Enzyme Immunoassay of the Glycoprotein Tropic Hormones—Choriogonadotropin, Lutropin, Thyrotropin—with Solid-Phase Monoclonal Antibody for the α-Subunit and Enzyme-Coupled Monoclonal Antibody Specific for the β-Subunit

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Monoclonal antibody technology has made it possible to produce homogeneous populations of antibodies to discrete determinants on an antigen surface. We have produced monoclonal antibodies to the α-subunit and β-subunits of the glycoprotein hormones choriogonadotropin, lutropin, and thyrotropin, and developed two-site simultaneous enzyme-linked immunospecific assays for these hormones. The anti-α-subunit monoclonal antibody was used as the solid-phase (coated tube) capture antibody for all three hormones; the anti-β-subunit monoclonal antibodies were coupled to horseradish peroxidase (EC 1.11.1.7). Cross reactions between the closely related choriogonadotropin and lutropin were apparently greater in this method than in RIA, with use of the same antibodies. Kd of the antibodies did not appear to be as critical to sensitivity of the sandwich assay as it was for RIA. The lower limit of detection was 0.2 μg/L after a 2-h incubation with serum sample at room temperature and a 30-min incubation with enzyme substrate at room temperature after washing away excess enzyme conjugate. Within-assay precision (CV) was very good, <6%.

Additional Keyphrases: enzyme-linked immunospecific assay • peroxidase • pregnancy

The quantitative analysis of biological fluids for the glycoprotein tropic hormones—human choriogonadotropin (hCG), lutropin (luteinizing hormone, LH), follitropin (follicle-stimulating hormone, FSH), and thyrotropin (thyroid-stimulating hormone, TSH)—has been accomplished largely by radioimmunoassay techniques (1). These methods are highly sensitive and can be specific for hCG, FSH, or TSH when anti-β-subunit antisera are used (2, 3). However, RIAs for LH are affected by cross reaction with the closely related structure of hCG (4).

The disadvantages of radioactive materials, namely, disposal of waste and short shelf-life of reagents, have led to the development of enzyme-linked immunospecific assay (ELISA) techniques as an alternative to RIA (5–7). Van Weeman and Schuurs described a competitive ELISA for hCG that involved enzyme-labeled antigen (8). They compared a competitive enzyme immunoassay with the two-site "sandwich" ELISA (9), in which enzyme-labeled antibody is used, and concluded that the competitive assay was 20-fold more sensitive than the sandwich assay. The advantage of the sandwich assay is that the enzyme-labeled antibody can be prepared easily and economically; preparation of enzyme-labeled antigen is costly because of the need for purified antigens from human sources.

By use of the hybridoma technology developed by Milstein and Kohler (10), monoclonal antibodies to the α- and β-subunits of the glycoprotein hormones have been prepared (11, 12). We have incorporated these antibodies into the two-site, sandwich ELISA methodology in developing assays for hCG, TSH, and LH. These assays are similar to the two-site ELISA for α-fetoprotein described by Uotila et al. (13), in that monoclonal antibodies are used. The anti-α-subunit monoclonal antibody, used as the capture antibody, is coated onto polystyrene tubes. Because of the structurally and immunologically similar nature of the α-subunits from the glycoprotein hormones (14), only one solid-phase anti-α-subunit monoclonal antibody is required. The anti-β-subunit monoclonal antibodies, directed against the hormone-specific determinants on the β-subunit, are coupled to horse radish peroxidase (EC 1.11.1.7) and confer specificity to the test systems. Serum-based samples and enzyme–antibody conjugate are incubated simultaneously in the antibody-coated tubes. We use a single wash step to remove excess conjugate before assaying for antigen-bound enzyme activity.

Evaluation of the performance of the hCG, LH, and TSH assays indicates that highly sensitive and reproducible two-site, sandwich ELISAs are possible, based on the use of monoclonal antibodies to the α- and β-subunits. Here we further characterize these assays and discuss their clinical applications.

Materials and Methods

Monoclonal antibodies. The monoclonal antibodies with specificity for the various glycoprotein hormones were produced by stable clones of cell hybrids, isolated from Balb/c mouse spleen cell NS-1 fusions according to Milstein and Kohler (10). The clone producing anti-β-hCG no. 5004 was isolated by Schröder et al. (11). Anti-β-LH no. 0205 was isolated by Federici et al. (12). Anti-β-TSH no. 55G2 and anti-α-TSH no. A57B5 were isolated from the same fusion with TSH immunogen (manuscript in preparation).

We purified the antibodies from ascites fluid harvested from inoculated Balb/c mice by passage through protein A–Sepharose columns (15). The purified monoclonal antibodies were all found to be monoclonal IgG1 by immunoelectrophoresis and by double diffusion in agarose gels with subclass-specific antisera.

Characterization of antibodies by RIA. The purified monoclonal antibodies were evaluated in competitive RIA systems by sequential additions of unlabeled hormone in serum and tracer to antibody, overnight incubation at room temperature, and precipitation with polyethylene glycol 6000, 200 g/L, in place of second antibody (16).
The association constants ($K_a$) were calculated by Scatchard analysis (17) of the data from RIA displacement experiments. Cross reactions of the related hormones in the RIA systems were determined by displacement of labeled hormone by cross-reacting hormone. Percent cross reactivity was calculated from amount of hormone causing half displacement ($D_{50}$) of tracer-labeled hormone on a weight-ratio basis: i.e., $[D_{50} \text{ hCG (ng)/}[D_{50} \text{ LH (ng)}] \times 100 = \text{ percent cross reactivity of LH in hCG assay system.}$

**Coupling of antibodies to horseradish peroxidase.** The purified monoclonal antibodies anti-$\beta$-hCG no. 5004, anti-$\beta$-TSH no. 55G2, and anti-$\beta$-LH no. 0205 were coupled to horseradish peroxidase, type VI (Sigma Chemical Co., St. Louis, MO 63178), by the method of Wilson and Nakane (18), in which sodium periodate oxidation activates the horseradish peroxidase for coupling. The enzyme-coupled antibody was chromatographed on a gel-filtration column, ACA-34 (LKB-Produktor AB, Sweden), in phosphate-buffered saline (PBS; per liter, 10 mmol of Na$_2$PO$_4$ and 9 g of NaCl), pH 7.0, and the eluted fraction containing the conjugate (as determined by enzyme activity) free of uncoupled antibody was pooled and stored at $-20^\circ$C.

**Coating of tubes with anti-$\alpha$-subunit antibody.** Polystyrene tubes, 12 $\times$ 75 mm, were coated with monoclonal anti-$\alpha$-TSH no. A67B5 in phosphate-buffered saline at 1–10 mg/L, according to the procedures described by Parsons (19). Excess coating antibody was removed by several washes with the buffer, and the tubes were stored at 2–8 $^\circ$C, either wet or after drying by desiccation.

**Antigens.** Purified hormones were obtained from Radioassay Systems Labs., Carson, CA 90746. Partly purified hCG was prepared from crude hCG isolated from pregnancy urine by chromatography on Sephadex G-100 (Pharmacia Fine Chemicals AB, Bromma, Sweden). This hCG preparation was calibrated (by RIA and the described ELISA procedure) against the World Health Organization First International Reference Preparation (WHO no. 75/537). The antigens were diluted in horse serum (Irvine Scientific, Irvine, CA 92705), 250 mL/L; in PBS; or in hormone-free human male serum (AMS-Biological and Diagnostics Co., Seguin, TX 78155).

**Two-site, simultaneous sandwich ELISA protocol.** The assay protocols for hCG, TSH, and LH were very similar. The enzyme-conjugate dilution and the incubation times were adjusted to optimize sensitivity and decrease the cross reactivity with the conjugated hormones.

Dilute the enzyme conjugate (anti-$\beta$-subunit for hCG, TSH, or LH) 500- to 2000-fold in conjugate buffer: PBS, pH 7.5, containing 1 g of twice-crystallized bovine serum albumin (Sigma Chemical Co.) and 0.4 mL of Tween-20 (Sigma Chemical Co.) per liter. Add 300 mL of the diluted conjugate to anti-$\alpha$-subunit-coated tubes followed by 25 or 50 mL of serum-based samples. After agitating the tubes, incubate at room temperature for 1–2 h. Remove excess enzyme conjugate by filling and decanting with three 4-mL washes of, per liter, 9 g of NaCl and 0.4 mL of Tween-20. This is most easily done by using a Repipet, to dispense wash solution, and a foam tube rack (Scientific Manufacturing Industries, Emeryville, CA 94608) to hold the tubes for decanting. Allow the wash solution to drain from the tubes, then add 500 mL of freshly prepared substrate solution (K$_2$HPO$_4$, 10 mmol/L, pH 6.0, containing 0.5 g of o-phenylenediamine and 300 mL of hydrogen peroxide, 300 g/L solution, per liter). Allow the enzymatic reaction to proceed at room temperature for 30 min, then terminate the reaction by adding 0.2 mL of 2.5 mol/L H$_2$SO$_4$. Read the endpoint absorbance at 490 nm, using a spectrophotometer equipped with a flow-through cuvette (e.g., Gilford Stasar III; Gilford Instrument Labs. Inc., Oberlin, OH 44074).

Plot the data for reference curves on linear graph paper. A calculator can also be used to store reference curves by using a four-parameter logistic curve-fitting program (20). Regression analysis of the data points for calculated dose response vs experimental values indicated a correlation coefficient ($r$) of 0.999 or better.

**Results and Discussion**

**Preparation of Horseradish Peroxidase—Monoclonal Antibody Conjugates**

We found the peridode method of Wilson and Nakane (18) to be effective for coupling horseradish peroxidase and monoclonal antibodies. Suitable enzyme/antibody molar ratios were estimated as 1:1 to 2:1 by $A_{490}$ and $A_{280}$ measurements. As noted by Wilson and Nakane, lower ratios gave conjugates with low sensitivity and higher ratios could produce high backgrounds.

We also noted that the optimal coupling conditions varied from one monoclonal antibody to another, probably because of the different physical properties of the antibodies as displayed by their varied electrophoretic mobilities in immunoelectrophoresis.

**Two-Site Sandwich Assays**

**Adjustment of enzyme-conjugate dilution.** When hCG and anti-$\beta$-hCG enzyme conjugate were simultaneously incubated in anti-$\alpha$-subunit-coated tubes, a "sandwich" formed between the solid-phase anti-$\alpha$-subunit, the hCG molecule, and the anti-$\beta$-enzyme conjugate. The amount of conjugate bound to the tube well depended on the concentration of hormone, the concentration of enzyme conjugate, and the incubation time in the coated tube. By adjusting the dilution of enzyme conjugate, we adjusted the sensitivity of the assay. Figure 1 shows a titration of enzyme conjugate for two concentrations of hCG and a zero calibrator (50 mL sample volume). By selecting a dilution such that the $A_{490}$ was 1.0 to 1.5 for an assay having a value of 100 int. units/L (2-h incubation), we obtained a highly sensitive assay having a low background for the zero calibrator and an upper limit of 200 int. units/L ($A_{490} < 3.0$) for the assay range.

Figure 2 shows the time course of an experiment in which the incubation time is varied from 0.5 to 24 h at room temperature. At the various concentrations of antigen used, the sandwich formation reaction followed pseudo first-order reaction kinetics for about the first 8 h. Correlation coefficients

**Fig. 1. Titration of enzyme conjugate**

Two concentrations of hCG (50 (O) and 100 (B) int. units/L, and a zero calibrator (A) were tested as described in Materials and Methods with 250- to 2000-fold diluted anti-$\beta$-hCG/ enzyme conjugate dilutions. Sample volume, 50 mL; 2-h incubation with sample.
obtained from linear regression analysis of the data (hCG, 25 to 100 int. units/L) were 0.990 to 0.999 for the first-order rate equation, \( k_t = \ln(1/1 - y) \). Comparisons between equilibrium assays for hCG incubated for 18 h and kinetic assays incubated for 2 h indicated that although absorbance values increased, other performance characteristics such as detection limit and coefficient of variation were similar. Thus we chose the 2-h incubation period.

The TSH and LH assays were similarly optimized. The TSH–enzyme conjugate was adjusted to give approximately 1.5 \( A_{490} \) at 100 milli-int. units of TSH per liter in a 2-h incubation. The LH–enzyme conjugate was adjusted to give approximately 1.0 \( A_{490} \) at 160 int. units of LH per liter in a 1-h incubation.

**Assay performance characteristics.** A representative standard curve for the hCG assay in serum-based media is presented in Figure 3. The curve appears to be linear between 0 and 10 \( \mu \)g/L, then begins to plateau. Analysis with curve-fitting programs indicated that the best fit for the experimental points was achieved by using a four-parameter logistic curve-fitting program as described by Rodbard (21, 22). Use of linear regression to evaluate the accuracy of the four-parameter logistic curve fit for the hCG standard curve yielded a correlation coefficient of 0.999 or better. These results were analogous to the immunoradiometric sandwich assay (21), for which logistic curve fitting has been empirically demonstrated to be applicable (22).

Similar standard curves were obtained for the TSH and LH assays (Figures 4 and 5).

The sensitivities of these assays were characterized by using 10 replicates of a zero calibrator to calculate the 2 SD range of the negative sample. The lower limit of detection was 0.2 \( \mu \)g/L (1 int. unit/L) for hCG, 0.2 \( \mu \)g/L (1 milli-int. unit/L) for TSH, and 0.5 \( \mu \)g/L (4 int. units/L) for LH.

Comparison of the sensitivities of the sandwich ELISA methods with the competitive RIAs for the respective hor- mones, with use of the monoclonal antibodies in both assays, indicated the highest affinity antibody in competitive RIA, anti-\( \beta \)-hCG (see Table 1), gave a highly sensitive ELISA, while the lower-affinity (by RIA) anti-\( \beta \)-TSH gave an ELISA as sensitive as the ELISA for hCG. This result for TSH may be explained by the presence of excess anti-\( \beta \)-TSH enzyme conjugate, which appears to compensate for lower antibody affinity. Therefore, the specificity of the monoclonal antibody may be more relevant than antibody affinity in determining the performance of simultaneous sandwich assays. The lower

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**Fig. 2. Kinetics of "sandwich" formation**

For hCG standard curve calibrators, the incubation time of sample (50 \( \mu \)L) with 1000-fold diluted enzyme conjugate in anti-\( \alpha \)-subunit-coated tubes was varied from 0.5 to 24 h at 22 °C. Nos. for each line indicate hCG concentration (int. units/L).

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**Fig. 3. Standard curve for hCG and cross reactivity with LH, FSH, and TSH**

Calibrators for the hCG assay were tested by using 50-\( \mu \)L sample volume and 2-h sample incubation (Figures 3-5). The hCG potency was 5 milli-int. units/ng, yielding an assay range of 10 to 500 int. units/L. FSH and TSH (\( \Delta \)) were tested for cross reactivity in the hCG assay at various concentrations and each had <1% cross reactivity. Cross reactivity of LH (O) corresponded to \( \approx 6\% \) over the range tested.

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**Fig. 4. Standard curve for TSH and cross reactivity with hCG, LH, and FSH**

Calibrators for the TSH assay were tested by using 50-\( \mu \)L sample volume and 2-h sample incubation (Figures 3-5). The TSH potency was 5.0 micro-int. units/ng, yielding an assay range of 2.5–50 milli-int. units/L. hCG and LH (\( \Delta \)) were tested for cross reactivity in the TSH assay at various concentrations and gave essentially no cross reaction. Cross reactivity of FSH (O) in the TSH assay was \( \approx 7\% \) over the concentrations tested.

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**Fig. 5. Standard curve for LH and cross reactivity with hCG**

Calibrators for the LH assay were tested by using 50-\( \mu \)L sample volume and 1-h sample incubation (Figures 3-5). The LH potency was 8 milli-int. units/ng, yielding an assay range of 8–160 int. units/L. Cross reactivity of hCG (O) in the LH assay was \( \approx 80\% \)
Table 1. Monoclonal Antibody Affinities and Specificity by Competitive RIA

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<th>$K_{a}$</th>
<th>L/mol</th>
<th>% cross reactivity</th>
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<tr>
<td>Anti-$\alpha$-subunit</td>
<td>$1 \times 10^{10}$</td>
<td>100</td>
<td>100 100 100 100</td>
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<tr>
<td>Anti-$\beta$-hCG</td>
<td>$2 \times 10^{11}$</td>
<td>100</td>
<td>&lt;1 &lt;0.1 0.5</td>
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<tr>
<td>Anti-$\beta$-TSH</td>
<td>$3.5 \times 10^{10}$</td>
<td>&lt;0.2</td>
<td>&lt;0.5 100 2.8</td>
</tr>
<tr>
<td>Anti-$\beta$-LH</td>
<td>$1 \times 10^{11}$</td>
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<td>100 6 &lt;1</td>
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sensitivity of the LH assays was likely due to the shorter incubation time.

To evaluate the precision of the hCG assay, we tested 10 replicates of the standard curve calibrators. The within-assay precision (CV) of the hCG assay (2-h incubation) was similar to that of RIA methods (3) and other enzyme immunoassay methods (23), being <6% throughout the assay range of 5–200 int. units/L (Figure 6).

Evaluation of LH, TSH, and FSH cross-reactivity in the hCG assay system. The cross-reactivity of the related glycoprotein hormones in the hCG assay was tested in two ways. First we added the cross-reacting hormones to the serum-based zero calibrator and assayed. The results (Figure 3) show very little cross-reactivity of FSH and TSH with hCG (<1%). The percent cross-reactivity was calculated for the highest concentration of hormone tested: FSH, 22 μg/L (200 int. units/L); TSH, 30 μg/L (200 milli-int. units/L). The equation used for calculating cross-reactivity in ELISA assays was:

$$\frac{\text{assay concn (μg/L)}}{\text{actual concn (μg/L)}} \times 100 = \% \text{ cross reactivity.}$$

Cross-reactivity of LH in the hCG assay was higher than expected, 8.3% for LH at 24 μg/L (200 int. units/L); but in competitive RIA with the same monoclonal anti-β-hCG, the cross-reactivity of LH with hCG was <1% (Table 1). This increase in cross-reactivity in the sandwich ELISA may be due to the antibody excess in the assay. For example, in the competitive RIA procedure, we observed that increasing antibody concentrations increased cross-reactivity (unpublished data). We also observed that use of lower concentrations of conjugate gave lower cross-reactivity values in the ELISA assays.

The second method for evaluating cross-reactivity was to determine what effect added FSH and TSH had on hCG recovery in human serum. We did not include LH in this test because it would not be a factor in pregnancy, and its cross-reactivity was already established in the LH-supplemented, hCG-negative samples. At the upper limits of physiological ranges (200 int. units of FSH and 680 milli-int. units of TSH per liter), the analytical recovery of hCG at 25 or 50 int. units/L was 94% or greater. Thus, although hCG must compete with TSH, and perhaps LH and FSH in abnormal circumstances, for anti-α-subunit solid-phase antibody, there is no significant interference in hCG measurement at physiological concentrations of the other hormones in serum. The only circumstance where the competition for antibody would become significant would be in the TSH assay during pregnancy when concentrations of hCG are high.

To evaluate cross-reactivity of hCG, LH, and FSH in the TSH assay system, we added the appropriate hormones to TSH-free serum (see Figure 4). We detected no cross-reactivity with hCG [<0.1% at 400 μg/L (2000 int. units/L)], or with LH [<1% at 400 μg/L (3328 int. units/L)]. Only FSH cross reacted significantly: 6.9% at 100–200 μg/L (900–1800 int. units/L). These results were consistent with the RIA results, in which the TSH assay had a 2.8% cross-reactivity with FSH and <1% with hCG and LH.

We tested the LH assay system only for cross-reactivity with hCG (Figure 5). Cross reaction with hCG was high, approximately 60% at 10 μg/L. Further refinements in the system should increase the specificity of the LH assay.

Clinical Applications of Two-Site Simultaneous ELISA

The major application of the two-site simultaneous ELISA for hCG is for the early diagnosis of pregnancy. In pregnancy, the concentrations of hCG may range from <0.025 to >100 kilo-int. units/L (24, 25). Because of this potential for very high values, we evaluated the hCG assay for the "hook" effect (nonlinear response to antigen excess) with extremely high concentrations of hCG. We found that the dose response does hook at high values but remains above the highest concentration calibrator up to 220 kilo-int. units/L (44 mg/L), given a 25-μL sample size. This qualitative assay range is adequate for normal pregnancy testing because hCG concentration peaks at 163 (SD 16) kilo-int. units/L at 8–10 weeks after conception (25).

At 440 kilo-int. units/L, the dose response begins to fall below the high standard (200 int. units/L). If hCG concentrations greater than this are suspected, as in the abnormal conditions of hydatidiform mole or metastatic choriocarcinoma (26), dilution of the serum by 100-fold or more may be necessary for qualitative, as well as quantitative, results. Alternatively, a sequential protocol could be used, incorporating pre-incubation with sample and a wash step to remove excess antigen before incubation with enzyme conjugate. The sequential protocol would obviate the question of antigen excess causing falsely low or negative results.

The LH assay has a potential application as an aid for infertility testing, the pre-ovulatory increase in serum LH being a good indicator of the beginning of the ovulatory phase of the menstrual cycle (27). The two-site simultaneous ELISA with monoclonal antibodies has the requisite sensitivity for this determination.

The TSH assay demonstrated the potential usefulness of this sensitive test system in measuring serum concentrations of TSH; however, several concerns reduce the clinical usefulness of this test system. Concentrations of hCG as high as encountered during pregnancy interfered with this test by competing for anti-α-subunit solid-phase antibody, and cross-reactivity with FSH was 6.9%, too great for the highly sensitive assay required for TSH quantification. We are currently exploring several modifications of the test system to overcome these problems.

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References


