Assays for Thyrotropin-Receptor Binding and Thyroid-Stimulating Antibodies in Sera from Patients with Graves' Disease

Catherine Massart, Bernard Hody, Luc Mouchel, Gilles Edan, and Marc Nicol

We compared the activities of thyroid-stimulating antibodies (TSAb) measured in cultures of human thyrocytes and the values for thyrotropin-receptor antibodies (TRAB) as measured with a commercial kit based on use of radiolabeled receptors. Sera were obtained from patients with Graves' disease before, during, and after therapy with carbimazole (1-methyl-2-thio-3-carbethoxyimidazole). We found a significant correlation between the measurements of these two antibodies in patients: before treatment ($r = 0.74, p < 0.01, n = 44$), after three months of treatment ($r = 0.76, p < 0.01, n = 21$), and during relapse after the drug was discontinued ($r = 0.64, p < 0.01, n = 19$). In all three situations, our TSAb technique was more sensitive than the TRAB method. We conclude that, even though the TRAB technique is simpler and quicker, this commercial kit is too insensitive to replace measurement of TSAb in fresh human thyrocyte cultures for management of drug therapy of patients with Graves' disease.

Additional Keyphrases: radioreceptor assay · "kit" method · culture of human thyrocytes · monitoring therapy · autoantibodies

Graves' disease is an autoimmune disease involving immunoglobulins (Ig) that act against thyrotropin receptors in the thyroid gland (1-3). The binding of these antibodies to the thyrotropin receptors activates the adenylate cyclase (EC 4.6.1.1) system, bringing about an increase in secretion of thyroid hormones. Among the many different methods proposed for assaying these Ig are those based on inhibition of binding of radiolabeled thyrotropin to specific receptors (TRAb assay) (3-5) and procedures measuring the stimulation of adenylate cyclase system in slices of thyroid (6, 7), in thyrocyte membranes (7-9), or in cultures of thyrocytes (10, 11) (TSAb assay).

Recently, several groups (12, 13) have advocated TSAb assay in thyrocyte cultures as a highly sensitive method. We adapted the TSAb assay for use with cultures of human thyrocytes and have reported (14), as Grant et al. (15) did, its usefulness in managing patients with Graves' disease who are being treated with drugs; i.e., the optimal time to discontinue the drug is when TSAb become undetectable. Unfortunately, because the assay is long and difficult, it is unsuitable for use with large numbers of samples. We therefore tested a rapid, simple commercial TRAB assay kit and compared results with those of the TSAb assay: analytically, by precise quality controls of the two methods; and clinically, by evaluating the correlations of results observed for patients with Graves' disease before, during, and after drug therapy.

Materials and Methods

Patients

We studied 44 patients (13 men, 31 women, average ages 46 and 42 years, respectively) with Graves' disease. Diagnosis was based on the usual clinical criteria: goiter, occasional exophthalmus, and increased concentrations of the free thyroid hormones, triiodothyronine and thyroxin. Toxic nodules were excluded by computer tomographic scan.

Forty-three sex- and age-matched healthy blood donors served as controls.

We assayed the antibodies in the patients' sera before drug therapy with carbimazole (1-methyl-2-thio-3-carbethoxyimidazole) was instituted during the treatment course (after about three months), and after the drug was discontinued. Blood was sampled into tubes containing no anticoagulant, then centrifuged at 1000 x g for 10 min at 4 °C. The serum was decanted and divided into 600-μL aliquots for storage at -20 °C until assay.

TSAb Assay

We assayed TSAb in human thyrocyte cultures according to the method described by Madec et al. (16), with the following modifications:

We cultured cells from human thyroid glands taken from patients free of thyroid disease—e.g., during excision of a cold nodule or subtotal laryngectomy. We also tested thyroid tissue from patients with thyroiditis. Enzyme dispersion of the cells was produced by "Dispase II" (5 g/L), a neutral Bacillus polymyx protease (EC 3.4.24.4) from Boehringer-Mannheim, F.R.G., in the method described by Stöckle et al. (13). Cellular reactivity (cAMP production) was assessed by stimulation with bovine thyrotropin provided by Sigma Chemical Co., St. Louis, MO.

To measure the total free cAMP produced during 48 h of incubation, we used a commercially available protein-binding assay (Amersham International, Amersham, Bucks, U.K.). Finally, we tested cryopreserved (-80 °C) thyrocyte cultures, to evaluate their reactivity after having been rewarmed to 37 °C and recultured. Aliquots of cell suspensions, 10 x 10⁶ cells in Ham's solution F12 ( Gibco Laboratories, Grand Island, NY), which contained 100 mL of fetal calf serum per liter, were placed in cryopreservation tubes, cooled to 4 °C, mixed with 100 mL of dimethyl sulfoxide (Sigma) per liter, then frozen at -80 °C for one week to one month. Suspensions to be recultured were warmed to 37 °C, then centrifuged at 500 x g for 5 min. The sediment was recovered in Ham's F12 solution containing fetal calf serum (100 mL/L) for culturing. All determinations were made in at least two separate assays, with three determinations per assay. TSAb activity was expressed as a percentage of thyrocyte stimulation (determined by measuring cAMP production) during 48-h culture as compared with the stimulation observed for pooled equal volumes of the 43 control sera. In the control sera, the variations in basal

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TSAb values ranged from 78 to 123% (100 ± 11%, mean ± 1SD). TSAb values >122% were thus designated as positive.

TRAb Assay

We measured TRAb activity with a commercial radio-receptor kit provided by Byk-Mallinckrodt France ("Thybias", Mallinckrodt Diagnostica, F.R.G.). The technique as described by the manufacturer is as follows: Mix 50 μL of serum (normal serum furnished in the kit or patient's serum) with 100 μL of a solution of porcine thyrotropin receptors (dissolved in a buffer solution, pH 7.5, containing 10 mmol of NaH2PO4 and 50 mmol of NaCl per liter). Incubate for 20 min at room temperature, then add 100 μL of 125I-labeled bovine thyrotropin and incubate for 2 h at room temperature. Add 1000 μL of polyethylene glycol 6000, and centrifuge at 2500 × g for 30 min. Discard the supernate and count the radioactivity of the sediment.

Total binding of 125I-labeled thyrotropin to thyrotropin receptors was 31.4% (SEM 3.8%); nonspecific binding was 8.3% (SEM 1.1%). To decrease the nonspecific binding, we added an additional step to the procedure, gently washing the sediment with 1 mL of a 40 g/L solution of polyethylene glycol 6000 before counting the radioactivity; the nonspecific binding was reduced to 3.4% (SEM 1.1%).

We expressed the amount of TRAb present as a percentage of displacement, as suggested by several authors (5, 15):

% displacement = 100 × (thyrotropin specifically bound in sample serum/thyrotropin bound in normal serum)

Results

Analytical Variables

CAMP assay to evaluate the amounts of TSAb present. The lowest detectable amount of cAMP, calculated as the mean +3 SD of the measurement for 0 TSAb, is 0.2 pmol of cAMP per 50-μL original sample.

Repeatability, as evaluated from the within-run CV for 12 assays of two control sera with average cAMP concentrations of 3.1 and 7.5 pmol/mL, was 7.1 and 8.9%, respectively.

Reproducibility was determined in 21 series of assays: the between-run CVs, calculated from assays of samples cited above, were 7.4 and 10.3%, respectively.

Reactivity of cultured human thyrocytes. CAMP concentrations produced after stimulation by increasing concentrations of bovine thyrotropin in fresh and cryopreserved human thyrocytes are shown in Figure 1. Thyrocytes cultured from normal glands responded with an increase in cAMP that was linearly related to concentrations of bovine thyrotropin from 0 to 300 milli-int-units/L. In fresh thyrocytes the cAMP concentration stimulated by maximum bovine thyrotropin (300 milli-int-units/L) was about sixfold the prestimulation value. Thyrocytes stored for 20 and 106 days at −80 °C and then stimulated in culture produced 50 and 70% less increase in cAMP, respectively, than did fresh thyrocytes. Linear curves for thyrocyte stimulation were also observed in thyrocyte cultures coming from a diseased gland, whether assayed in fresh tissue or after freezing. However, cryopreserved thyrocytes were so fragile that we found freshly prepared cultures to be more suitable. Although these findings suggest a similar response in cultures obtained from normal or thyroiditis-affected glands, we chose to assay TSAb only in cell cultures originating in healthy individuals, because all the stimulation tests with diseased tissue came from one gland and could not be renewed.

Precision and parallelism of the TSAb and TRAb assays. Between- and within-run precision was satisfactory for both TSAb and TRAb assays, the CV being <16% (Table 1). Dilution curves for TSAb and TRAb assays, prepared from data on sera from patients with Graves' disease, were parallel straight lines.

TSAb and TRAb Activity in Normal Controls and Patients

These data are reported in Figure 2. The mean TRAb activity in the 43 control sera was 93% (SD 4%). TRAb values <85% were thus considered to be positive. Positive TSAb results were observed in 41 of the 44 (93%) patients with Graves' disease, before therapy, the mean activity being 450% (SD 325%) of normal values. Although TRAb results were positive in only 30, sera of these (68%), with

Fig. 1. CAMP concentrations after stimulation by bovine thyrotropin in thyrocytes from four disease-free glands (a) and from one gland affected by thyroiditis (b)

Bars indicate the mean ± SD of six incubations. ▲, ▲, fresh thyrocytes. ☆, O, thyrocytes cryopreserved (−80 °C) for 20 days. ■, thyrocytes cryopreserved for 106 days.

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Table 1. Precision of the TSAb and TRAb Assays

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mean activity of 76% (SD 13%), the TSAb and TRAb activity correlated significantly \( r = 0.74, p < 0.01 \). Both assays gave positive results for 23 of the 44 sera. TRAb was negative and TSAb was positive for 12 sera, and one serum was negative for TSAb and positive for TRAb.

TSAb results were positive in 16 of the 21 (76%) patients who had been receiving drug therapy for three months. TRAb results were positive in only seven (33%) of these patients. The two activities were significantly correlated \( r = 0.76, p < 0.01 \).

Positive TSAb values were found in 16 of the 19 (84%) patients who relapsed after discontinuing the drug. TRAb values were positive in only seven (36%) of these patients. The TSAb and TRAb activities were also correlated \( r = 0.64, p < 0.01 \).

The TSAb activities were weakly positive in two of the 15 patients remaining in remission after discontinuation of the drug for at least six months. TRAb results were negative in these 15 patients.

Discussion

We show here a difference in the sensitivity of TSAb and TRAb assays. We used fresh human thyroid cells for TSAb assay; cells cryopreserved at \(-80^\circ C\) are more convenient for routine assays, but these cells alter rapidly. Several groups have cryopreserved cells at \(-80^\circ C\) for a few days, then stored them in liquid nitrogen before use for TSAb assays. Their results, however, vary widely: Grant et al. (15), Hinds et al. (17), and Davies et al. (18) found 96%, 83%, and 71% of their untreated patients with positive TSAb, respectively.

On the other hand, authors reporting techniques involving fresh thyrocyte cultures have found results in agreement with our findings:

- 80 to 100% positive TSAb results were observed in patients with Graves' disease before therapy (11-13, 19-21).
- both TSAb and TRAb values declined during drug therapy (21).
- In case of relapse, Creagh et al. (19) found 72% patients with positive TSAb.

Our findings show the relatively poor sensitivity of TRAb assay. All the reported assays involving crude membrane receptors or buffer solutions of receptors gave positive TRAb results in 50 to 76% patients with Graves' disease before therapy (5, 15, 22-25). Our results agree with these authors' findings. Smith et al., however, obtained a higher sensitivity (96% patients with positive TRAb) by use of detergent-solubilized receptors (27, 28).

We found a satisfactory correlation between TSAb and TRAb in all sera assayed. Conflicting results have been reported: many investigators (4, 22, 23, 29, 30) found no correlation between TSAb and TRAb activity; others (31, 32) found a good correlation. It has been suggested that the poor sensitivity of the TSAb methods involving measuring
the stimulation of the adenylate cyclase system in slices of thyroid or thyrocyte membranes could explain these differences. Nevertheless Creagh et al. (19) and Grant et al. (15), who used very sensitive TSAb assays in thyrocyte cultures, also reported conflicting results. Creagh et al. found, as we have, a good correlation between TSAb and TRAb values for treated and untreated patients. Grant et al. found no correlation, but they did not report on the analytical quality control of their methods.

We conclude that a rapid and simple commercial TRAb assay kit gives results closely correlated with TSAb values but has a poor sensitivity. Thus this kit does not give results that are reliable enough for management of drug therapy of patients with Graves' disease.

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References