An Attempt to Analyze Various Thyroid Stimulators by the Receptor Assay using hTSH Radioimmunoassay

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Synopsis

In an attempt to analyze thyroid stimulators in serum we developed an assay procedure using hTSH radioimmunoassay (RIA) in combination with receptor competition. The principle of this method is the determination by RIA of hTSH displaced by other thyroid stimulators from a thyroidal receptor preparation which previously bound unlabelled hTSH. Practically 4 μU of hTSH were bound with human or bovine receptor, and then hTSH displaced by addition of test serum (0.1 ml) or samples dissolved in serum (0.1 ml) was measured by RIA.

This assay can determine the thyroid stimulators other than hTSH in serum that has the displacement activity of 0.5–4.0 μU of hTSH in the useful range, such as μU/ml level of bovine TSH or rat TSH. Cholera toxin that has the thyroid stimulating activity like TSH also showed the displacement of the bound hTSH.

This assay is not applicable for the human serum with more than 5 μU/ml of TSH, because the assay value is over estimated by the free hTSH derived from the test serum. On the other hand, eighteen sera with high LATS activity and 42 sera with negative LATS activity from patients with untreated hyperthyroidism did not show any displacement. This might be due to the lower binding activity of LATS with hTSH receptor or the lower sensitivity of this assay method.

Although it is difficult to use this assay clinically because of its low sensitivity, increased TSH in animal serum can be determined by this assay. The principle of this method may be also useful for examining the receptor binding of other peptide hormone that can be determined by an RIA method.

Evidence has been accumulated that polypeptide hormones bind to specific high-affinity receptors located on the cell surface of each target organ. There are several reports of radio-receptor assays of TSH and thyroid stimulating immunoglobulin (TSI) of Graves' disease (Amir et al., 1973; Manley et al., 1974; Smith and Hall, 1974; Verrier et al., 1974; Mehl and Nussey, 1975; Mukhtar et al., 1975; Schleussner et al., 1975; Teng et al., 1975; Endo et al., 1976). In these assays labeled TSH binds to the specific receptor preparation in a membrane or solubilized receptor preparation and is displaced by unlabeled TSH or TSI. However, the sensitivity of the assay was reported to be limited due to the fact that the extent of binding of the labeled hormone to the membranes was affected by the presence of serum proteins or of salt or bivalent cations in the incubation medium.

The authors attempted to analyze animal TSH or other thyroid stimulators by radioimmunoassay (RIA) of unlabeled hTSH which was displaced from thyroid membranes by the thyroid stimulators to be assayed. This was expected to avoid the
loss of biological activity of the hormone by the labeling procedure and the non-specific influence of proteins in the conventional receptor assay. We would like to describe the experimental results by this receptor assay method.

Materials and Methods

LATS bioassay

Bioassay was performed by a minor modification of the Mckenzie method (Ochi et al., 1977).

Receptor fractions from human and bovine thyroid

Thyroid glands obtained at operation from patients with Graves' disease (Kuma Thyroid Clinic, Kobe) were homogenized very gently with 5 volumes of 10 mM tris-HCl buffer, pH 7.6, in ice water. The homogenate was centrifuged at 800 × g for 15 min. The supernatant fraction was centrifuged at 10,000 × g for 30 min, and the pellet was used as crude receptor fraction. Receptor fraction from bovine thyroid was obtained by a similar method. Purified plasma membrane (receptor fraction) was obtained according to Nagai's method (1977). The fraction between 600 × g for 10 min and 20,000 × g for 12.5 min was obtained. Then, the fraction was applied to the upper layer containing two sucrose solutions of 0.32 M and 1.2 M. After centrifugation at 81,500 × g for 60 min, the milky band at the interface between two sucrose layers were collected. These receptor fractions were stored at -20°C until used.

Assay procedure for thyroid stimulator

All assays were performed in duplicate according to the following procedure. In the receptor assay, 0.6 ml of 0.15 M, pH 7.8 phosphate buffer (PB) containing the receptor fraction (0-100 mg protein) was mixed with 0.2 ml buffer containing 8 μU of human TSH (hTSH, Daiichi-radiisotope Lab., Tokyo), and incubated for 60 min at room temperature. Then 0.2 ml of test serum or normal human serum (NHS; 1 μU/ml by TSH RIA) was added and the incubation continued for the following several hours (0.5, 1, 2, 3 and 6 hrs). After centrifugation at 15,000 × g for 30 min at 5°C, the supernatant fraction (SF) was divided and each 0.5 ml fraction was used for hTSH RIA as follows.

hTSH-RIA

The standard curve of RIA for hTSH using 0.1 ml of test serum was set up according to the design of the NIH TSH kit. The incubation medium was consisted of 0.5 ml of SF, 0.2 ml of PB, containing 2% normal rabbit serum (NRS), 0.1 ml of rabbit antiserum for hTSH (supplied by NIAMDD) (1: 20,000 dilution) and 0.1 ml of 125I-hTSH. A standard curve was set up using 0.5 ml of PB containing 0.1 ml of NHS and hTSH instead of the SF. The first incubation was performed for 3 days at 5°C, and then a second incubation (24 hr) was performed after adding the second antibody.

Absorption of LATS activity by the PM

Thirty mg of crude PM and 8 mg of purified PM of human thyroid enough to absorb 4 μU of hTSH were incubated with 0.1 ml of LATS positive sera for 30 min at room temperature. After centrifugation at 15,000 × g for 30 min, the supernatant fraction was assayed by the Mckenzie bioassay (Ochi et al., 1977). Assay samples were injected ip in a volume of 0.5 ml and blood was obtained at 9 and 24 hrs after the injection of test sample.

Results

A) Preliminary experiment

Components in assay system; A typical standard curve of the RIA for determination of hTSH, using 0.1 ml of test serum is shown in Fig. 1. This figure represents

Fig. 1. Standard curve of RIA for hTSH in test serum (ml) by double antibody method.
the average in 3 experiments. The inter-assay coefficient of variations (CV's) from 6 assays for each sample corresponding to 2, 5, 10, 20 and 40 μU TSH/ml was 18, 15, 14, 12 and 17%, respectively. The intraassay CV's for each of the above samples measured in duplicate through 8 assays were 10, 8, 8, 7 and 8%, respectively. The curve was steep up to 40 μU/ml (i.e., 4 μU/0.1 ml of test serum) and then flattened gradually. Therefore, suitable amounts of the receptor fraction to absorb 4 μU of hTSH were examined.

Absorbed hTSH by the receptor fraction was determined from the supernatant fraction after centrifugation of the incubated medium containing the varying quantities of the receptor fraction and 4 μU of hTSH.

As shown in Fig. 2, absorbed hTSH was augmented by increasing amounts of receptor fraction and a dose-response relationship was obtained. More than 20 mg of the crude receptor fraction obtained from bovine thyroid showed almost complete absorbing activity. Human thyroid had less absorbing activity. When the purified receptor of human thyroid was used, 8 mg of the receptor fraction was necessary to absorb 4 μU of hTSH completely.

**Conditions for assay**; The practical application of the receptor assay was performed with the crude receptor fraction of 20 mg of bovine thyroid or 30 mg of human thyroid, or the purified receptor fraction of 8 mg of human thyroid for absorbing 4 μU of hTSH. Then the effect of 0.1 ml test serum on displacing hTSH from the receptor was observed. When NHS (TSH, 1 μU/ml by RIA) was used, no significant displacement was observed within 6 hr at room temperature. However, more than 5 μU of bTSH containing NHS (0.1 ml) showed a strong displacement at 30 min incubation. Displacing activity did not change within 6 hr incubation. Therefore, the displacement activity of test serum was examined at 30 min.

**Calculation of displacement activity by test serum**; The amounts of hTSH displaced from the thyroid receptor by test serum (0.1 ml) were calculated by the subtraction of the amounts of displaced hTSH in the normal control serum (NHS) that were less than 0.3 μU. Thus it is possible to measure the thyroid stimulator having the displacement activity of less than 4 μU of hTSH from the receptor by 0.1 ml test serum.

**B) Application**

**Animal TSH and cholera toxin**; Various amounts of bovine TSH (bTSH, Thytopar, Armour Comp., Chicago) in 0.1 ml NHS

![Fig. 2. Absorption of hTSH by the crude or purified receptor fraction from bovine thyroid or Graves' thyroid.](image)

**Upper**: Crude PM from bovine thyroid or Graves' thyroid was used for absorption of hTSH.

**Lower**: Purified PM from Graves' thyroid was used for absorption of hTSH.

All values are expressed as mean±SD.
were assayed. The bTSH was not detected by hTSH-RIA. In the typical experiment, more than 5 mU of bTSH completely displaced hTSH from the crude receptor of human thyroid. One mU of bTSH displaced 2.2 μU of hTSH from bovine receptor, while the control (NHS) was 0.3 μU. Thus the displaced activity of 1 mU bTSH was calculated as 1.9 μU hTSH (2.2-0.3) as shown in Table 1. In the other experiment, hTSH displaced from human receptor by bTSH or cholera toxin (Schwartz-Mann, N. Y.) was examined (Fig. 3). The dose-dependent displacement of hTSH was observed by bTSH. More than 1 μg of cholera toxin showed the displacement activity of 4 μU of hTSH from the receptor. However, cholera toxin did not show any cross reaction with hTSH.

When the serum of methyl-thiouracil treated rats containing increased TSH (Ochi et al., 1974) was assayed after dilution with normal rabbit serum (NRS), rat TSH also produced complete displacement of hTSH (Table 1).

Sera of untreated hyperthyroid patients; Test sera (TSH < 5 μU/ml by RIA) were

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4 μU hTSH were routinely incubated with the crude receptor, then the displacement activity was determined by adding bovine or rat TSH.

*: Armour TSH preparation containing NHS (0.1 ml) was assayed.

**: Value determined by RIA for rat TSH (Ochi et al., 1974).
examined in this receptor assay*. Eighteen sera from untreated hyperthyroid patients which were highly positive (>250% at 9 hrs in assay response) in LATS bioassay, were examined for displacement activity with the receptor fraction of human thyroid or bovine thyroid. However, no positive displacement was observed in any of these cases (Fig. 4). When 42 LATS negative sera were tested, no significant displacement was observed.

**Fig. 4.** Receptor assay value in LATS positive sera of untreated hyperthyroidism. Displacement activity of hTSH from the crude receptor of human (open circle) or bovine thyroid (closed circle) by LATS.

**Fig. 5.** Absorption of the LATS activity in LATS positive sera by the PM. Crude or purified PM of human thyroid was used for absorption of LATS activity in 4 LATS positive sera. Each experiment was performed in 4 mice. All values are expressed as mean±SD.

Discussion

Several investigators have suggested that two thyroid stimulators, TSH and LATS might interact with the same receptor site on thyroid in the radio-ligand receptor assay (Manley et al., 1974; Mukhtar et al., 1975; Schleussner et al., 1975; Teng et al., 1975). However, there is also reports that TSH and LATS do not bind to the same receptor site (Amir et al., 1973). Mehdi's paper (1975) suggested that the receptor site on the thyroid membrane for TSH and LATS were intimately related, if both were different. The questions are remained open whether LATS and TSH have the same receptor site.

The discrepancy in these previous reports may be due to the inaccuracy of the method of receptor assay. Several problems may upset the receptor assay. One is the loss of receptor binding activity during the preparation of the thyroid tissue. An other is the loss of binding ability of the receptor due to loss of biological activity of the hormone by radioiodine labeling. Another is the influence of the protein concentration in the incubation medium on

* Test sera having 40~200 μU/ml of hTSH-RIA (diluted properly with NHS) was examined by the receptor assay. The assay values were similar to values obtained by RIA. When the values by receptor assay was compared to the value by RIA, a high correlation was observed (r=0.93). This means that the determination of increased hTSH appears to be meaningless and that sera with increased TSH (more than 5 μU/ml) should not be used for this receptor assay.
the binding of labeled hormone.

Schleussner et al. (1975) found that false positive results occurred if the protein concentration in TSH receptor assay system was changed. It is impossible to determine thyroid stimulating substances in the serum directly by the receptor assay. Most investigators isolate the gamma-globulin fraction from test serum (usually 1 mg) for the assay.

In this experiment, to avoid the loss of biological activity of TSH by radioiodine labeling 4 µU of a hTSH preparation with biological activity was incubated directly with the receptor fraction. Then, displacement of hTSH from the receptor was observed by adding 0.1 ml of test serum to avoid the nonspecific effect of protein. The displaced hTSH was determined by RIA. This receptor assay can be detected theoretically, the thyroid stimulators having the displacement activity of less than 40 µU of hTSH in 1 ml of test serum. NHS or NRS did not show any significant displacement usually. More than 0.3 mU of hTSH or 100 ng of rat TSH had a potent displacing activity. Eighteen sera with high LATS activity did not show any positive displacement of hTSH from the PM. This fact might be due to the low binding affinity of LATS with the TSH receptor in human thyroid. The binding of LATS with the PM was observed from the absorption experiment in which the dose of PM was enough to absorb 4 µU of hTSH. LATS activity was absorbed less than 25% by 8 mg of purified PM. This means that the amount of PM is not enough to absorb the LATS activity, and that the large amounts of PM are needed to absorb the LATS activity completely.

TSH is known to have the thyroid stimulating action to bind with high affinity to specific receptor on human thyroid membrane and to increase the production of cyclic AMP. In the mean while LATS is determined by the stimulatory action of mouse thyroid in the Mckeenzie bioassay. The stimulatory action may be caused by the specific binding of LATS with mouse thyroid membrane. There are several evidences that LATS has less stimulating activity for human thyroid (Adams, 1975; Mckeenzie and Zakarija, 1977). Smith's data (1974) that showed no significant correlation between the values obtained by receptor assay and LATS biological activity might be explained by the lack of specificity of LATS for the human thyroid receptor.

In this receptor assay it is impossible to draw conclusion whether LATS does compete for the same receptor as TSH. However, it is interesting that the bovine and rat TSH which do not react immunologically with hTSH have the displacement activity of hTSH from the receptor.

Cholera toxin is known as the universal stimulator of adenyl cyclase in various organs. Mullin et al. (1976) reported that cholera toxin stimulated adenyl cyclase and also bound with the same receptor of TSH in the thyroid. In the previous paper we demonstrated the thyroid stimulating action of cholera toxin (Ochi et al., 1977) and in the present experiment we confirmed an evidence for the binding of both cholera toxin and TSH to the same receptor.

Although this receptor assay is not sensitive enough for the clinical use, the fundamental basis of this method may provide as a tool for the study of hormone receptor.

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