Bioassay of Thyroid-Stimulating Immunoglobulin in Cryopreserved Human Thyroid Cells: Optimization and Clinical Evaluation

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We have explored the method of Rapoport et al. (J Clin Endocrinol Metab 1984;58:332–8) for the bioassay of thyroid-stimulating immunoglobulin (TSI) in cultured human thyroid cells, to optimize the assay and to evaluate its utility in clinical diagnosis and management of patients with autoimmune thyroid disease. Here we describe the procedure ultimately adopted, its major properties, and the results it has yielded in various clinical states. Clinical sensitivity of the assay was established by demonstrating TSI activity in all of 60 cases of active Graves' disease. We observed in these patients a nonlinear correlation between concentrations of TSI and of triiodothyronine, as well as between TSI concentration and the clinical severity of the thyrotoxicosis. Specificity of the assay was demonstrated by finding no TSI bioactivity in 13 patients with toxic adenoma, five with cold nodule, and 18 of 19 with nontoxic goiter. Remission of Graves' disease in 25 patients was invariably accompanied by undetectable concentrations of TSI; evidently this assay may be useful in identifying patients who are likely to go into remission. TSI activity was present in eight of 11 patients with euthyroid ophthalmopathy (unilateral and bilateral) associated with a normal response to the thyrotropin-releasing hormone test and absence of increased titers of antithyroid antibodies, suggesting that this assay may provide a powerful tool in the clinical diagnosis of this disorder.

Additional Keyphrases: autoimmune disease · Graves' disease · thyrotoxicosis · hyperthyroidism · TSI bioassay · Graves' disease in remission · euthyroid ophthalmopathy

According to current concepts, autoantibodies to thyrotropin (TSH; thyroid-stimulating hormone) receptor, which contribute to the pathogenesis of autoimmune thyroid disorders, are actually a heterogeneous population composed of stimulatory and inhibitory immunoglobulins (1, 2).1 When the patients are thyrotoxic, as in active Graves' disease, the stimulatory antibodies predominate, whereas blocking antibodies may prevail in some cases of hypothyroidism (1, 2). Recent years have witnessed a proliferation of in vitro systems for assaying one of the best characterized of such autoantibodies: thyroid-stimulating immunoglobulin (TSI), as is reviewed in references 1–3. Of these, the development by Rapoport et al. (4, 5) of a bioassay of TSI, with use of cryopreserved human thyroid cells, represents one of the most sensitive and convenient assays.

We describe modifications of the Rapoport technique and define optimal conditions based on our experience with the assay during three years. We further describe our findings, using this assay, for a large series of patients with and without autoimmune thyroid disorders. We also document the clinical utility of the system for the diagnosis and management of various aspects of thyroid disease.

Materials and Methods

Materials

Materials were from the following sources: culture media from Biological Industries, Beth Haemek, Israel; collagenase (type IV), trypsin (type III), and 3-isobutyl-1-methylxanthine from Sigma Chemical Co., St. Louis, MO; "Dispace" (grade II; neutral protease) from Boehringer Mannheim, Mannheim, F.R.G.; 125I-labeled cAMP from Amersham International, Amersham, Bucks, U.K.; cAMP antisera from Bio-Yeda, Rehovot, Israel; bovine TSH from Armour, Eastbourne, U.K. "Medical Research Council standard B" for long-acting human thyroid stimulator (LATS), which served as a TSI reference preparation, was kindly supplied by the National Institute for Biological Standards and Control, London, U.K.

TSI Bioassay

The assay, based on the use of cultured human thyroid cells, preserved in liquid nitrogen, was performed as described by Rapoport et al. (4, 5), with the following modifications:

- We used colloid-goiter tissue (from nodular-goiter patients) obtained at thyroidectomy.
- For follicle dispersion we used either collagenase (1 mg/mL) or "Dispace" (neutral protease, 5 mg/mL), as recommended in the earlier version of the Rapoport method [Hinds et al. (6)].
- The primary culture interval was restricted to three or four days.
- Serum samples, after precipitation with ammonium sulfate, 1.6 mol/L final concentration (5), were tested at a concentration of 9 mg of immunoglobulin (Ig) per milliliter (1.8 mg of Ig in 0.2 mL of medium, in 0.28 cm² microtiter wells).
- Total cAMP accumulation, both intracellular and that released into the hypotonic medium [sodium chloride-free Hank's balanced salt solution containing, per liter, 20 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 2 mmol of 3-isobutyl-1-methylxanthine, and 3 g of bovine serum albumin, pH 7.4], was measured after thyroid cells (25 000 cells per well) were exposed to Ig samples as follows. We aspirated the hypotonic medium after 4 h of incubation at 37 °C in air, then added 0.4 mL of cold (4 °C) absolute ethanol to the medium. We then added 0.2 mL of cold (4 °C) absolute ethanol to each well, and stored the multiiwell plate overnight at −20 °C. The cells were then scraped from the wells, combined with the previously aspirated medium, and centrifuged at 700 × g for 10 min at room temperature. The supernatant fluid was air-dried and the residue reconstituted in 2.5 mL of sodium acetate buffer (50 mmol/L, pH 6.2). We acetylated 100-μL aliquots of this

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1 Nonstandard abbreviations: TSH, thyrotropin (thyroid-stimulating hormone); TSI, thyroid-stimulating immunoglobulin; TRH, thyrotropin-releasing hormone; Ig, immunoglobulins; LATS, long-acting thyroid stimulator; and T3, triiodothyronine.

Received July 27, 1987; accepted November 10, 1987.

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and measured cAMP by RIA as described by Rapoport et al. (5).

TSI activity in a sample was expressed as a percentage of the basal activity, as follows:

\[
\text{TSI} = \frac{\text{cAMP accumulated in presence of test Ig}}{\text{cAMP accumulated in presence of normal Ig}} \times 100%. 
\]

Normal Ig was prepared from a pool of normal subjects. All samples were tested in duplicate wells.

Patients

Serum samples were obtained from patients with various thyroid disorders, who were attending the outpatient clinic of the hospital. The patients were categorized according to the following diagnostic criteria:

Active Graves’ disease: Clinical and laboratory evidence of hyperthyroidism, diffuse homogeneous distribution of technetium on scintiscan, and increased radioiodine thyroid uptake, with or without ophthalmopathy.

Graves’ disease in remission: Clinical and laboratory evidence of euthyroidism for at least 1½ years after discontinuation of antithyroid drug therapy for hyperthyroidism due to Graves’ disease. No relapse has yet been noted in any of the patients.

Hashimoto’s thyroiditis: Clinical signs of hypothyroidism, diffuse goiter, increased serum TSH, and increased titers of antimicrosomal or antithyroglobulin antibody in serum.

Primary myxedema: Same as Hashimoto’s thyroiditis, but with no goiter palpable.

Euthyroid ophthalmopathy: Clinical and laboratory evidence of euthyroidism, normal TSH response to the thyrotropin-releasing hormone (TRH) test (7), and unilateral or bilateral ophthalmopathy. A nonendocrine cause of the ophthalmopathy, such as local tumor or vascular lesion, and congenital exophthalmos were excluded by computed-tomographic scan or orbital ultrasonography, or both.

Hypofunctioning “cold” nodule: Clinical and laboratory evidence of euthyroidism, in association with a solitary nodule (determined by palpation) that failed to take up technetium (as determined by scintiscan).

Nontoxic goiter: Clinical and laboratory evidence of euthyroidism, in association with a multinodular or diffuse goiter on palpation and scintiscan.

Toxic adenoma: Clinical and laboratory evidence of hyperthyroidism, in association with a solitary nodule by palpation, and scintiscan exhibiting localization of technetium in a single nodule (“hot nodule”).

Serum samples were obtained at or near the time of diagnosis and before any treatment was given. Informed consent was obtained from the patients and the investigation was approved by the hospital’s human-research committee.

Controls consisted of normal subjects with no history of thyroid disease or clinical and laboratory evidence of euthyroidism. None had detectable titers of antimicrosomal or antithyroglobulin antibodies.

Results

System Characterization

Table 1 shows the yield and the response to TSH and TSI of thyroidal cell preparations of different origins. Colloid goiter tissue (from nodular goiter patients) is clearly superi-...
25% of the original amount of TSH in the serum, as measured by RIA (8). This observation, together with the high sensitivity of the thyroid cell bioassay to TSH, necessitates ascertaining—especially in sera with high concentrations of TSH such as are present in Hashimoto's thyroiditis and primary myxedema—that the biological activity observed can indeed be ascribed to immunoglobulins and not to possible contamination with TSH. For this purpose, we used the thyroid cell bioassay to test 23 sera from patients with Hashimoto's thyroiditis, primary myxedema, or thyroid cancer post-thyroidectomy, in which TSH concentrations ranged from 5 to 160 milli-int. units/L, as well as control serum samples supplemented with TSH concentrations in the range of 10–120 milli-int. units/L. All had no stimulatory activity.

We performed an electron-microscopic study of thyroid cells before and after freezing, to determine the influence of cryopreservation in liquid nitrogen, as routinely performed for the preparation of thyroid cells for the TSH bioassay, on the ultrastructural morphology of human thyroid cells. Only minor intracellular changes, restricted to the mitochondria, were apparently induced by the freeze–thaw procedure. This is consistent with the high viability and responsiveness of the cryopreserved cells to the stimulatory agents (9).

TSI Activity in Thyroid Diseases (Figure 3)

The mean (+2 SD) percentage change from baseline cAMP of normal control Ig samples was 160% (dashed line in Figure 3). This value was therefore taken as the cutoff point demarcating the presence or absence of TSI activity. Such activity was demonstrable in the sera of all of the 60 patients with untreated active Graves' disease that we assayed. Only 25% of such patients had TSI values between 160% and 250% of basal activity. The rest exhibited higher TSI values, which enabled much more distinct separation from controls. Graves' disease patients in remission (n = 25) after treatment with antithyroid drugs were devoid of TSI bioactivity. Non-autoimmune thyroid disease—cold nodule, non-toxic goiter (with a single exception in this group), and toxic adenoma patients—were found to be devoid of TSI bioactivity.

Of the 50 patients with hypothyroid autoimmune thyroiditis (Hashimoto's thyroiditis and primary myxedema), 42 had undetectable TSI while eight (16%) showed detectable, albeit low (<250%), TSI activity. Of 11 patients with euthyroid ophthalmopathy, TSI was positive in eight instances, although here again the activity was relatively low, less than 250% of normal, except for two patients with values of 262% and 410%. None of the patients tested in this group had above-normal titers of antimicrosomal or antithyroglobulin antibodies.

Relationship of TSI Activity to Thyroid Function and Clinical Status in Active Graves' Disease

No significant linear correlation (P > 0.05, linear regression analysis) was observed, in active untreated Graves' disease patients, between TSI values and values for triiodothyronine (T3) (r = 0.258, n = 42) or free thyroxin (r = 0.087, n = 42) in serum or radiiodine uptake at 2 (r = 0.273, n = 31), 4 (r = 0.239, n = 32), and 24 (r = 0.283, n = 31) hours. However, when the patients were classified according to their level of TSI activity, T3 concentration, and degree of severity of clinical manifestations, a clear trend emerged (Table 2). Relatively mild increases in TSI (160–250%) were seen with both mild and marked increases in T3, whereas marked increases in TSI (>250%) were almost exclusively associated with marked increased in T3 (P < 0.05, x^2 test). Similarly, mild thyrotoxic symptoms (grades I and II in Table 2) were associated with mildly increased TSI, whereas severe clinical symptoms (grade III in Table 2) were only seen when TSI values exceeded 250% of normal (P < 0.05, x^2 test).

Discussion

The introduction by Rapoport and his group of a bioassay for TSI involving the use of cryopreserved human thyroid cells (6), together with their subsequent modification (4, 5) of the technique by performing the cell stimulation in hypotonic medium (50), offered a significant advance in TSI assay methodology. This assay overcame the major drawbacks inherent to the other widely used TSI methods,
Table 2. Correlation of TSI Activity in Patients with Active Graves' Disease and Concentrations of Trilodothyronine (T₃) in Serum, and with Clinical Severity of the Disease

<table>
<thead>
<tr>
<th>TSI, % of basal activity</th>
<th>No. of patients</th>
<th>With serum T₃⁺ of</th>
<th>With symptoms and signs of grade*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.71–3.0 µg/L</td>
<td>&gt;3.0 µg/L</td>
</tr>
<tr>
<td>160–250</td>
<td>12</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>251–1000</td>
<td>21</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>9</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Normal values: 0.7–1.7 µg/L. * The clinical degree of severity of thyrotoxicosis was evaluated by two experienced thyroidologists, who took into consideration the full clinical presentation, and was classified as mild (I), moderate (II), or severe (III).

namely, assays based on inhibition of TSH receptor binding, which also measure non-stimulating antibodies, and techniques involving FRTL-5 cells—a rat thyroid cell line with limitations imposed by species specificity (11–13). We have further developed this method and have attempted to optimize the system.

The data presented here allow us to draw the following conclusions regarding this assay:

1. Compared with Graves' and normal thyroid tissue, colloid-goiter tissue affords a higher yield of cells, which also are more responsive to both TSI and TSH. This finding—not previously reported—presents obvious advantages in that colloid tissue is more abundant, both in terms of frequency with which surgery is done and the amount of tissue available at each such operation. Furthermore, the higher cell yield and responsiveness, as well as the possibility of cryopreservation, allows many assays to be performed on a single tissue preparation, with use of relatively few cells, in microtiter multi-well plates. Thus, the reproducibility and convenience of the assay are significantly enhanced and potential problems of species specificity (such as with the FRTL-5 rat cell line) are obviated without compromising the excellent sensitivity and specificity of the assay.

2. We found the efficacy of collagenase for follicular dispersion to be variable. Our observation that Dispase can be effectively and safely used in those cases that are resistant to collagenase further increases cell yield.

3. Although previous reports suggest that a primary culture of five to seven days is needed, our finding that three or four days suffices simplifies and shortens the procedure of preparing the cells and decreases the risk of fibroblast proliferation or microbial contamination.

4. By measuring total cAMP accumulation (i.e., in both medium and cells) rather than in medium alone, one need not assume that for all TSI concentrations the cell:medium cAMP ratio is constant. Furthermore, this modification permits the increase of total Ig concentration to 9 mg/mL, a concentration at which the cell:medium cAMP ratio has been shown to be inconstant (4). Both of these factors further enhance the sensitivity of the assay without compromising its specificity.

5. Many investigators have used the TSI bioassay, not only in patients with increased thyroid function where the endogenous TSH concentrations are low, but also in studying patients with hypothyroidism. Therefore, our demonstration that high TSH concentrations in serum do not interfere with the interpretation of the assay results is a necessary validation procedure that has not been previously reported.

6. Although expressing the TSI activity in terms of TSH equivalents may appear more logical, we confirm here the findings of others (7) that expression of the results in terms of percent of basal activity enhances the assay's ability to distinguish between autoimmune and non-autoimmune thyroid disease.

Having made these modifications, we have demonstrated the sensitivity and specificity of the assay by finding increased TSI in 100% of 60 patients with active Graves' disease (75% of them markedly increased, >250% of basal activity), and in only 3% (of 37) of patients with non-autoimmune thyroid disease (cold nodule, nontoxic goiter, and toxic adenoma). Moreover, none of the 25 patients with Graves' disease in remission had increased values for TSI, a finding with obvious clinical implications. In all, the assay's performance seems unsurpassed by other procedures in which cultured human thyroid cells are used (5, 10, 14–18).

No significant linear correlation was found in active Graves' disease between TSI values and:

(a) radioiodine uptake, as Bech and Nistrup Madsen (19) and Macchia et al. (20) found, but unlike Mukhtar et al. (21), Clague et al. (22), Endo et al. (23), and Kuzuya et al. (24);

(b) thyroxin free in serum, in agreement with Orgiazzi et al. (25), Endo et al. (23), Bech and Nistrup Madsen (19), Kuzuya et al. (24), and Macchia et al. (20);

(c) T₃ concentration, as also found by Orgiazzi et al. (25), Endo et al. (23), Kuzuya et al. (24), and Macchia et al. (20), but unlike McGregor et al. (26), Bech and Nistrup Madsen (19), and Grant et al. (18).

However, when the degree of thyrotoxicosis was classified as mild, moderate, or severe in terms of T₃ concentration or in terms of clinical severity of symptoms, a significant association was found with the degree of increase in TSI activity. The lack of a stricter correlation between TSI titers and the degree of thyrotoxicosis may be explained by the diversity of biologically active antibodies—stimulating, blocking, as well as destructive—that modulate the hormonal response of the thyroid target organ and determine the final clinical expression.

Having established the sensitivity and specificity of the assay, and having examined its utility in the evaluation of Graves' disease, we then proceeded to study other clinical entities whose autoimmune etiology and (or) pathophysiology are less well described via-a-vis TSI bioactivity.

While a large majority (84%) of our patients with hypothyroid autoimmune thyroiditis (Hashimoto's thyroiditis and primary myxedema) had undetectable TSI, 16% (eight of 50) of such patients—without co-existent ophthalmopathy—exhibited positive, albeit low, TSI values. Similar findings were also reported by Grant et al. (18), who used a cultured human thyroid cell method (1/9 TSI-positive), and by Vitti et al. (27), who used the FRTL-5 rat thyroid cell procedure (3/7 TSI-positive). The presence of TSI activity—the hallmark of Graves' disease—in some patients with autoimmune thyroiditis is consistent with certain other shared immunological and clinical characteristics of these
autoimmune thyroid disorders: the finding of antimicrosomal and antithyroglobulin antibodies in both disorders, occurrence of co-existing disease ("Hashitoxosis" or "semi-Hashi"), familial disease clustering, and the occasional onset of one disease before the other (28, 29).

TSI was detectable in 73% (eight of 11 patients) with euthyroid ophthalmopathy in the absence of any other characteristic sign of thyroid disease. No significant difference ($P > 0.05, \chi^2$ test) was found in the ratio of unilateral to bilateral ophthalmopathy in the TSI-positive (three unilateral, five bilateral) and negative patients (two unilateral, one bilateral). In contrast, Rapoport et al. (5) reported that TSI was non-detectable by the human cell culture procedure in all five of their cases with unilateral euthyroid ophthalmopathy. The fact that, in our study, TSI activity was present in such a high percentage of euthyroid ophthalmopathy patients, all of whom had a normal response to the TRH stimulation test, and in the absence of concurrent antimicrosomal or antithyroglobulin antibodies, is of critical importance. Zakarija and McKenzie (30), using a slice assay, could only detect TSI (six of 10 patients) in euthyroid ophthalmopathy patients associated with either an abnormal response to the TRH stimulation or $T_2$ suppression test and presence of antithyroid antibodies; the four TSI-negative patients in that study had a normal TRH test result. Smyth et al. (31), using a cytotoxic assay, could measure TSI in euthyroid ophthalmopathy patients who did not concomitantly have increased antimicrosomal or antithyroglobulin antibodies, but with a decreased prevalence in those patients who responded normally in the TRH test compared with those who had impaired responses (three of 13 compared to 11 of 13 TSI-positive patients, respectively). Our finding of a positive TSI value in these patients can be expected to significantly aid the clinician confronted with the difficulty of arriving at a correct diagnosis of euthyroid ophthalmopathy based exclusively—as it is today—on elimination of other causes and on nonspecific signs. The findings of TSI at relatively low titers, and/or the coexistence of antibodies with agonist and antagonist properties and a decreased thyroid reserve, may explain the euthyroid status despite positive results for TSI seen in these patients.

In conclusion: we present modifications of a well-defined TSI bioassay and describe optimal conditions that increase the sensitivity and convenience while maintaining a high degree of specificity. Clinical use of the assay showed: (a) improved sensitivity in the diagnosis of active Graves' disease, (b) absence of such activity in patients in remission of the disease, and (c) high prevalence of TSI activity in patients with euthyroid ophthalmopathy (unilateral and bilateral) associated with a normal TRH test result and absence of increased antithyroid antibodies.

We are much indebted to Dr. B. Rapoport for his continued and most valuable help in setting up the cell culture technique. We also gratefully acknowledge the Endocrine Laboratory, Lin Clinics, for performing the thyroid hormone and TSH estimations; the Immunology Laboratory, Carmel Hospital, for the antimicrosomal and antithyroglobulin assays; the Endocrine Institute, Lin Clinics, for the serum samples; the Surgery and Pathology Departments, Carmel Hospital, for the human thyroid tissue samples; and the Nuclear Medicine Department, Carmel Hospital, for the thyroid scintiscans.

This work was supported by a grant from the Chief Scientist's Office, Ministry of Health, Israel. The National Institute for Biological Standards and Control, London, U.K., kindly supplied the LTS standard used in the study.

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