Importance of the Detection Method for Thyroglobulin Antibodies for the Validity of Thyroglobulin Measurements in Sera from Patients with Graves Disease

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Background: The use of recovery tests has been proposed to disclose interferences from anti-thyroglobulin antibodies (TgAbs) in thyroglobulin (Tg) assays. We studied the value of a recovery test in Tg measurement by a new commercial IRMA.

Methods: Blood samples were collected from 153 patients with untreated Graves disease. Tg and TgAbs were measured by IRMA and RIA, respectively (Dynotest Tg-plus and Dynotest anti-Tgn; Brahms Diagnostica). The recoveries of added amounts of Tg were calculated for each serum.

Results: TgAbs were detected in 72 of the 153 patients (47%). The recovery test results for the 81 TgAb-negative sera (median, 101%; range, 80–115%) were identical to the results for the 91 controls (median, 102%; range, 80–124%). By contrast, significantly lower recovery test results were observed for the 72 TgAb-positive sera (median, 79%; range, 60–103%; Z = 8.36; P < 0.0001). In the 34 of the 72 TgAb-positive sera with a normal recovery test, Tg concentrations were significantly lower (median Tg, 13.6 μg/L; range, 1.1–360 μg/L) than those measured in the TgAb-negative sera (median, 107 μg/L; range, 1.2–700 μg/L; Z = −3.79; P < 0.0001).

Conclusions: Tg values were decreased in TgAb-positive sera even when the results of the recovery tests were normal. This test should not be used alone to determine the validity of a serum Tg measurement in Graves disease.

Graves disease is an autoimmune disorder characterized by hyperthyroidism attributable to thyroid-stimulating hormone (TSH) receptor autoantibodies with thyroid-stimulating activity (1–3). The measurement of TSH receptor-binding antibodies in serum can be used to follow the course of Graves disease (4, 5). However, this single marker cannot predict the outcome for individual Graves patients (6) even when TSH receptor-binding antibodies are assayed with a sensitive radioreceptor assay (7). Because the serum thyroglobulin (Tg) concentration reflects the magnitude of TSH receptor stimulation (8), it has been reported that measurement of serum Tg can provide useful information for monitoring the course of Graves disease and for managing therapeutic treatment (9–12).

Measurement of Tg in serum is hampered by the presence of circulating interfering factors, especially Tg antibodies (TgAbs), which are frequent in Graves disease and which affect the reliability of Tg assays (8, 13). Because most current RIA methods do not reliably measure serum total Tg, IRMAs using monoclonal TgAbs that do not cross-react with endogenous TgAbs have been developed (14, 15). These IRMAs do not necessarily provide any substantial advantage over a conventional polyclonal IRMA in detecting Tg in TgAb-positive sera (16).

It has been suggested (17) that quantitative assessment of Tg in TgAb-positive sera may be achieved by determination of the recovery of known amounts of Tg added to TgAb-positive samples. We used a new commercially available IRMA incorporating both polyclonal and monoclonal TgAbs to test TgAb interference and the value of the recovery test in sera from nontreated Graves patients.

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Materials and Methods

Patients
The study involved 153 patients (125 women and 28 men; median age, 42 years; range, 17–65 years) with Graves disease diagnosed from typical clinical signs: hyperthyroidism, vascular and homogeneous goiter, occasional exophthalmos, increased free thyroid hormone concentrations (free triiodothyronine >8.9 pmol/L and free thyroxine >23.4 pmol/L), and undetectable TSH (<0.05 mIU/L). Patients showing toxic nodules were excluded.

Ninety-one apparently healthy euthyroid individuals (82 women and 9 men; median age, 41 years; range, 20–60 years) served as controls. These individuals did not smoke and had no goiter or no personal or family history of thyroid disease, and thyroid autoantibodies were negative.

We assayed Tg and TgAbs in the 153 Graves patients before treatment. Blood samples were collected in anticoagulant-free tubes and centrifuged at 1000g for 10 min at 4°C. Sera were decanted for storage at −20°C until assay.

Tg Assay
Tg was measured by IRMA using a new commercial reagent set (Dynotest Tg-plus; Brahms Diagnostica, Berlin, Germany). The method as described by the manufacturer is as follows: Standard or patient’s serum (100 μL) is pipetted into test tubes coated with polyclonal TgAb. The tubes are incubated for 18 h at room temperature and then washed twice with 2 mL of washing solution. The tubes are turned upside down on blotting paper for at least 10 min. After the tubes are again turned right side up, 200 μL of 125I-labeled monoclonal TgAb is added. The tubes are incubated for 2–3 h at room temperature with shaking (300–400 rpm), after which they are washed twice with 2 mL of washing solution and then turned upside down for at least 10 min on blotting paper. The radioactivity of each tube is then measured.

Sera with high Tg concentrations were diluted with the buffer included in the reagent set. Two dilutions (undiluted and 1:10) were prepared for each specimen with an increased Tg concentration 10-fold above the upper limit of the assay range (>220 μg/L). Interference from autoantibodies was assessed by direct measurement of TgAb with a RIA (Dynotest anti-Tgn; Brahms Diagnostica); all values <60 kilounits/L were considered negative. The Tg IRMA method also contained an estimate, by routine determination, of recovery that was carried out by adding the 50 μg/L Tg calibrator to each serum tested. The recovery test was also performed by adding 1 μg/L Tg to several sera containing a low concentration of Tg (<3 μg/L). Recovery (%) was calculated as:

\[
\frac{\mu g \text{Tg/L (A)}}{50 \text{ (or 1) } \mu g \text{Tg/L (B)}} \times 100
\]

where A is the supplemented serum sample, and B is the unsupplemented serum sample.

All determinations were performed in duplicate. The Tg standard was calibrated with the International Standard Certified Reference Material 457 in the following ratio: 1 ng of Certified Reference Material was equal to 0.5 ng of the Dynotest Tg-plus standard.

Statistical Analysis
Quantitative variables were analyzed using nonparametric tests. The Mann–Whitney test was used to compare the variables in the different groups. Correlation analyses were performed using the Spearman test.

Results

Tg Method
The imprecision of the assay (CV) was <3% at 3–335 μg/L (Table 1). The functional assay sensitivity, defined as an interassay CV <20% during a 6-month period (8), was 0.2 μg/L. Dilution curves for serum samples paralleled the calibration curve (Fig. 1).

Among 91 samples from healthy volunteers, 4 had TgAb concentrations ≥60 kilounits/L. These four samples were eliminated from the estimation of reference values. The Tg values for the remaining 87 serum samples showed a logarithmic distribution (mean, 7.6 μg/L; range, 2.45–22 μg/L).

Evaluation of Recovery
For 30 sera with low Tg values (<3 μg/L), the recovery test was performed by adding 1 μg/L Tg; for other sera, we added 50 μg/L. For the 87 control samples considered negative for TgAbs (<60 kilounits/L), the median recovery was 102% (range, 80–124%) compared with 82% (range, 65–98%) for the 4 TgAb-positive samples.

In the 153 sera from patients with Graves disease, median recovery was 90% (range, 62–113%), which was significantly lower than that obtained for the controls (Z = −5.606; P = 0.0001). Recovery was <80% for 39 (25%) of the 153 sera.

For 81 samples considered negative for TgAbs (<60 kilounits/L), median recovery was 101% (range, 80–115%), which was identical to the recovery for control sera (Z = −1.134; P >0.05). By contrast, significantly lower

<table>
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<tr>
<th>Table 1. Precision of the Tg assay. a</th>
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<tr>
<td>Intraassay</td>
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<tr>
<td>Mean ± SD, μg/L</td>
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<tr>
<td>1.75 ± 0.13</td>
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<tr>
<td>12.3 ± 0.2</td>
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<tr>
<td>43.0 ± 0.66</td>
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<tr>
<td>335.0 ± 7.6</td>
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<tr>
<td>Interassay</td>
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<tr>
<td>Mean ± SD, μg/L</td>
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<tr>
<td>3.36 ± 0.096</td>
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<tr>
<td>12.5 ± 0.29</td>
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<tr>
<td>43.5 ± 1</td>
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<td>332.0 ± 6.9</td>
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a) Intra- and interassay variations were calculated from duplicate Tg measurements of four pooled sera (n represents the number of assays in the same series for intraassay precision and the number of series for interassay precision).
recoveries were observed in the 72 samples with TgAb concentrations $\geq 60$ kilounits/L (median, 79%; range, 60–103%; $Z = -8.363; P < 0.0001$).

Recovery values and TgAb activities were significantly correlated ($r = -0.602; P < 10^{-4}$). Except for one sample, all sera with a negative TgAb activity had a normal recovery ($\geq 80$%; Fig. 2A). In 38 (53%) of the 72 TgAb-positive sera, a recovery $< 80$% was observed, whereas 34 (47%) of the 72 sera had a normal recovery test (Fig. 2B).

Tg values in the 72 TgAb-positive sera (median, 7 $\mu$g/L; range, 0.16–277 $\mu$g/L) were significantly lower than those obtained in the 81 TgAb-negative sera (median, 114 $\mu$g/L; range, 1.2–700 $\mu$g/L; $Z = -4.788; P < 0.0001$; Fig. 3A). Moreover, in the 34 of the 72 TgAb-positive sera with a normal recovery test ($\geq 80$%), Tg values were also significantly lower (median Tg, 13.6 $\mu$g/L; range, 1.1–360 $\mu$g/L) than those obtained in the TgAb-negative samples with a correct recovery (median, 107 $\mu$g/L; range, 1.2–700 $\mu$g/L; $Z = -3.797; P < 0.0001$; Fig. 3B).

Thirty-one (79%) of the 39 sera with a low recovery test result had Tg values $< 22$ $\mu$g/L (Fig. 4).

**Discussion**

Tg assays in serum are used in the follow-up of patients with thyroid diseases such as thyroid carcinoma and Graves disease (12, 18, 19). Tg assays also contribute to the diagnosis of factitious hyperthyroidism (20) and to the detection of functioning tissue in cases of congenital hypothyroidism (21). Investigators using competitive RIA methods based on polyclonal antibodies have reported interference caused by TgAbs (22, 23). More recently, IRMAs using monoclonal TgAbs that do not cross-react with endogenous TgAbs have been developed in an attempt to overcome TgAb interference (14, 15, 24, 25). However, the use of a monoclonal antibody IRMA for serum Tg, although less susceptible to in vitro TgAb interference, would not necessarily provide any substantial advantage (16). These interferences have led to an underestimation of Tg concentrations as well as decreased recovery of a known concentration of Tg added to the serum (17). The commercial availability of a new IRMA method that uses both a polyclonal coated TgAb and a labeled monoclonal TgAb as a tracer enabled us to study TgAb interference in the assay.
The Tg concentrations obtained in controls agreed with other reports on Tg reference intervals (26, 27). To determine the extent of this interference, we evaluated the TgAb concentrations and the recovery of added Tg. For TgAb measurement, we used a very sensitive RIA because it has been shown that TgAbs should be measured quantitatively in every specimen by a specific immunoassay and not an insensitive qualitative hemagglutination test (8, 26). We chose 80% recovery of added Tg as the cutoff for a normal test because all except one of the samples tested that had no measurable TgAbs gave a recovery value >80%. This cutoff is different from that recommended by the manufacturer, who considers all values ≥70% as normal. However, our lower limit of correct recovery was identical to that obtained in the literature (26, 27). The poor recovery for the one sample may be attributable to other interfering factors in serum, as suggested previously (26). We found no recovery rates >130%, as reported in some studies and for which no clear explanation has been put forward (26, 28). The inability of the assay to detect concentrations >80% of added Tg in 47% of the samples containing TgAbs indicates that some interference from TgAbs was present in the assay. This percentage was similar to the 41% obtained by Erali et al. (27). The proportion of sera with a low recovery test result (25%) was higher than that obtained with monoclonal IRMAs. However, TgAbs are found more frequently in patients with autoimmune diseases than in patients with differentiated thyroid cancer, as reported in extensive studies in the literature (26, 28).

Our data support the findings (17, 28, 29) that TgAb interference leads to decreased Tg values because the median Tg was lower in TgAb-positive sera than in sera without TgAbs. Thus, an underestimation of the Tg concentrations is possible in the presence of TgAbs. This underestimation of Tg may produce false negatives during cancer monitoring. Patients with thyroid carcinoma typically have TgAbs with broader-based epitope specificities than do patients with autoimmune thyroid diseases (30). However, the different TgAbs found in sera from patients with thyroid cancer and Graves disease recognize mainly region II and, occasionally, region IV on the human Tg molecule (31). Thus, TgAbs could also interfere with Tg in sera from patients with thyroid carcinoma.

Recovery tests should not be used in attempts to correct for TgAb effects when measuring serum Tg. With TgAbs, poor recovery rates spread out in the low range of Tg values (<22 µg/L). However, 8 of the 39 sera in our data...
study with recovery rates <80% did not display low Tg concentrations. Moreover, Tg values remained significantly reduced in TgAb-positive sera despite normal recovery test results. Thus, inappropriately low Tg results were the consequence of in vitro TgAb interference, which recovery tests sometimes failed to detect. The TgAb measurement is a more suitable tool to disclose these interferences. Tg concentrations should be interpreted with respect to the presence or absence of TgAbs. These results are in agreement with those of Spencer et al. (8), who reported poor recovery test values in six Tg assays performed with other commercial RIA or IRMA methods. Finding a lower than expected serum Tg value in TgAb-positive sera may indicate a truly low Tg concentration, possibly because of accelerated Tg metabolic clearance (16). Measurement of TgAbs remains an absolute prerequisite for the correct interpretation of serum Tg assays.

In conclusion, with this new IRMA, Tg results were affected by TgAbs in Graves disease sera although the recovery test results were normal. Consequently, the Tg recovery test is not a reliable way to detect interference in all TgAb-positive sera; it should not be used alone to determine the validity of a serum Tg measurement in the monitoring of patients with Graves disease. A similar study on sera from patients with differentiated thyroid carcinoma would be useful.

We thank Brahms Diagnostica (Berlin, Germany) for the gift of Dynotest Tg-plus and Dynotest anti-Tgn reagent sets.

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