A Rapid, Sensitive Enzyme-Linked Immunoassay for Human Thyrotropin

Yueh-Chu Tseng, Kenneth D. Burman, James R. Baker, Jr., and Leonard Wartofsky

In this enzyme-linked immunoassay for human thyrotropin (TSH) in unextracted serum we use 96-well immunoenzymometric assay plates, first coated with polyclonal antibody to TSH, then incubated with the serum samples and reacted with mouse monoclonal antibody to human TSH. After incubation with alkaline phosphatase-labeled antibody against mouse IgG, disodium p-nitrophenyl phosphate is added and the color change is measured spectrophotometrically. Assay sensitivity is 0.1 milli-int. unit/L. Cross reactivity with lutropin, follitropin, or choriogonadotropin was negligible. TSH concentrations ranged from 0.4 to 4.1 milli-int. units/L in 43 normal subjects (mean 2.0, SD 1.0), and were uniformly <0.3 milli-int. unit/L in 23 patients with hyperthyroidism. Features which make this assay advantageous to the clinical laboratory include ease of set-up, ability to assay many samples at a time, high sensitivity, rapid turnaround time (8 h), and absence of requirements for radioactive materials.

Additional Keyphrases: immunoenzymometric assay \thryotropin

The principal desirable characteristics of a TSH assay that will distinguish normal subjects from those with hypothalamic or hyperthyroidism include rapidity, convenience, and high sensitivity. The use of various technical modifications of standard TSH radioimmunoassay (RIA) methods has improved sensitivity, but most of these methods require special antisera or involve laborious extraction techniques. The development of technology for immunoenzymometric assay, commonly known as enzyme-linked immunosorbent assay (ELISA or EIA) (1, 2), and the ability to produce specific monoclonal antibodies to TSH have led to the generation of new assays with the potential to meet the above criteria. The assay we describe here has sufficient sensitivity and is easily enough performed to be readily adaptable for use in any research or clinical laboratory.

Materials and Methods

Reagents

Rabbit antibody to human TSH (Pacific Biotech Inc., San Diego, CA) was diluted 1000-fold with sodium carbonate/bicarbonate buffer (0.1 mol/L, pH 9.8 at 25°). Mouse monoclonal antibody to human TSH (Hybritech Inc., San Diego, CA) was diluted 1000-fold with a 2 g/L solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 10 mmol phosphate and 9 g of NaCl per liter, pH 7.3). Alkaline phosphatase-labeled antibody to mouse IgG, 0.1 mg/mL (Kirkegaard and Perry Labs., Inc., Gaithersburg, MD) was diluted 250-fold with a 2 g/L solution of BSA in PBS buffer. The substrate for alkaline phosphatase (EC 3.1.3.1) was prepared by dissolving 1 g of disodium p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 1 L of 0.1 mol/L diethanolamine buffer containing, per liter, 0.5 mmol/L of magnesium chloride and 0.2 g of sodium azide, titrated with 1.0 mol/L hydrochloric acid to pH 9.8. Blocking solution was prepared by adding 10 g of BSA per liter to PBS buffer. Wash solution contained 1 g of BSA and 0.2 g of Tween 20 [polyoxyethylene (20) sorbitan monolaurate] per liter of PBS buffer. Serum devoid of TSH was prepared by passing outdated pooled human serum initially through a column of concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ) to remove TSH and other glycoproteins (3), and then through a column of staphylococcal Protein A (Pharmacia Fine Chemicals) to remove interfering immunoglobulins (4). This "stripped" serum was used to dilute the TSH standard (Pacific Biotech Inc., San Diego, CA) solutions to concentrations of 0 to 15 milli-int. units/L. The standard solutions were calibrated against WHO International Reference Preparation 69/38.

For an examination of cross reactivity with other pituitary glycoprotein hormones, we used human follitropin, 0–200 int. units/L (NIAMDD, FSH-2; National Pituitary Agency, Baltimore, MD). Its activity was calibrated against WHO International Reference Preparation 70/45. The activities of human choriogonadotropin (0–1 000 000 int. units/L) and human lutropin (0–200 int. units/L), both from Boehringer-Mannheim Biochemicals, Indianapolis, IN, were calibrated against the Second International Reference Preparation.

Patients

Serum was sampled from 43 euthyroid subjects shown to have normal values for serum thyroxin, triiodothyronine resin uptake, and free-thyroxin index. Serum was also obtained from 23 clinically thyrotoxic and 10 clinically hypothyroid patients who had above- and below-normal values, respectively, for these tests. Pooled normal serum was obtained by combining sera from euthyroid patients and volunteers.

We also assayed sera from two groups of post-menopausal women who were being treated by L-thyroxin replacement therapy. From their values for serum thyroxin and their clinical status, all of these women were considered to be taking a slight overdosage of thyroxin, yet the concentrations of TSH in their serum were clearly measurable by the kit assay routinely used in our clinical laboratory (New England Nuclear, Boston, MA). A single serum sample for TSH determination by our ELA technique was available in the first group of 12 postmenopausal patients, while serum samples at zero time and 15 and 30 min after injection of thyrotropin-releasing factor (TRF) were examined in a
second group of 11 similar patients. The issue being addressed in these subjects was whether their increased concentrations of circulating gonadotropins might be responsible for the inappropriate TSH concentrations (in the face of high concentrations of thyroxin in serum) measured by the clinical laboratory assay.

Immunoenzymometric Assay Procedure

Rabbit antibody to human TSH (50 μL/well) was adsorbed to a 96-well "Immulon II" immunoenzymometric assay plate (Dynatech Labs., Inc., Alexandria, VA) overnight at 28 °C. Unbound sites in the wells were blocked with 250 μL of blocking solution per well for 30 min at 28 °C. The plates were then emptied and washed twice with 300 μL of the wash solution per well. Standard or patient's serum (50 μL) was then placed in each well and reacted for 1.5 h at 28 °C. The plates were then emptied and washed twice with 300 μL per well of the wash solution. Monoclonal antibody to TSH (50 μL per well) was then added to each well and allowed to react for 1.5 h at 28 °C. After emptying and washing the plate twice, we added 50 μL of the phosphatase-linked antibody to mouse IgG, and incubated the plate at 28 °C for 1 h, then emptied it, washed it five times with wash solution, and added 100 μL of the substrate for alkaline phosphatase to each well. The plate was allowed to stand in the dark at room temperature, and was read 2 and 16 h later with an automatic "Titertek Multiskan" plate reader (Flow Labs., McLean, VA) with use of a 405-nm filter. Standards and serum samples were assayed in triplicate. Each plate included a set of standards and two control serum samples with known TSH values of 1.8 and 4.9 milli-int. units/L as internal standards.

For correlative purposes, the same sera also were analyzed for TSH by the Nichols Institute Reference Laboratory, San Juan Capistrano, CA, by their highly sensitive TSH radioimmunoassay. The latter is a sequential assay in which rabbit antihuman TSH and highly purified 125I-labeled TSH are used. For bound/free separation, second antibody is used, accelerated by polyethylene glycol, and the sensitivity of the assay is 0.3 milli-int. unit/L at 90% binding.

Results

Dose-response correlation: Figure 1 illustrates the changes in measured TSH vs changes in absorbance after the substrate is added. We saw no difference in absorbance for TSH values at or below 0.1 milli-int. unit/L; greater concentrations gave increased absorbance readings. A comparison of absorbance readings at 2 and 16 h gave parallel results. Table 1 summarizes inter- and intra-assay variation as evaluated from six determinations on three pooled human sera with different TSH concentrations.

TSH concentrations: In 43 euthyroid subjects the TSH concentrations ranged from 0.4 to 4.1 milli-int. units/L and averaged 2.0 (SD 1.0) milli-int. units/L. In all 23 of the hyperthyroid patients we examined, the serum TSH concentration was uniformly <0.3 milli-int. unit/L, with some samples reading below the detectable level of 0.1 milli-int. unit/L. In 10 hypothyroid patients, TSH concentrations ranged from 42 to 306 milli-int. units/L, and averaged 155 (SD 91) milli-int. units/L. There was an excellent correlation (r = 0.92) between TSH values as obtained with this ELA for 51 patients and values obtained for the same samples by the reference laboratory (Figure 2).

Cross reactivity: We observed very minimal cross reactivity when we assessed the effect of the other pituitary glycoprotein hormones on TSH measurements. Concentrations of 200 and 1,000,000 int. units of chorionic gonadotropin

Fig. 1. Absorbance vs TSH concentration in the present assay

Table 1. Inter- and Intra-Assay Variation of TSH As Measured by the Present Assay in Three Sera

<table>
<thead>
<tr>
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<th>intra-assay</th>
<th>interassay</th>
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<tr>
<td>TSH, milli-int. units/L</td>
<td>0.83 ± 0.06 (8%)</td>
<td>0.86 ± 0.07 (7%)</td>
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<tr>
<td></td>
<td>2.55 ± 0.08 (3%)</td>
<td>2.58 ± 0.13 (5%)</td>
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<td></td>
<td>6.65 ± 0.26 (4%)</td>
<td>6.75 ± 0.38 (6%)</td>
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* n = 6, throughout. Values are means ± SD (and CV).

Fig. 2. Correlation of values for TSH in serum from 51 patients, as measured by Nichols Institute RIA (sensitivity 0.3 milli-int. unit/L) and by the present method (sensitivity 0.1 milli-int. unit/L).
eller liter gave TSH readings of 0.0 and 0.38 milli-int. unit/L, respectively; 200 int. units of follitropin and lutropin gave SH readings of 0.5 and 2.0 milli-int. units/L, respectively.

In view of our observation that values for TSH as measured routinely with the New England Nuclear RIA kit appeared to be spuriously increased for samples from postmenopausal women who were receiving treatment with tyrroxin, we compared TSH values obtained with this kit to 12 postmenopausal women (average age 55.5 years) with values obtained with the present assay. As noted above, these women were clinically euthyroid, but most of them had values for the free thyroxin index that were in the upper-normal to frankly above normal, averaging 68 (SD 1.75), the normal range being 1.60 to 4.96. TSH concentration averaged 5.18 (SD 1.5) milli-int. units/L by the kit method and 0.8 (SD 0.5) milli-int. unit/L with our assay. Because basal TSH should have been predictably low or undetectable in these chemically hyperthyroid women, the detectable "TSH" measurable with the kit must in fact represent gonadotropins; indeed, these were shown to be increased in these patients by specific assays for lutropin and follitropin.

Whether the difference between values measured in the two assays was attributable to a greater degree of gonadotropin cross-reactivity in the kit was examined further by comparing results of stimulation of TSH release by TRF in a second group of 11 postmenopausal patients. Mean (and SD) TSH values for serum at zero time and 15 and 30 min after TRF administration were 2.9 (0.6), 3.3 (0.7), and 3.2 (0.7) milli-int. units/L, respectively, with the kit; 0.4 (0.1), 0.4 (0.1), and 0.5 (0.1) milli-int. unit/L by the reference laboratory; and 0.2 (0.1), 0.2 (0.1), and 0.2 (0.1) milli-int. unit/L by our assay (we assigned a value of 0.1 milli-int. unit/L to samples in which TSH was undetectable). Thus, the failure of apparent "TSH" as measured with the kit to increase after TRF suggests the artifactual nature of this "TSH," a likelihood supported by the extremely low concentrations of TSH seen in all samples with both the highly sensitive reference laboratory RIA and our assay.

Discussion

Generally available RIAs for human TSH will reliably discriminate between the low values seen in normal subjects and the above-normal ones characteristic of primary hypothyroidism. Concentrations of TSH in serum from patients with either pituitary hypofunction or hyperthyroidism of gest etiologies are undetectable in such assays, and thus are clearly distinguishable from normal. With various technical modifications, including the use of higher-affinity antisera, the sensitivity of the routine assay may be improved (5). Further modifications have led to a sensitivity adequate to demonstrate that TSH concentrations usually are clearly lower in hyperthyroid than in normal individuals (6–8). However, the differentiation by RIA requires a sensitivity that can be attained only by tedious and time-consumning techniques, involving concentration of the TSH from serum by extraction onto concanavalin A linked to Sepharose. Less-tedious methods such as use of a longer antigen-antibody incubation time may increase sensitivity, but not deceptively to clearly discriminate normal from thyrotoxic sera (9).

Problems inherent in the RIA methodology—in addition to the long incubation times and insufficient sensitivity—include the immunological and radiochemical instability of human TSH after 125I labeling by the Chloramine T technique with consequent increasing "damage" that further reduces sensitivity and augments interassay variation. Moreover, effects of serum on precipitability of bound labeled antigen by second antibody have been described (8). To eliminate these problems, several workers have recently reported immunoradiometric assays of shorter duration and higher sensitivity. While improving sensitivity over the RIA, the use of radiolabel perpetuated the damage problem and served to similarly impair immunoreactivity (11, 12). A recent and exciting technical refinement (13) is an immunocholinoluminometric assay that involves reaction of serum samples with monoclonal antibodies to TSH that have been labeled with a chemiluminescent ester. Subsequent reaction with a solid-phase polyclonal antibody to TSH is followed by measurement of chemiluminescence in a luminometer. Values for normal subjects ranged from 0.4 to 4 milli-int. units/L, while patients with "overt" hyperthyroidism measured <0.05 milli-int. unit/L. Although a promising improvement, the latter method, which requires luminometric measurement of photon emission, is tedious and time-dependent, and also requires the individual and sequential handling of each sample. Additional reports of modified monoclonal-antibody immunoradiometric TSH assays have since appeared (14–16). These assays are more sensitive and faster, but the need to use radioisotopes—with their limited shelf life and attendant disposal problems—remains a significant drawback.

Previous attempts to measure TSH by EIA have either failed in terms of sufficient sensitivity (17, 18) or were sufficiently complex and difficult (although very sensitive), to preclude routine use in a clinical laboratory (19). In the latter report, sensitivity was increased by the use of affinity-purified anti-human TSH, which was then passed through a Sepharose 4B column. In addition to this complexity, this EIA (19) is also more time consuming than the present assay. The tedious and expensive cost of preparing enzyme-labeled antigen was recognized by Wada et al. (20), who developed instead an EIA involving monoclonal antibody technology. Although their methodology was indeed simplified, their assay still was not as sensitive and specific as one would wish.

In the present EIA for TSH—unlike the RIA method, which depends upon competitive binding to antibody between free TSH and free 125I-labeled TSH—a double-antibody "sandwich" technique is used, in which detection of TSH depends directly upon antibody/antigen/antibody binding. The linked enzyme serves to amplify the substrate color change, which greatly increases the sensitivity of the assay such that a low range of TSH values, heretofore unapproachable to most clinical laboratories, can be measured. Moreover, binding and cross-reactivity of the other glycoprotein hormones is lessened, thus eliminating this interference problem so frequently associated with RIA of TSH.

While other laboratories may wish to substitute other polyclonal antibodies, we would emphasize that this assay probably depends upon the antibody pair possessing different binding sites to the human TSH molecule; thus the polyclonal/monoclonal antibody pairs combination must be properly screened for maximum assay sensitivity. However, we have obtained similar standard curves and results on using polyclonal antibody to human TSH from another source (National Pituitary Agency, Baltimore, MD; NIADDK—anti-human TSH-3), diluted 100-fold, to coat the immunoenzymometric assay plates.

In addition to the ease of assay set-up, the present EIA has the additional advantages that radioactive materials are not used, and that the reagents have long shelf lives under appropriate storage conditions. Further, given that multiple samples can be analyzed simultaneously, with results available within 8 h, this technique has outstanding potential for routine clinical application.
Availability of a highly sensitive and specific assay for TSH will have widespread application to the routine diagnostic evaluation of patients with suspected thyroid dysfunction. The importance of the greater sensitivity relates to eliminating any overlap of TSH values between normal subjects and those with hyperthyroidism, a feature that will facilitate earlier diagnosis of thyrotoxic patients who have only marginally increased serum iodothyronines, as well as the refinement of dosage modification and adjustment in patients who are receiving therapy with thyroxin for purposes of either replacement or suppression. The greater sensitivity of the assay will also facilitate investigations into effects of various illnesses and drugs (e.g.) on serum TSH concentrations or the TSH response to TRF in normal or euthyroid individuals in whom detection of TSH suppression was not previously possible.

The question of cross reactivity is of special importance with respect to postmenopausal women who are receiving thyroxin for suppression but who often have persistently measurable concentrations of apparent TSH by most routine TSH radioassays. Such women can be falsely diagnosed as hypothyroid when measured "TSH" due to high serum gonadotropins is inappropriately interpreted. With the present EIA, the demonstration of adequate TSH suppression to concentrations <1.0 milli-int. unit/L in such patients would avoid unnecessary—and potentially dangerous—increments in their thyroxin dosage.

Finally, the improved sensitivity of this assay may obviate the need for the TRF tests in selected patients, as recently suggested by others (10). For example, the presence of a basal serum TSH value between 0.4 and 1.5 milli-int. units/L in our assay will exclude hyperthyroidism, whereas a TRF test might have been required to do so in a patient with suggestive clinical and/or laboratory values and TSH undetectable by a routine laboratory assay.

Addendum: Since submission of our manuscript, a letter to the editor has appeared (Roddis et al., Lancet i: 277, 1985) describing an immunoenzymometric assay for TSH with sensitivity and specificity similar to that reported herein.

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