Highly Sensitive Immunoenzymometric Assay for Human Thyrotropin

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A sensitive assay procedure for immunoenzymometric assay of serum thyrotropin (TSH) was developed by making several modifications of the Enzymun-Test® TSH kit (Boehringer, Mannheim GmbH). Serum samples were first incubated in plastic tubes precoated with monoclonal antibodies specific to the beta subunit of human TSH. After the tubes were washed, the TSH bound to the tubes was detected with peroxidase-conjugated polyclonal antibodies to TSH. The sensitivity of the assay was 0.2 milli-int. unit/L, and the intra- and interassay CVs were <10%. Analytical recovery was 96 to 106%. The normal basal range of TSH was 0.5 to 4.8 milli-int. units/L. The basal levels of TSH in all but one of 48 thyrotoxic patients with Graves' disease were less than 0.2 milli-int. unit/L, clearly different from those of normal subjects. Thyrotoxic patients in early normal pregnancy showed TSH concentrations of 1.7 to 2.9 milli-int. units/L by conventional double-antibody radioimmunoassay, possibly from cross-reactivity with human chorionic gonadotropin, but undetectable TSH by this method. Measurement of basal TSH by this sensitive assay can be used as an initial screening test for thyroid dysfunction.

Additional Keyphrases: "kit" method · thyroid status · pregnancy · Graves' disease · monoclonal antibodies

Measurement of serum thyrotropin (TSH) is useful for the detection of various thyroid conditions, especially mild or latent primary hypothyroidism (1–3), in which TSH is increased in serum and thus easily detected with currently available routine RIA or enzyme immunoassay kits. However, in patients with thyrotoxicosis, serum TSH is suppressed by excess thyroid hormones through a negative-feedback mechanism (1, 4, 5). This makes it difficult to differentiate the basal concentrations of TSH in sera of euthyroid subjects from those of thyrotoxic patients by using commercially available kits with low sensitivity. Therefore, tests with thyrotripsin (thyrotropin-releasing hormone) have been widely used to diagnose borderline or mild cases of thyrotoxicosis (4, 5).

In this study we developed a sensitive enzyme immunoassay for TSH in which we made several modifications of a commercially available kit (Enzymun-Test® TSH) (6). We also report here the clinical application of the procedure.

Materials and Methods

Subjects: We measured basal TSH concentrations in sera of 100 normal subjects (69 women, 21–43 years old; 31 men, 22–35 years old) and 48 untreated thyrotoxic patients with Graves' disease, including five patients in early normal pregnancy. Cases of subclinical autoimmune thyroiditis were excluded from the group of normal subjects by measurements of anti-thyroid antibodies (7). Graves' disease was diagnosed on the basis of clinical symptoms of thyrotoxicity and laboratory findings of increased thyroxin (T4) and triiodothyronine (T3) in serum, and macroaggregated albumin T3 uptake (8).

Conventional radioimmunoassay: A commercially available double-antibody RIA kit (Daichi Radioisotope Laboratory, Tokyo, Japan) was used, with minor modifications. In brief, 100 μL of serum sample or standard TSH solution was mixed with 100 μL of anti-human TSH rabbit antibody, and incubated for 3 h at room temperature. Then 100 μL of 129I-labeled TSH was added and the mixture was incubated for 20 h at room temperature. Finally, 100 μL of anti-rabbit γ-globulin goat serum was added and the mixture was incubated for 5 h at room temperature. This was centrifuged at 2000 × g for 30 min and the radioactivity in the precipitate was counted. The concentration of standard hTSH was calibrated with WHO standard MRC 68/38. The sensitivity of this assay was 1.0 milli-int. unit of TSH per liter.

Immunoenzymometric assay: We used a commercially available enzyme immunoassay kit (Enzymun-Test® TSH; Boehringer Mannheim GmbH, Mannheim, F.R.G.), with several modifications. We incubated 200 μL of serum test sample or standard TSH serum (calibrated with WHO Standard MRC 68/38) with 1 mL of phosphate buffer (16 mmol/L, pH 6.9) for 17 h at 4 °C (instead of 1 h at room temperature as in the manufacturer's protocol), in a plastic tube precoated with monoclonal antibodies specific to the beta subunit of the human TSH molecule. We washed the tube once with Tris HCl buffer (50 mmol/L, pH 7.4) instead of with tap water, and we incubated the tube with 1 mL of horseradish peroxidase-conjugated polyclonal antibodies against human TSH for 2 h (instead of 1 h) at room temperature. The antibody-peroxidase conjugate had been diluted 200-fold (instead of 100-fold) with phosphate buffer (36 mmol/L, pH 6.9) 1 h before use.

After incubation, we rinsed the tube three times with the Tris HCl buffer containing 0.5 mol of NaCl and 0.5 mL of Tween 20 per liter, instead of rinsing once with tap water as the manufacturer recommended. After adding 1 mL of substrate solution, we incubated the mixture for 1 h at room temperature in the dark. For the substrate we used o-phenylenediamine instead of the kit-supplied ABTS® (di-ammonium 2,2'-azino-di-(3-ethylbenzthiazoline)-6-sulfonate). The substrate solution consisted of 10 mg of o-phenylenediamine, 10 μL of H2O2 (300 mL/L solution), and 25 mL of citrate buffer (0.1 mol/L, pH 5.0). After this incubation, we stopped the reaction by adding 0.2 mL of 4 mol/L sulfuric acid and measured the absorbance of the solution at 492 nm, in a spectrophotometer. To measure the lower concentrations of TSH, we prepared fresh TSH standard in sera at concentrations of 0.2 and 0.5 milli-int. unit/L by mixing TSH-free serum with standard TSH serum (1.0 milli-int. unit/L) from the manufacturer.

Measurement of thyroid hormones: Serum T4 and T3 were measured by radioimmunoassay as described previously (8). T3 uptake (T3U) was determined with a kit for measuring...
macro-aggregated albumin uptake (Amersham International, Amersham, Bucks., U.K.). The free-T₄ index and free-T₃ index were calculated as $T_4 \times T_3 U$; mean normal $T_3 U$ and $T_3 \times T_3 U$; mean normal $T_3 U$, respectively, according to the modified method of Clark and Horn (9).

**Results**

Figure 1 shows the standard curve for sensitive immunoenzymometric assay for human TSH. The absorbance corresponding to a TSH concentration of 0.2 milli-int. unit/L was significantly different ($p < 0.001$) from that at 0 milli-int. unit/L; thus the sensitivity of this assay is 0.2 milli-int. unit/L. The within-assay CVs for samples containing TSH at 1.0 and 15.0 milli-int. units/L were 8.0% and 5.0%, respectively. The between-assay CVs for TSH at 1.0 and 14.0 milli-int. units/L were 10.0% and 5.8%, respectively. Analytical recovery in the assay was 96% to 106%.

As shown in Figure 2, all but one normal subject had detectable basal concentrations of TSH. TSH values in normal subjects showed a normal logarithmic distribution; the reference interval (95% confidence limits) was 0.5–4.3 milli-int. units/L. All but one patient with thyrotoxic Graves' disease had a low (<0.2 milli-int. unit/L) basal concentration of TSH. Thus, by using this sensitive enzyme immunoassay to measure basal TSH one can clearly distinguish normal subjects from thyrotoxic patients with Graves' disease. The one exception we saw was a patient with Graves' disease whose TSH was 0.9 milli-int. unit/L.

Table 1 summarizes the results for this assay when applied to five thyrotoxic patients with Graves' disease who were also pregnant. All five patients had clinical symptoms of thyrotoxicosis and increased concentrations of thyroid hormones, but the conventional double-antibody radioimmunoassay measured detectable TSH in their serum. However, the TSH beta-subunit-specific sensitive enzyme immunoassay clearly showed that the TSH concentrations in all these patients were <0.2 milli-int. unit/L.

**Discussion**

The sensitivity of an immunoassay is affected by many factors. Among the methods used to increase the sensitivity are the introduction of high-affinity antibodies, delayed addition of labeled antigen, prolongation of incubation, utilization of immunometric assays rather than competitive immunoassays, and application of sensitively detectable tracer. Delayed addition of labeled TSH and prolonged incubation have been used to increase the sensitivity of immunoassay for TSH (10–12) but these modifications are not suitable for use in routine tests. Very recently, Weeks et al. (13) reported a highly sensitive immunochemiluminescent assay using monoclonal antibody and a sensitive chemiluminescent tracer. More recently, a sensitive radioimmunoassay of TSH was developed by using a combination of immunometric assay and monoclonal antibodies (14, 15).

Immunoenzymoassay of TSH has long been used (16–18), but one problem in this assay is sensitivity. Recently, Imagawa et al. (19) developed a sensitive immunoenzymoassay for TSH in which affinity-purified polyclonal anti-human TSH IgG conjugated with $\beta$-galactosidase was used. However, this method has not been used widely as a routine method. All these reported methods may give false values for TSH in patients with extremely high concentrations of human chorionic gonadotropin, such as those in early pregnancy.
The sensitivity of our immunoenzymometric assay was 0.2 milli-int. unit/L, five- to 10-fold the sensitivity of conventional competitive radioimmunoassays. This high sensitivity might be the result of use of a combination of monoclonal antibodies specific to the beta subunit of TSH and polyclonal antibodies and to the lowering of the nonspecific binding background. The reproducibility of the procedure was also satisfactory for routine use, and patients with Graves' disease could be clearly distinguished from normal subjects. Basal TSH in serum of patients with thyrotoxicosis, measured with a highly sensitive radioassay and a chemiluminescent assay, have been reported as <0.2 milli-int. unit/L (14, 15) and <0.05 milli-int. unit/L (13), respectively. The reason why one of our thyrotoxic patients had detectable TSH (0.9 milli-int. unit/L) is not known.

Because thyroid disease is common in women of child-bearing age, it is often necessary to examine thyroid function in patients who are pregnant. During pregnancy the concentrations of thyroxin-binding globulin, T₄, and T₃ in serum are increased; the free-T₄ or free-T₃ index should thus be measured to evaluate thyroid function (20). However, normal values for free T₄ differ markedly, depending on theRIA method used (21). Furthermore, Graves’ disease is frequently aggravated in early pregnancy (22), making especially difficult the accurate evaluation of thyroid function, particularly in early pregnancy, where the choriogonadotropin concentration is often increased to as much as 200 000 int. units/L (20).

As shown in Table 1, in all of our pregnant thyrotoxic patients with Graves’ disease, serum TSH was falsely detectable by the conventional RIA, but was <0.2 milli-int. unit/L by the modified immunoenzymoassay. This discrepancy possibly is ascribable to cross reactivity of choriogonadotropin in the conventional RIA. For assay of low concentrations of pituitary peptide hormones, monoclonal antibodies specific to the beta subunit of the peptide should be used.

During pregnancy, the thyrotoxin test is not permitted for thyroid evaluation, and there is still much controversy about the “normal” concentration of free T₄ in sera of pregnant subjects. Therefore, a highly sensitive assay for TSH should certainly be useful in these cases. Measurement of basal TSH is the first choice as a thyroid-function test if a highly sensitive assay is available, as suggested by Allen and Watson (14) and Alexander et al. (15).

This work was supported by a research grant from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare; by a Grant-in-Aid for Scientific Research 56870261 (to N.A.) from the Ministry of Education, Science, and Culture of Japan; and by a grant from the Clinical Pathology Research Foundation of Japan.

References

Table 1. Thyroid Hormone Values in Serum from Five Pregnant Patients with Graves’ Disease

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<th>T₄</th>
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<th>Free T₄ Index</th>
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Serum samples were obtained at nine to 13 weeks of pregnancy. MT₄/JL, macroaggregated albumin T₃ uptake.