M., 1968) was added and the mixture was incubated at 4°C for 1 hr and centrifuged at 3000 rpm for 15 minutes to separate “bound” and “free” forms. Then, using 2 ml of 100% ethanol, the precipitate was washed and centrifuged and the radioactivity of the resulting pellet was determined with an autowell.
gamma counter (Aloka ARC-503) (Fig. 3).

7. Samples
Samples included sera from 76 non-pregnant healthy female subjects and 80 patients with trophoblastic diseases in the remission stage and 2 patients under follow-up. Serum samples, prepared by immediate centrifugation of the collected blood, were all carefully stored at a constant stable temperature of $-20^\circ\text{C}$ until use.

8. Reproducibility
In order to examine the reproducibility of the present assay method, two frozen samples were subjected to within-assay and between-assay. In addition, 24 frozen samples were subjected to correlation between the present assay method and RIA using anti $\beta$-hCG antibody. The accuracy of the assay system was verified by parallelism and recovery tests.

9. Assay of serum hLH
Serum hLH levels in non-pregnant healthy female subjects were assayed by a RIA method using anti-hCG rabbit polyclonal antibody and $[^{125}\text{I}]$ hCG (Tomoda Y. and Hreshchyshyn M.M., 1968).

Results
The rate of adsorption of a standard hormone preparation on the solid-phase monoclonal antibody was greater than 84.8%, as shown in Table 1, at any hCG level used in the present experiment, but the rate of adsorption of hLH was as low as 1.7–14%. The Standard curve of hCG in the present study and its cross-reactivity with hLH are shown in Fig. 4. The curve was obtained for hCG levels between 0.38 and 100 mIU/ml. Cross-reactivity between hCG and hLH was found to be 0.4% and the sensitivity was set at 0.5 mIU/ml (Table 2). Reproducibility of the present hCG assay method is shown in Table 3. Assay precision was 5.6–12.9% and 6.6–9.4% for within-assay
Table 1. Adsorption of hCG and hLH to monoclonal antibody-coupled Sepharose 4B

<table>
<thead>
<tr>
<th>Incubated Gonadotropins (A) mIU/ml</th>
<th>Supernatant (B) mIU/ml</th>
<th>Adsorbed Gonadotropins (A - B)/A mIU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG 100</td>
<td>8.0±2.8</td>
<td>92.0±2.8</td>
</tr>
<tr>
<td>50</td>
<td>6.3±1.5</td>
<td>87.4±3.0</td>
</tr>
<tr>
<td>25</td>
<td>&lt;3.8</td>
<td>&gt;84.8</td>
</tr>
<tr>
<td>hLH 100</td>
<td>98.3±1.5</td>
<td>1.7±1.5</td>
</tr>
<tr>
<td>50</td>
<td>47.9±3.0</td>
<td>4.2±6.0</td>
</tr>
<tr>
<td>25</td>
<td>21.5±1.1</td>
<td>14.0±4.4</td>
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</tbody>
</table>

Table 2. Comparison of cross-reactivity and sensitivity in RIA using conventional antiserum to β-hCG and new hCG-RIA

<table>
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<tr>
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<th>RIA with conventional antiserum</th>
<th>New RIA</th>
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<tbody>
<tr>
<td>Cross-reactivity with hLH</td>
<td>9.3%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>5 mIU/ml</td>
<td>0.5 mIU/ml</td>
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Table 3. Reproducibility of hCG assay in serum

<table>
<thead>
<tr>
<th>Within-assay</th>
<th>Between-assay</th>
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<tbody>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>mean±SD (mIU/ml)</td>
<td>mean±SD (mIU/ml)</td>
</tr>
<tr>
<td>Sample A</td>
<td>5.57±0.72</td>
</tr>
<tr>
<td>Sample B</td>
<td>22.98±1.30</td>
</tr>
</tbody>
</table>

and between-assay, respectively. Correlation between the present assay method and RIA using anti β-hCG antibody was 0.857 (γ). The result of recovery experiment was 89.2–109.2% (n=7, mean±SD=98.18±8.29). Dilution curves of all samples were about a straight line through the origin of the coordinate axes (Fig. 5).

Serum hCG levels in 76 non-pregnant healthy female subjects, as determined by the present method, are shown on the ordinate in Fig. 6. Serum hLH levels in the same samples, determined by an RIA method using anti-hCG rabbit polyclonal antibody, are shown on the abscissa. Levels of hCG in the 6 subjects with high titers of hLH were 0.5 to 1.5 mIU/ml, but hCG levels in the remaining 70 subjects (92.1%) were well short of 0.5 mIU/ml. Also, the levels of hCG in 72 out of 80 (90.0%) trophoblastic patients in the remission stage were less than 0.5 mIU/ml.

The following two patients, who had been hospitalized at the Department of Obstetrics and Gynecology, Nagoya University School of Medicine, and followed up after discharge, were screened by the present assay method. The first case was a 45-year-old patient with trophoblastic disease who had undergone
total hysterectomy. After 4 courses of chemotherapy with Methotrexate, the hCG level was lower than 5 mIU/ml as determined by RIA using β-hCG (Fig. 7), but, as shown by the dotted line in the same figure, hCG measured by the present assay method still showed levels of 2.4 and 1.2 mIU/ml. After as many as 6 courses of chemotherapy it dropped below 0.5 mIU/ml and this level was maintained unchanged when determined twice after that. The second case was also a 36-year-old patient with trophoblastic disease. Postchemotherapy hCG levels, determined by RIA using anti β-hCG antibody, were lower than 5 mIU/ml, but, by using the present assay method, lower levels of hCG could be assayed (Fig. 8).

Discussion

A New RIA for hCG was developed using murine monoclonal antibody to β-hCG. The sensitivity for hCG in this assay system was 0.5 mIU/ml serum and the cross-reactivity between hCG and hLH was 0.4%.

The most characteristic feature of the present method of hCG assay is the application in the assay system of and-β-hCG monoclonal antibody, obtained by chance, which specifically binds with hCG and β-hCG and has cross-reactivity between hCG and LH of less than 3% by EIA. Another unique characteristic of this anti-β-hCG monoclonal antibody is that it does not react with 125I-β-hCG as confirmed by RIA (Furuhashi Y. et al., 1983). This interesting fact suggests that some epitopes expressed on β-hCG (Sieigfried S. et al., 1986) were modified by the iodination technique and lost affinity for the antibody. Thus, in the present assay method, the solid phase monoclonal antibody permitted specific concentration and
Fig. 7. Case 1.
45-year-old patient with trophoblastic disease who had undergone total hysterectomy, after 4 courses of chemotherapy.
- - - - -: Hemagglutination assay using anti-hCG rabbit serum
△-△-△: Radioimmunoassay with anti-β-hCG rabbit serum
○-○-○: New radioimmunoassay

Fig. 8. Case 2.
36-year-old patient with trophoblastic disease
adsorption of hCG in the sample without reacting with $^{125}$I-β-hCG used in the competitive reaction in the second step.

This assay system is based on the following two separate steps: in the first step the use of anti-β-hCG monoclonal antibody as solid phase antibody permitted specific adsorption and concentration of hCG in the sample (2 ml of serum). In the second step, assay of hCG adsorbed on solid phase antibody in a competitive RIA system using highly specific conventional anti-β-hCG antibody, which has hLH cross-reactivity of less than 10%, further increased the sensitivity of this assay method. The results obtained support the fact that the sensitivity of this assay method, in which 2 ml of serum sample is used, should theoretically be ten times as high as the conventional anti-β-hCG antibody RIA, in which 0.2 ml of serum sample is used.

The microassay of hCG, described in the literature up to now, employs the method of concentrating hCG from a large volume of samples. Apart from the fact that complicated procedures make this method less practical for routine hCG assay, the chemical reactions involved bear the disadvantage of inevitable concomitant extraction and concentration of glycoprotein hormones similar to hCG, making it impossible for hCG alone to be selectively concentrated and extracted (Ayala AR, 1978). In the present method, however, the use of monoclonal antibody specific to hCG helped further increase the specificity of the assay at the concentration step.

Many authors have reported various immunoassay methods using solid-phase antibody (Schuurs A. H. W. M. and Van Weemen B. K., 1977), but the fact that the use of Sepharose, as a solid phase antibody, markedly increases the nonspecific adsorption has resulted in many failures in attempts to increase the sensitivity of hCG assay (Van Weemen B. K. and Schuurs A. H. W. M., 1974). In an attempt to increase the rate of hCG adsorption, we used Sepharose 4B as a solid phase antibody (Armstrong E. G. et al., 1984).

We believe that our application of alcohol precipitation for separation of the bound and free hCG after the second reaction might have played an important role in increasing the efficiency of this assay method. According to Tomoda et al. (1968), good solubility of $[^{125}$I] β-hCG in 6% ammonium acetate in 60% ethanol may have been an important factor in decreasing the nonspecific adsorption, and this precipitation procedure contributes to the excellent reproducibility and simplicity of the present assay procedure.

The present method was applied to hCG assay in actual clinical cases. Minute serum hCG levels, which were impossible to detect by conventional assay methods, could be assayed by the present assay method (Fig. 7, 8). However, as shown in Fig. 6, the fact that effects of crossreactivity were seen, even though only slightly, in cases exhibiting high levels of hLH, indicates that the specificity of the monoclonal antibody employed is still not quite satisfactory and that further studies are required to find an even more specific antibody.

This assay system showed a high sensitivity for hCG and little cross-reactivity between hCG and hLH. This method could be limited in clinical application by the extraction procedure and long-time incubation. Further study will be necessary to simplify this assay system.

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References


Van Weemen B. K. and A. H. W. M. Schuurs
