Sensitive, Practical Bioassay of Thyrotropin, with Use of FRTL-5 Thyroid Cells and Magnetizable Solid-Phase Antibodies

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We have developed a new method for assessing the bioactivity of thyrotropin (TSH) in human serum by measuring cAMP production in FRTL-5 thyroid cells as an index of stimulation. To eliminate serum inhibitors of the cAMP response to TSH, we purified samples by mixing them with anti-TSH antibodies coupled to magnetizable particles. This method of immunofinity purification was simple and suitable for the measurement of a large number of samples. The detection limit of the bioassay was 3.13 milli-int. units of human TSH per liter. Intra-assay and interassay coefficients of variation ranged from 5.4 to 8.6% and from 12.6 to 21.5%, respectively. The concentrations of bioassayable TSH closely correlated with those of immunoassayable TSH, both in the immunofinity-purified samples (r = 0.946, P < 0.001) and in the serum samples (r = 0.945, P < 0.001) from 14 euthyroid subjects and 53 hypothyroid patients with Hashimoto's thyroiditis. The bioactivity-to-immunoreactivity ratio was almost constant (0.94 ± 0.30; mean ± SD) over the range of concentrations of immunoassayable TSH in serum, 6.3 to 177 milli-int. units/L. We also demonstrated that the technique is applicable to measurements of bioactivities of circulating forms of immunoreactive TSH in different pathophysiological conditions.

Additional Keyphrases: immunofinity purification · immunoassay compared · hypothyroidism · cAMP

A sensitive immunoradiometric assay (IRMA) for thyrotropin (thyroid-stimulating hormone, TSH) in serum is now widely used as the first-line test of thyroid function (1–5). However, some evidence indicates that TSH immunoreactivity and bioactivity are not necessarily synonymous (4–11). We therefore performed this study to evaluate both the immunoreactivity and the bioactivity of TSH in human sera.

Numerous bioassays for TSH have been developed. The early in vivo bioassay developed by McKenzie (12) was of limited sensitivity and precision, and was subject to many interferences (13–15). The assay measuring adenylate cyclase activity in human thyroid membrane fractions as an index of stimulation (4, 8–10) also is of limited sensitivity. On the other hand, the development of a sensitive cytochemical bioassay (16, 17) has permitted estimation of TSH bioactivity in small volumes of unfractionated serum. The assay is, however, technically difficult; one must measure serum samples at various dilutions and incubation times to avoid intra-assay interference from thyroid-stimulating antibodies (TSAb), because the incubation time at which maximum stimulation occurs in the assay depends not only on the type of the stimulators (TSH or TSAb) but also on their concentrations (18). Moreover, this assay may also be subject to interference by a blocking type of TSH-binding inhibitor immunoglobulins (TBII), which have been detected in some patients with Hashimoto's thyroiditis and primary myxedema (19, 20).

Rapoport and Adams (21) first used the cAMP response to TSH in cultured thyroid cells to assay TSH bioactivity. They eliminated the serum inhibitors of the cAMP response to TSH by using Sephacryl S-200 gel filtration. Recently, Dahlberg et al. (II) developed a bioassay for TSH, measuring cAMP production in FRTL-5 thyroid cells exposed to TSH partly purified from serum by immunofinity chromatography with an anti-α subunit column. These methods, however, seem unsuitable for assaying TSH bioactivity in a large number of serum samples.

Here, we have modified the method developed by Dahlberg et al. (II), making it simpler and more practical, by purifying TSH from each serum sample with anti-TSH antibodies coupled to magnetizable particles. We have compared the results of the bioassay with measurements of immunoreactive TSH in serum from euthyroid subjects and patients with primary hypothyroidism.

Materials and Methods

Samples

Serum samples from 14 euthyroid and 53 hypothyroid patients with Hashimoto's thyroiditis were analyzed for TSH bioactivity. The diagnosis of Hashimoto's thyroiditis was based on diffuse goiter, increased titers of antithyroglobulin antibody and antimicrosomal antibody, and histological findings obtained by needle biopsy. The diagnosis of hypothyroidism was based on low concentrations of free thyroxin and high concentrations of TSH in serum. TSH concentrations in serum from hypothyroid patients ranged from 6.3 to 177 milli-int. units/L (normal range, 0.31–3.90 milli-int. units/L).

To evaluate the specificity of the bioassay, we tested the following samples with the bioassay: (a) three sera from pregnant women and one serum from a patient with hydatidiform mole, all known to have high concentrations of human choriogonadotropin (hCG) in serum (8.6–10.7 and 1440 kilo-int. units/L, respectively); (b) six sera from postmenopausal women, with high concentrations of lutropin (LH; 100–380 int. units/L) and follitropin (FSH; 40–300 int. units/L) after stimulation with lutein; and (c) eight sera from patients with Graves' disease, who had detectable TSAb (per liter, 91–224 milli-int. units of human TSH equivalent). To study the effect of a blocking type of TBII on the assay result, we used 1 mg of TBII-positive IgG (obtained from a patient with primary hypothyroidism, then passed through a column of Protein A–Sepharose), potent enough to inhibit cAMP production completely in FRTL-5 cells stimulated by TSH; we added this before the bioassay to a serum containing a high concentration of TSH (50 milli-int. units/L).

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Materials

Anti-TSH antibodies coupled to magnetizable particles and a magnetic separator were kindly supplied by Corning Medical, Corning Glass Works, Medfield, MA. Coon's modified Ham's F-12 medium for cell culture was obtained from KC Biological, Lenexa, KS. Calf serum was obtained from Gibco Laboratories, Grand Island, NY. Insulin, hydrocorti-
sone, transferrin, glycy1-L-histidyl-L-lysine acetate, somato-
statin, and 3-isobutyl-1-methylxanthine were all purchased from Sigma Chemical Co., St. Louis, MO; purified hCG from UCB-Bioproducts S.A., Braine-L'Alleud, Belgium; bovine
TSH from Armour Pharmaceutical, Kankakee, IL. Human
TSH (WHO, 2nd IRP, 80/558) was kindly supplied by Dr.
A. F. Bristow (National Institute for Biological Standards
and Control, Holly Hill, London, U.K.). [*ITSH was
provided by Daiichi Radioisotope Institute, Tokyo, Japan.

Assay kits were obtained from the following sources:
cAMP RIA kit from Yamasa Shoyu Chemical Institute,
Chiba, Japan; TSH IRMA kit "RIA-ghost hTSH" from
Hoechst, Tokyo, Japan (originally from Behring Laboratory,
Marburg, F.R.G.); LH and FSH RIA kits "Daiichi" from
Daiichi Radioisotope Institute; hCG RIA kit from Green
Cross Co., Osaka, Japan (originally from Compagnie
Industrie S.A., France).

Procedures

Immunopurification of serum TSH. To purify TSH in
serum, we used polyclonal antibodies against human TSH (WHO 68/38), coupled to magnetizable particles, as follows.
We placed 1-mL aliquots of the anti-TSH antibodies solution
(provided in the "Magnetic TSH RIA" kit, Corning Medical)
in a magnetic separator. In a few minutes, all magnetizable
particles were attached to the side wall of the tubes. After aspirating the supernate and washing once with 1.5 mL
of isotonic saline (NaCl 150 mmol/L), we added 2.4-mL serum
tubes to the samples, incubated for 30 min at room tempera-
ture, then placed the tubes and contents in the magnetic
separator again. After aspiration and washing, we added to each tube 2 mL of 0.1 mol/L glycine-
HCl solution (pH 2.0). After incubation for 30 min, we
transferred to another tube the supernate, which contained
TSH dissociated from the anti-TSH antibodies, neutralized
it with a few drops of 1 mol/L NaOH, and dialyzed this
against 0.5 mmol/L phosphate buffer. The dialyzed samples
were divided into two aliquots, both of which were freeze-
dried and stored at -20 °C until we used them to measure
the immunoactivity and bioactivity of TSH.

In some experiments, the dialyzed samples were not
divided: we measured only their TSH bioactivity.

TSH bioassay. The continuously proliferating and partly
differentiated rat thyroid cell strain, FRTL-5, established by
Ambesi-Impiombato et al. (22), was kindly supplied by Dr.
L. D. Kohn (National Institutes of Health, Bethesda, MD).
The FRTL-5 cells were grown in Coon's modified Ham's F-
12 medium supplemented with calf serum (50 mL/L) plus
the following mixture: per liter (final concentration), 10 mg
of insulin, 10 mmol of hydrocortisone, 5 mg of transferrin, 10
µg of glycy1-L-histidyl-L-lysine acetate, 10 µg of somato-
statin, and 1 int. unit of bovine TSH. This medium is hereinafter referred to as "6H medium." The bioassay was
performed, as we discussed elsewhere (23). Briefly, when
the cells had grown to confluence in a 24-well culture plate,
we replaced the 6H medium with the same medium minus TSH
(5H medium). After seven days in 5H medium, the freeze-
dried samples mentioned above were dissolved in 1 mL of
Hanks' medium (without NaCl) containing, per liter, 15 g of
bovine serum albumin, 20 mmol of 4-(2-hydroxyethyl)-1-
piiperazineethanesulfonic acid, and 0.5 mmol of 3-isobutyl-1-
methylxanthine. We performed the assay in triplicate, by
incubating the cells with the immunopurified TSH dissolved
in 300 µL of the hypotonic Hanks' medium. After a 2-h
incubation at 37 °C, we transferred 100-µL aliquots of the
medium to plastic tubes containing 900 µL of distilled
water, then stored these at -20 °C until used for cAMP
measurement.

TSH bioactivity was expressed as human TSH equivalent,
determined from the dose-response curve of cAMP produc-
tion obtained with various concentrations (1.56, 3.13, 6.25,
12.5, 25, 50, and 100 milli-int. units/L) of human TSH.

TSH immunoassay. The remaining freeze-dried aliquots of
samples were dissolved in 1 mL of Dulbecco's PBS (without
bovine serum albumin (1 g/L). We assayed their
immunoactivity by using TSH IRMA kits standardized with
2nd IRP (80/558). The TSH values obtained did not differ
significantly (P > 0.05) whether the freeze-dried samples
were dissolved in Dulbecco's PBS (—) or in TSH-free serum
(data not shown). The minimal detectable concentration
of TSH was 0.03 milli-int. unit/L, defined as the mean ± 2SD
of zero standard values (n = 5). We also determined the
concentrations of TSH in serum with the IRMA kits.

Statistics. Data were analyzed for statistical significance
by Student's t-test.

Results

TSH concentrations in serum measured by IRMA
decreased from 156-326 to 3.3-4.7 milli-int. units/L after
treatment with the anti-TSH antibodies, indicating that,
in serum, TSH was almost completely bound to the antibodies.
The percent dissociation of [*ITSH, which had been bound
to the solid-phase, into the 0.1 mol/L glycine HCl buffer, pH
2, was 89.2%, much greater than that obtained at pH 3 or 4
(47.8 and 1.6%, respectively). Gel chromatography (Sephadex
G-100) of the supernate after treatment with 0.1 mol/L
glycine HCl revealed neither leakage of the antibodies nor
apparent dissociation of the hormone into its α and β
subunits (data not shown).

The mean cAMP responses to various concentrations
of human TSH in six different assays are shown in Figure 1.
There was a significant (P < 0.05) difference between
the values for cAMP production in triplicate determinations at 0

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Dose-response curve of cAMP production stimulated by different concentrations of human TSH, as a percentage of that produced by the buffer control

*Vertical bars indicate mean ± SD of six different assays*
and 3.13 milli-int. units/L concentrations of human TSH in all six assays, whereas the difference between the values at 0 and 1.56 milli-int. units/L concentrations of human TSH was not always statistically significant. Therefore, we defined the detection limit of the assay as 3.13 milli-int. units of human TSH per liter. Precision of the bioassay was satisfactory, with intra-assay and interassay CVs of 5.4–8.6% and 12.6–21.5%, respectively (Table 1).

We tested the bioassay for cross-reactivity with hCG, LH, and FSH. Addition of purified hCG at concentrations of 30, 100, and 300 kilo-int. units/L increased cAMP production by 2.3, 6.9, and 16.5 times. However, three serum samples from pregnant women with hCG concentrations of 8.6–10.7 kilo-int. units/L (determined by RIA) and from a patient with hydatidiform mole, whose serum hCG was 1440 kilo-int. units/L, demonstrated no stimulatory activity in the present bioassay. The negative bioassay results may be explained by the limited binding capacity for hCG of the anti-TSH antibodies, because treating pregnancy sera with the anti-TSH antibodies decreased the hCG concentrations by nearly 1000 int. units/L. Also showing no stimulatory activities were eight serum samples from patients with Graves’ disease, which were positive for TSAb activity. This suggests that TSAb were completely removed during the purification process. Addition of a blocking type of TBII to serum did not affect the concentrations of bioassayable TSH (data not shown). Serum samples containing high concentrations of LH (100–380 int. units/L; n = 6) or FSH (40–300 int. units/L; n = 6), as determined by RIA, also gave negative bioassay results.

Concentrations of immunoassayable TSH in the freeze-dried samples correlated very closely with those in serum samples, as shown in Figure 2. These results indicate a stable recovery of TSH by purifying, dialyzing, and freeze drying. The mean analytical recovery rate, calculated from the values for all samples, was 49.9 (SD 16.9)%.

Ratios of bioactivity to immunoactivity (B/I) in the freeze-dried samples were almost constant (0.84 ± 0.30; mean ± SD) in 53 patients with primary hypothyroidism, and were not significantly different among the following groups: 0.89 ± 0.27 in 16 subclinically hypothyroid patients with 6.3–20 milli-int. units/L concentrations of immunoassayable TSH in serum; 0.78 ± 0.26 in 29 mildly or moderately hypothyroid patients with TSH in serum exceeding 100 milli-int. units/L; and 0.78 ± 0.17 in eight severely hypothyroid patients with TSH in serum exceeding 100 milli-int. units/L.

Dilution of sera containing TSH >100 milli-int. units/L (determined by RIA) with TSH-free serum caused no apparent dissociation between concentrations of immunoassayable TSH and those of bioassayable TSH (data not shown).

**Table 1. Precision of the TSH Bioassay**

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<th>Interassay Mean</th>
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**Fig. 2.** Relationship between concentrations of immunoassayable TSH in sera from euthyroid and hypothyroid patients with Hashimoto’s thyroiditis and those in the freeze-dried samples obtained from the patients’ sera after immunoaffinity purification.

Considering that the freeze-dried sample obtained from 1.2 mL of the original serum was dissolved in PBS (—) and that 50% of TSH in serum was recovered, one would estimate the ratio of y to x to be 0.5. This is in good agreement with the slope of the regression line (0.567).

The correlation between concentrations of immunoassayable TSH and those of bioassayable TSH was somewhat rough, when concentrations of TSH in serum were low (r = 0.754, in 26 sera with TSH <15 milli-int. units/L vs r = 0.946 in all 67 sera in Figure 3). Concentrating the samples twofold before the bioassay gave much closer correlations between these two measurements (Figure 4).

**Discussion**

We have developed a practical, precise, and sensitive in vitro bioassay of TSH, and used it to ascertain a close correlation between TSH immunoactivity and bioactivity in sera from patients with primary hypothyroidism. To measure TSH bioactivity, we used a clonal rat thyroid cell line (FRTL-5) that displays more constant response of cAMP and is more readily available than thyroid cells in primary culture or thyroid membrane preparations. In addition, purifying TSH in serum by use of solid-phase-bound anti-TSH antibodies is simple, and allows measurement of a large number of samples. The recovery rate of TSH during the purification process was stable in the range of 2–177 milli-int. units/L original concentration in serum.

The present bioassay is not only practical but also sensitive with a significant increase in cAMP observed in the presence of 3.13 milli-int. units of human TSH per liter. Rapoport and Adams (21), using cultured dog thyroid cells, reported a detection limit of 10 milli-int. units/L for bovine TSH, which was equivalent to ~100 milli-int. units/L for human TSH. Dahlberg et al. (11), who used the same cell line that we did, reported a detection limit of 20 milli-int. units/L for human TSH, much higher than ours. This difference in sensitivity may be partly due to heterogeneity in substrains of FRTL-5 cells.

Specificity of the cAMP response to TSH was demonstrable by the lack of effect from sera containing high concentrations of hCG, LH, and FSH, or potent activity of TSAb. The precision of the measurements was excellent for a bioassay. However, given the detection limit of 3.13 milli-int. units/L (human TSH) and the ~50% recovery of TSH, we recommend concentrating TSH in serum for evaluating low bioactivities, as shown in Figure 4.

Variations in TSH bioactivity and B/I ratios in hypo-
bioactivity of TSH, and a direct relationship between concentrations of TSH in serum and B/I ratios in euthyroid subjects and patients with primary hypothyroidism. They proposed a hypothesis that thyroid hormones regulated glycosylation and alterations in carbohydrate structure of TSH, which might be responsible for the variation in TSH bioactivity. However, in contrast to their findings, we demonstrated a highly significant correlation between concentrations of immunoassayable and bioassayable TSH, and a constant B/I ratio in patients with various degrees of primary hypothyroidism. The B/I ratio in patients with subclinical hypothyroidism in the present study was much higher than that in their study (0.89 vs 0.25, respectively), whereas no remarkable difference was observed in B/I ratios in moderately or severely hypothyroid patients. These differences might be related to the different methods used. Dahlberg et al. determined B/I ratios in subjects with low concentrations of TSH by assaysing samples that had been extensively concentrated by reduced-pressure dialysis. Differences in the anti-TSH antibodies used for purification of TSH (their anti-α subunit polyclonal antibodies vs our anti-TSH polyclonal antibodies) and for the TSH immunoas-
say (anti-TSH polyclonal antibodies vs anti-β subunit monoclonal antibodies, respectively) are probably also responsible.

Rapoport and Adams (21) reported that discrepancies between concentrations of immunoassayable and bioas-
sayable TSH were also shown in some euthyroid subjects and patients with primary hypothyroidism. However, unlike Dahlberg et al., they did not find a correlation between concentrations of TSH in serum and B/I ratios.

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Clinical and Analytical Evaluation of Two Immunoassays for Direct Measurement of Creatine Kinase MB with Monoclonal Anti-CK-MB Antibodies

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We examined the clinical and analytical performance of two immunoassays (Becton Dickinson CK-MB; Ciba-Corning Magic Lite CK-MB) in which monoclonal anti-CK-MB antibodies are used for directly measuring creatine kinase (EC 2.7.3.2) isoenzyme MB (CK-MB) in serum, and also one electrophoretic method (Ciba-Corning). Within- and between-assay precision for both immunoassays was good at the upper reference limits (<10% CV). Analytical recoveries ranged from 102 to 114%. Both immunoassays were free from interference by CK-BB, mitochondrial-CK, macro-CK, adenylate kinase, and CK-MM. Retrospectively, we evaluated four categories of patients, using both immunoassays and electrophoresis: normal controls, acute myocardial infarction (AMI) patients, severe skeletal muscle trauma patients, and acutely ill patients known not to have AMI. In general, there were excellent correlations among all three methods. CK-MB activity (U/L) measured by the Becton Dickinson immunoassay was ~50% of the mass concentration (µg/L) of the Magic Lite immunoassay and 50% of the activity concentration (U/L) determined by electrophoresis. Both immunoassays were easy to perform and sensitive to the low CK-MB concentrations often found with low CK activity.

Measurement of creatine kinase (EC 2.7.3.2) isoenzyme MB (CK-MB) in serum is still the critical laboratory test in biochemical diagnosis of acute myocardial infarction (AMI) (1, 2). Numerous reports have described the performance and shortcomings of current methods for quantification of CK-MB (3, 4). While several immunoassays have incorporated antibodies directed against subunits CK-M or CK-B, or both, analytical problems have been identified (3-5). Recently, two independent groups of researchers (6, 7) described the use of a unique monoclonal antibody that reacts only to CK-MB, and not with CK-BB, CK-MM, mitochondrial-CK, or immunoglobulin-bound CK-BB (macro-CK). Incorporating these specific antibodies into an assay allowed for the direct measurement of CK-MB in serum.

The purpose of the present study was to examine the clinical and analytical performance of two commercial immunoassays (Becton Dickinson CK-MB; Ciba-Corning Magic Lite CK-MB) in which a monoclonal anti-CK-MB antibody is used for direct measurement of CK-MB in serum.

Materials and Methods

Procedures

For the Becton Dickinson (BD) and Ciba-Corning (Magic Lite) CK-MB assays, we followed the recommended package-insert directions and instrument applications as provided by the manufacturer. A single lot of reagent was used for each kit for the entire study. The BD assay used in this study has not yet been placed on the market, and our evaluation thus constitutes a premarketing trial. For the BD CK-MB immunoassay we used the premarketing system essentially described by the manufacturer. In the assay, run in duplicate, a total sample volume of 400 µL is used, and it measures the activity concentration. The Ciba-Corning “Magic Lite” CK-MB immunoassay was performed according to the manufacturer's guidelines. The assay, run in duplicate, requires a total sample volume of 200 µL, and it measures mass concentration. For electrophoresis, we used the Ciba-Corning Electrophoresis system (Special Purpose agarose film) essentially as described by the manufacturer. Specimens with total CK activity exceeding 500 U/L were diluted before analysis. The percentage CK-MB multiplied by the total CK activity gives the activity of CK-MB. We measured total CK at 37°C in a Paramax analyzer according to the guidelines of the manufacturer (Baxter Clini...