A sensitive and practical assay for thyroid-stimulating antibodies using FRTL-5 thyroid cells

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Abstract. A sensitive, precise and practical assay for thyroid stimulating antibodies was developed in which poorly differentiated rat thyroid cells (FRTL-5) were exposed to crude immunoglobulin fractions precipitated from serum with 15% polyethylene glycol under hypotonic conditions. After the incubation at 37°C for 2 h, cAMP released into Hank’s medium without NaCl was determined by radioimmunoassay. The removal of NaCl from the isotonic Hank’s medium greatly enhanced cAMP production in response to both TSH and thyroid stimulating antibodies. The assay was sensitive enough to elicit an approximately 30-fold increase in cAMP at 10 mU/l bovine TSH. Thyroid stimulating activities measured using FRTL-5 cells significantly correlated with those measured using cultured porcine (r = 0.918, N = 72) or human (r = 0.830, N = 23) thyroid cells. Thyroid stimulating activities were detected in all of the 50 patients with hyperthyroid Graves’ disease, the 14 patients with recurrent hyperthyroid Graves’ disease, and the 25 patients with ophthalmic Graves’ disease. Thyroid stimulating activity was also detected in some patients (9/24, 37.5%) with Hashimoto’s thyroiditis whose serum TSH concentrations were higher than 30 mU/l. However, it was completely abolished by pre-treatment of the sera with anti-TSH antibodies. Although thyroid stimulating activities were detected in one of the patients with simple goitre (N = 10) and in one with thyroid cancer (N = 10), none of the patients with silent thyroiditis (N = 7), adenomatous goitre (N = 11), and thyroid adenoma (N = 9) were positive for thyroid stimulating antibodies.

Thyroid stimulating antibodies (TSAb) in the serum of patients with Graves’ disease are believed to stimulate thyroid activity through activation of adenylate cyclase (Orgiazzi et al. 1976; McKenzie et al. 1978; Kasagi et al. 1980). Since the development of a TSAb assay by Toccafondi et al. (1980), the cAMP increase in cultured thyroid cells has been commonly used as an index of stimulation. Assay sensitivity was enhanced by using a hypotonic assay medium (Kasagi et al. 1982; Rapoport et al. 1982; Zakarija et al. 1985). Furthermore, by measuring cAMP released into the assay medium instead of total cAMP (Rapoport et al. 1984) and adding crude immunoglobulin (Ig) fractions sedimented from serum with polyethylene glycol (PEG) directly into the cultured cells (Kasagi et al. 1986), the assay has recently been simplified and made suitable for clinical use. However, when we use thyroid cells in primary culture, a constant supply of target cells is not available. Recent isolation of a continuously proliferating and partially differentiated rat thyroid cell strain (FRTL-5) maintained in the presence of thyrotropin (Ambesi-Impiombato et al. 1980) has made the development of alternative assay systems possible. In the present study, we applied the modifications described above to the assay using FRTL-5 thyroid cells, and developed a new, practical and sensitive assay for TSAb.

Materials and Methods

1. Thyroid cell culture

FRTL-5 thyroid cells, kindly supplied by Dr L. D. Kohn (NIH, Bethesda, MD, USA), were grown according to
the procedure described by Vitti et al. (1983). The cells were dispersed in 24-well plates (Corning) and allowed to grow to confluency in Coon's modified Ham's F-12 medium containing 5% calf serum (Gibco Laboratories, Chagrin Falls, OH, USA) and a mixture of 1 U/l bovine TSH (bTSH; Thytropar, Armour Pharmaceutical, Phoenix, AZ, USA), $1.3 \times 10^{-6}$ mol/l insulin, $10^{-8}$ mol/l hydrocortisone, $6.3 \times 10^{-11}$ mol/l transferrin, $6.1 \times 10^{-9}$ mol/l somatostatin, and $2.5 \times 10^{-8}$ mol/l glycyl-L-histidyl-L-lysine (all from Sigma Chemical Co, St. Louis, MO, USA) (6H medium). Thereafter the medium was replaced with one composed of all constituents mentioned above except bTSH (5H medium). Unless otherwise noted, cells were used for the assay 7 days after the 5H medium had been added to the cultured cells.

2. Preparation of crude Ig fractions
Approximately 1 h before the assay, 0.5 ml serum samples were mixed with 1.5 ml 20% PEG (w/v; final concentration 15%; mol wt 4000), followed by centrifugation at 2800 x g for 20 min. The pellet was dissolved in 0.6 ml of Hank's medium containing 1.5% (w/v) BSA, 20 mmol/l Heps, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (modified Hank’s medium 1) or 0.6 ml Hank's medium without NaCl containing the same constituents as described above (modified Hank’s medium 2). The assay was performed in duplicate determinations using 0.3 ml aliquots of the medium in each well.

3. Thyroid cell cAMP measurement
After removing the 5H medium, the thyroid cells were incubated with the crude Ig fractions or bTSH dissolved in 0.3 ml of modified Hank’s medium 2 at 37°C for 2 h. After incubation, 100 μl aliquots of the medium were transferred to plastic tubes containing 900 μl of distilled water, frozen and stored at −20°C until used for cAMP measurement by radioimmunoassay (RIA).

The assay was sensitive enough to increase cAMP release 3.5 (± 1.5) (sd), 32.6 (± 15.8), 89.6 (± 33.1) and 134.3 (± 37.5) times at 1, 10, 100 and 1000 mU/l bTSH, respectively.

The inter-assay coefficient of variation of TSAb activity in 2 samples (cAMP release increased 19.5 and 109.2 times compared with the average of release in the presence of several normal samples) in 6 different assays was 21.3 and 34.9%, respectively. TSAb assay using porcine or human thyroid cultured cells was performed using the method previously described (Kasagi et al. 1982, 1986).

4. Patients
Blood samples were obtained from 31 normal subjects, 50 patients with untreated hyperthyroid Graves’ disease, 14 patients with recurrent hyperthyroid Graves' disease, 60 euthyroid patients with Graves' disease under treatment with a maintenance dose of an antithyroid drug (methimazole, 5–10 mg/day, or propylthiouracil, 50–100 mg/day), 25 patients with ophthalmic Graves’ disease, 24 patients with Hashimoto’s thyroiditis, 7 with silent thyroiditis, 10 with simple goitre, 11 with adenomatous goitre, 9 with thyroid adenoma, and 10 with thyroid cancer.

The diagnosis of Graves’ disease was based on the presence of diffuse goitre, thyrotoxic symptoms and signs, elevated serum thyroid hormone concentrations, elevated 30 min $^{99m}Tc$ thyroidal uptake, and detection of circulating antithyroid (thyroglobulin and microsomal) antibodies (Fujizoki, Inc, Tokyo, Japan). Ophthalmic Graves’ disease was defined as ophthalmopathy of Graves’ disease associated with thyroid autoimmunity such as diffuse goitre or detection of antithyroid antibodies in euthyroid or hypothyroid subjects. The diagnosis of Hashimoto’s thyroiditis was based on elevated antithyroid antibody titres in euthyroid or hypothyroid subjects with diffuse goitre and histological findings obtained by needle biopsy. The diagnosis of silent thyroiditis was based on diffuse goitre, hypothyroidism, and low $^{99m}Tc$ thyroidal uptake. The diagnoses of simple goitre and adenomatous goitre were based on diffuse and multinodular goitre, respectively, and histological findings obtained by needle biopsy. The diagnoses of thyroid adenoma and thyroid cancer were based on histological examination of tissue obtained at surgery.

Serum TSH concentrations measured by RIA were lower than 10 mU/l in all patients studied except those with Hashimoto’s thyroiditis (normal range < 5 mU/l). Among the 24 patients with Hashimoto’s thyroiditis, 11 had high serum TSH concentration (> 5 mU/l). In 3 hypothyroid patients with ophthalmic Graves’ disease, blood samples were obtained while they were euthyroid on T4 therapy.

Triiodothyronine (T3) suppression test was performed in all patients with ophthalmic Graves’ disease except 3 who were hypothyroid. $^{99m}Tc$ thyroidal uptake 30 min after the injection (normal range 0.4 – 3.0%) was assessed before and after treatment with T3 for 7 days (75 μg/day). T3 suppressibility was considered positive if the uptake after T3 was less than half the pre-treatment value and less than 1.0%.

Absorption of TSH in sera by antibodies against TSH was performed using the method previously described (Kasagi et al. 1986).

All data were analyzed for statistical significance by Student’s two-tailed t-test.

Results
The ability of an acute addition of TSAb to stimulate cAMP release into modified Hank’s medium 2 was compared on different days (1, 3, 5, 7, 10, 14).
Comparison of cAMP increases induced by TSH or TSAb using two different kinds of medium.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Magnitude (folds) of cAMP increase</th>
<th>Modified Hank's Medium 1 (with NaCl)</th>
<th>Modified Hank's Medium 2 (without NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>bTSH (mU/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.7</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>41.0</td>
<td>82.0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>97.1</td>
<td>251.1</td>
<td></td>
</tr>
<tr>
<td>10 000</td>
<td>114.4</td>
<td>167.8</td>
<td></td>
</tr>
</tbody>
</table>

Experiment 2

| (µL) | K. K. | 10 | 1.6 | 17.6 |
| 25 | 2.2 | 47.0 |
| 100 | 5.3 | 73.6 |
| 250 | 8.6 | 70.9 |
| S. H. | 10 | 1.2 | 1.7 |
| 25 | 1.6 | 4.5 |
| 100 | 2.1 | 23.3 |
| 250 | 3.2 | 51.6 |
| T. S. | 10 | 1.3 | 8.0 |
| 25 | 1.8 | 19.2 |
| 100 | 2.6 | 62.3 |
| 250 | 5.4 | 84.7 |
| F. H. | 250 | 1.3 | 3.9 |
| Y. O. | 250 | 2.5 | 6.0 |
| C. H. | 250 | 1.4 | 8.0 |
| T. S. | 250 | 1.4 | 12.9 |
| Y. K. | 250 | 1.7 | 13.0 |
| T. M. | 250 | 3.5 | 26.1 |
| H. I. | 250 | 7.3 | 47.0 |
| K. M. | 250 | 14.5 | 68.9 |
| S. K. | 250 | 7.2 | 151.2 |
| F. T. | 250 | 6.6 | 196.0 |
| C. T. | 250 | 14.0 | 275.6 |
| N. O. | 250 | 15.2 | 312.0 |

Average** 6.3 ± 4.8 (SD) 89.9 ± 96.2 (SD)

In experiment 2, crude Ig fractions precipitated with PEG from 10, 25, 100 or 250 µl of serum were assayed. Values are expressed in terms of magnitude (folds) of total (both intra- and extracellular) cAMP compared with the values for buffer controls in experiment 1 or with the average values for the PEG precipitates from 10 normal sera in experiment 2. Amount of cAMP was determined after extraction with trichloroacetic acid (Kasagi et al. 1982). *Original serum volume.

** Mean of TSAb activity in 15 crude Ig fractions prepared from 250 µl of sera.

And 10) after TSH withdrawal from 6H medium. Cells deprived of TSH for 7 days gave the highest response (data not shown).

When cAMP increments upon stimulation by increasing doses of TSH or TSAb in two different media (modified Hank's medium 1 and 2) were compared, the assay with the hypotonic medium (medium 2) gave constantly a higher response (Table 1). By using 250 µl of serum, which volume elicited the highest response among 4 different volumes tested (10, 25, 100 and 250 µl), TSAb activities in crude Ig fractions from 15 patients with Graves' disease were compared, and the cAMP increment in the hypotonic medium was about 14-fold greater than that in the isotonic medium (Table 1). Therefore, modified Hank's medium 2 was used in all subsequent experiments.

When the cells were exposed to TSH or Graves' Ig, the ratio of cAMP released into the medium to total (both intracellular and extracellular) cAMP significantly correlated with the total cAMP produced as shown in Fig. 1. The ratio obtained in the presence of TSH was not affected by the addition of the crude Ig fractions from normal controls. Because of the close correlation (r = 0.981) and in view of simplifying the assay procedure, only the amount of cAMP released into the medium was measured in subsequent experiments.

The TSAb activity obtained using FRTL-5 cells showed a significant correlation (r = 0.918, N = 72, P < 0.001) to that measured using cultured porcine cells (Fig. 2). A significant correlation was also observed between TSAb activity assessed using FRTL-5 cells and cultured human thyroid cells, although the number of samples studied was small (N = 23, r = 0.830, P < 0.001). FRTL-5 cells gave a slightly higher response than porcine (Fig. 2) or human cells (data not shown).

As shown in Fig. 3, only 1 out of 31 normal samples showed a value exceeding the upper normal limit (mean ± 2 SD of the normal values). TSAb were detected in all of the 50 patients with untreated hyperthyroid Graves' disease, the 14 patients with recurrent hyperthyroid Graves' disease, and in 42 (70.0%) of the 60 patients with Graves' disease who were euthyroid on a maintenance dose of an antithyroid drug. All 25 patients with ophthalmic Graves' disease including 3 hypothyroid patients and 4 euthyroid patients with positive T3 suppressibility were positive for
TSAb (Fig. 3). Thyroid stimulating activity was also detected in 9 (37.5%) of the 24 patients with Hashimoto's thyroiditis. However, it significantly correlated with the serum TSH levels ($r = 0.801$, $N = 24$, $P < 0.001$), and none of the samples from sera containing less than 30 mU/l was TSAb positive.

When sera from 5 hypothyroid patients were treated with anti-TSH antibodies, the serum TSH concentrations decreased from 33–210 mU/l to less than 10 mU/l, and the thyroid stimulating activity detected in the crude Ig fractions from

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**Fig. 1.**
Relationship between total cAMP generated in response to TSH or TSAb and the ratio of cAMP released into medium to total cAMP. The amount of cAMP in the cells or the medium was measured by the conventional extraction technique using trichloroacetic acid (Kasagi et al. 1982).

![Graph showing the relationship between cAMP generation and TSH or TSAb](image)

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**Fig. 2.**
Correlation between TSAb activity measured using cultured porcine thyroid cells and that using FRTL-5 thyroid cells. Values were expressed in terms of magnitude (folds) of cAMP increase compared with the average of the values for 10 normal samples.
these sera became undetectable. TSAb activity in the crude Ig fractions from patients with Graves' disease, on the other hand, did not change significantly before or after absorption of the sera with anti-TSH antibodies (data not shown). In other thyroid diseases, the incidence of TSAb was 10.0% (1/10) in simple goitre and thyroid cancer and 0% in silent thyroiditis (N = 7), adenomatous goitre (N = 11), and thyroid adenoma (N = 9).

Discussion

Accumulation of cAMP in FRTL-5 thyroid cells after incubation with TSH or TSAb was first demonstrated by Vitti et al. (1982). In comparison with an assay for TSAb using thyroid cells in primary culture, the assay using this continuously proliferating thyroid cell strain (Vitti et al. 1983; Bidey et al. 1985), seems more promising as a routine clinical test, the cells showing more stable and reproducible cAMP responses to the thyroid stimulators. In the present study, we developed and evaluated a new TSAb assay using FRTL-5 cells in hypotonic medium as responders and crude Ig fractions prepared by PEG precipitation as stimulators. The reproducibility of the assay was satisfactory for a bioassay, the coefficient of variation obtained being similar to that reported by Bidey et al. (1985).

Removal of sodium chloride from the medium potentiated the cAMP response (Table 1), as was observed in assays using human (Kasagi et al. 1982; Rapoport et al. 1982, 1984; Zakarija et al. 1985) and porcine (Smith et al. 1985; Kasagi et al. 1986) thyroid cells. Although the mechanism by...
which salt alters the sensitivity of the cAMP response to TSH or TSAb still remains to be elucidated, the use of the hypotonic medium is considered useful for the precision of the assay especially for detecting weak stimulators. In the assay developed by Vitti et al. (1983), the incidence of TSAb in patients with active Graves' disease was 90% and only half of the TSAb-positive samples exhibited more than a 2-fold increase in cAMP, whereas in the present assay, TSAb were positive in all patients with hyperthyroid Graves' disease and four fifths of the samples exhibited more than a 10-fold increase.

The present assay is not only sensitive and precise, but also simple and practical. For the following reasons, therefore, it deserves a routine clinical application. First, unlike when cells in primary culture are used, combersome procedures such as digestion and cell dispersion are not necessary. Second, the amount of cAMP released into the hypotonic medium is directly measured by RIA, instead of intracellular cAMP levels being determined after an ether or alcohol extraction procedure. The ratio of extracellular cAMP to the total amount of cAMP, however, was not constant, but increased from 0.66 to 0.98 (Fig. 1). Therefore, we should keep in mind that the magnitude of cAMP increase calculated from the amount of cAMP release can be estimated to be up to 1.5 times greater than that calculated from the total cAMP. Third, the method of sample preparation using PEG is simple. It takes only one hour to prepare 100 samples. This method of Ig preparation had been found useful in an assay for TSH-binding inhibitor immunoglobulins (Shewring et al. 1982) and also in a TSAb assay using cultured porcine thyroid cells (Kasagi et al. 1986). The latter study revealed that the PEG precipitates from 250 µl of serum from patients with Graves' disease were more stimulatory than 3 mg of purified Igs.

It should be noted that the use of PEG precipitates as samples presents the problem of inclusion of a detectable amount of TSH in the case of serum with high concentrations of TSH (> 30 mU/l), as was previously demonstrated (Kasagi et al. 1986). Although it is rather seldom that TSAb measurement is required in such patients, the problem can be solved by the pre-treatment of the sera with anti-TSH antibodies. Thereby the assay is applicable to the detection of TSAb in hypothyroid patients.

The TSAb activity measured by using FRTL-5 cells significantly correlated with that measured by using porcine or human thyroid cells. This finding, together with the good correlation between TSAb activity measured by using porcine and human thyroid cells (Kasagi et al. 1986) suggests that there is no apparent species specificity among human, porcine and FRTL-5 (rat) thyroid cells as far as cAMP increase is concerned, and that FRTL-5 cells can be an alternative to human thyroid cells for the measurement of human thyroid stimulating activity.

TSAb were specifically detected in patients with Graves' disease except in one patient with simple goitre and one with thyroid cancer who had weakly positive TSAb (cAMP release increased 2.2 and 1.8 times, respectively). The positive TSAb activity detected in these two patients is unexplained at present and needs further studies. Since TSAb were undetectable in all patients with silent thyroiditis in contrast to 100% detectability in those with active Graves' disease, TSAb measurement in thyrotoxic patients can offer a good marker for the differential diagnosis of silent thyroiditis from Graves' disease.

Surprisingly, all patients with ophthalmic Graves' disease were also positive for TSAb. This finding not only indicates the importance of the TSAb assessment for the diagnosis of this condition, but also suggests a close relationship between ophthalmopathy and Graves' disease. This contrasts Solomon's concept (1977) that Graves' ophthalmopathy is an independent disease entity which is different from and frequently complicated with Graves' disease or Hashimoto's thyroiditis. Why do these patients not develop hyperthyroidism in spite of having TSAb? An impaired response of the thyroid to TSAb in ophthalmic Graves' disease, presumably owing to some destructive change, has been postulated (Teng et al. 1977) and is partly proven by the fact that some of the patients are hypothyroid (Christy et al. 1977). As another possibility, some qualitative difference between the TSH receptor antibodies in ophthalmic and thyrotoxic Graves' disease might be speculated, considering the reported lower incidence of TSH-binding inhibitor immunoglobulins in patients with ophthalmic than in those with thyrotoxic Graves' disease (Teng et al. 1977; Konishi et al. 1987). However, the in vivo mechanism by which TSAb induce clinical manifestation of hyperthyroidism still remains to be clarified.
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References


