Technical Evaluation of a New Immunoradiometric and a New Immunoluminometric Assay for Thyroglobulin

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Background: After removal of differentiated thyroid carcinoma (DTC), serum thyroglobulin (Tg) can indicate persistent or recurrent disease. We describe two novel two-step assays designed to measure low Tg concentrations.

Methods: We evaluated prototypes of the new IRMA, DYNOtest® Tg-pluS, and the new immunoluminometric assay (ILMA), LUMItest® Tg-pluS. In the first step, a high-salt incubation buffer leads to dissociation of Tg-Tg antibody complexes in serum and is intended to reduce nonspecific interference and interference of potential Tg autoantibodies in the system. We studied recovery of human Tg (from thyroid glands) added to horse serum. We also studied 58 patients with DTC in whom Tg values under thyroid-stimulating hormone (TSH) suppression and TSH stimulation (without thyroxine) were available.

Results: The detection limits were 0.04 μg/L Tg for the IRMA and 0.02 μg/L for the ILMA. Intraassay imprecision (CV) was <10% over the range of the calibration curve in both assays. The day-to-day CV was <20% at 0.2 μg/L for the IRMA and at 0.06 μg/L for the ILMA. No high-dose hook effect was seen with up to 200 000 μg/L added Tg or in dilutions of 12 patient sera with Tg values of 307–38 880 μg/L. Mean recovery of 50 μg Tg/L was 96% in those patients. Among 77 samples with Tg antibody values of 65.2–8150 kilounits/L, recovery by the IRMA was disturbed in 7 cases (9%) and by the ILMA in 9 cases (12%). Tg increased as measured in both assays in 50 of 58 patients after thyroxine withdrawal.

Conclusions: The new assays have improved precision for Tg <1 μg/L, and even low measured Tg concentrations respond physiologically to thyroxine withdrawal. The assays are free of a high-dose hook effect up to a Tg concentration of at least 38 000 μg/L and may further reduce Tg antibody interference.

The major clinical application for thyroglobulin (Tg) determination is the postoperative follow-up of patients with differentiated thyroid carcinoma (DTC), where any increase in serum Tg indicates residual thyroid tissue or persistent and recurrent disease (1–4). However, serum Tg measurements are one of the most difficult biochemical tests to maintain with high precision and reliability [see Spencer et al. (5) for a detailed review]. Several requirements are generally accepted for valid Tg determinations: high sensitivity, high interassay precision over a period of 6–12 months, no high-dose hook effect when measuring very high Tg concentrations (e.g., to monitor therapy), and the introduction of a common standardization system (6).

A particularly problematic issue is Tg detection in the serum of patients with Tg antibodies (Tg-Abs). Depending on the assay system, this can lead to either false-positive or -negative values (5). To detect interference in the Tg determination, a recovery test is usually performed using Tg, from thyroid glands, in concentrations of ~50 μg/L. Measurements with disturbed recovery are discarded and will not be used for clinical decisions. This practice is strongly debated. Whereas several groups find

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recovery studies mandatory (7–10), others question the rational (11) on the basis of different Tg immunoreactivities between the Tg present in a patient’s serum and the exogenous Tg from thyroid glands (12). Assay interference might not be detected because of differences between serum Tg in cancer patients and the Tg used for recovery. However, a positive Tg result in serum with Tg-Abs is considered valuable clinical information by some researchers (4), but others are adamant about rejecting any Tg detection in the presence of antibodies, regardless of whether Tg is detectable (13).

In the present study, we present two novel assays for Tg detection, using a stringent incubation protocol in high salt to reduce nonspecific and autoantibody-related interference.

Materials and Methods

We evaluated prototypes of the new IRMA, DYNOtest® Tg-pluS, and the new immunoluminometric assay (ILMA), LUMItest® Tg-pluS (B.R.A.H.M.S AG, Hennigsdorf, Germany) regarding technical features and preliminary clinical performance in 58 patients with DTC for whom Tg values under thyroid-stimulating hormone (TSH) suppression and TSH stimulation were available.

Clinical Information on Patients

We studied 58 patients with DTC who were routinely scheduled for a posttherapy whole-body 131I scan at the Nuclear Medicine outpatient department of the University Clinic Wuerzburg. Serum samples were taken before and after thyroxine (T4) withdrawal. All patients had initially been treated by total thyroidectomy followed by radioablation. The interference of Tg-Abs was studied in 77 patients with autoimmune thyroid disease (Hashimoto thyroiditis and atropic thyroiditis). Dilution studies were performed on 12 sera from patients with metastatic thyroid cancer. Informed consent was obtained from all study participants.

Assay Principle

The DYNOtest Tg-pluS (IRMA) and LUMItest Tg-pluS (ILMA) are two-step assays for the quantitative detection of Tg in human serum. In the first step, 100 μL of serum is added to tubes coated with affinity-purified rabbit polyclonal anti-Tg; 200 μL of high-salt incubation buffer (50 mmol/L Tris, pH 6.8, 1.5 mol/L NaCl, 5.0 mL/L Triton X-100, 20 g/L bovine serum albumin) is then added to each tube. The tubes are incubated without shaking at room temperature overnight. The high NaCl concentration is used to dissociate complexes of Tg and anti-Tg antibodies in the patient serum and to reduce their interference in the assay. After the tubes are washed twice with washing buffer (B.R.A.H.M.S AG), they are incubated for 2 h at room temperature with shaking. For the IRMA, we used 200 μL of a 125I-labeled murine anti-Tg monoclonal antibody in buffer (50 mmol/L sodium phosphate, pH 6.8, 25 mmol/L NaCl, 2.0 mL/L Triton X-100, 10 g/L bovine serum albumin) as tracer. For the ILMA, we used 200 μL of acridinium ester-labeled murine anti-Tg monoclonal antibody in the same buffer. After the tubes were washed three to four times, bound radioactivity was counted in a gamma counter for 1 min (IRMA) or the relative light units for the ILMA were detected in a luminometer. The cpm or relative light units were proportional to the Tg concentration of the patient serum.

Tg-Abs were determined in a commercially available competitive RIA, DYNOtest® anti-Tgs (B.R.A.H.M.S AG). The cutoff for positivity is 60 kilounits/L.

Calibrators and Controls

Tg concentrations were calculated using a calibration curve derived from human thyroid gland Tg in horse serum. Calibrators were adjusted to the current Tg IRMA, DYNOtest Tgs (B.R.A.H.M.S AG), to contain 0.15, 0.8, 4.0, 20, 100, and 250 μg/L Tg. Calibration with CRM 457 (6) was performed, and 1 μg/L CRM 457 was measured as 0.5 μg/L in the DYNOtest Tg-pluS or LUMItest Tg-pluS. Two controls containing a defined amount of human serum Tg were included in each analytical run.

Recovery

Tg recovery was calculated using 50 μg/L Tg (final concentration) from human thyroid glands added to horse serum.

Results

A typical calibration curve for the IRMA is shown in Fig. 1. The calibration curve for the ILMA was similar (not shown). Both assays responded to serum Tg from 0.15 to 250 μg/L.
intraassay, interassay, and interlaboratory CVs
The lower detection limit was 0.04 μg/L Tg for the IRMA and 0.02 μg/L for the ILMA. The intraassay CV (10 determinations per sample) was <10% over the range of the calibration curve for the IRMA (Fig. 2A) and <5% for the ILMA (Fig. 2B), respectively. The interassay CV was <20% (10 determinations per sample) for the IRMA at 0.1 μg/L Tg with fresh tracer (not shown) and at 0.2 μg/L Tg with tracer at the expiration date (Fig. 2C). The interassay CV for the ILMA was <10% over the range of the calibration curve and <20% at 0.06 μg/L (Fig. 2D). The CV between different reagent lots (interlot CV, 10 determinations per sample) was determined by five operators using two counters and three reagent lots over a period of 12 months. All samples had a CV <12% (Fig. 2E) or <15% (Fig. 2F), respectively.

CROSS-REACTIVITY AND RELIABILITY
There was no cross-reactivity to horse Tg up to a concentration of 100 000 μg/L in either assay. Assay linearity was confirmed using dilutions (1 part serum + 1 part diluent to 1 part serum + 31 parts diluent) of 10 different sera. All sera showed linear dilution in both assays, and the calculation of the original concentration in all dilutions was in agreement with the undiluted value. No pool effect was observed in the same 10 sera; the Tg determination in pooled sera produced the expected Tg concentrations.

NO HIGH-DOSE HOOK EFFECT
To exclude a high-dose hook effect, Tg calibration material was diluted in human serum (highest concentration, 200 000 μg/L). As shown in Fig. 3A, even at the highest values the calibration curve of the IRMA remained in the plateau and did not decrease to lower or even negative values. Additionally, a total of 12 patient serum samples with Tg values of 307–38 880 μg/L were diluted and measured in the assay. All sera showed linear dilution in the IRMA without a high-dose hook effect (Fig. 3B). Data for undiluted samples and 1:10 dilutions (1 part serum + 9 parts diluent) for some sera were extrapolated from the calibration curve (>250 μg/L). The precise Tg concentrations of these sera were calculated with the 1:100 (1 part serum + 99 parts diluent) and 1:1000 (1 part serum + 999 parts diluent) dilutions. Data for the ILMA were similar (not shown).

ROBUSTNESS AND INTERFEROENCES
Both assays were robust with respect to changes in incubation temperature (17-29 °C) and incubation time (18 ± 4 h for the first incubation step, 2 h ± 30 min for the second incubation step). To test whether the assays were influenced by hemolysis, serum lipids, or bilirubin, we diluted three sera in normal plasma, plasma containing lysed blood cells, plasma containing serum lipids, or plasma containing bilirubin. Both assays were unaffected by hemoglobin up to 60 g/L, serum lipids up to 3.2 g/L, or bilirubin up to 200 g/L.

COMPARISON WITH ESTABLISHED Tg ASSAY
Both new assays were compared with the DYNOtest Tg-S, using serum samples over the entire range of the calibration curve. Results of the regression analysis are shown in Fig. 4.

Fig. 2. Imprecision of the IRMA (A, C, and E) and ILMA (B, D, and F).
(A and B), intraassay CV (n = 10 runs); (C and D), interassay CV (n = 10 analytical runs); (E and F), CV between different reagent lots and operators (interlot CV; n = 10 analytical runs).
All sera with disturbed recoveries had Tg values above 130% of the added Tg. Except for one serum in the IRMA, considered undisturbed if measured Tg values were below 70% (these three sera had a recovery of 69%). Recovery was observed in one serum only in the IRMA, and three only in the ILMA. Of those sera, six were disturbed in both assays, two in the IRMA (12%), and in the IRMA in seven sera (9%), respectively. Among the 116 samples from patients receiving or not receiving T4, the mean recovery in the IRMA was 96% (Fig. 5) and autoantibodies were detected in 1:100 parts diluent; 1:1000 parts diluent; 1:10000 parts diluent; 1:100000 parts diluent.

INTERFERENCE FROM Tg-Abs

Interference from Tg-Abs was tested in 77 sera that had tested positive for Tg-Abs (minimum, 65.2 kilounits/L; maximum, 8150 kilounits/L; median, 181 kilounits/L). Recovery (50 µg/L) was disturbed in the ILMA in nine sera (12%), and in the IRMA in seven sera (9%), respectively. Of those sera, six were disturbed in both assays, one serum only in the IRMA, and three only in the ILMA (these three sera had a recovery of 69%). Recovery was considered undisturbed if measured Tg values were 70–130% of the added Tg. Except for one serum in the IRMA, all sera with disturbed recoveries had Tg values <1 µg/L (Fig. 5) and autoantibodies >800 kilounits/L (not shown).

Among the 116 samples from patients receiving or not receiving T4, the mean recovery in the IRMA was 96% (median, 97%).

Tg determination before and after T4 withdrawal

Evaluation of 58 paired thyroid cancer samples (with and without T4) demonstrated an increase in Tg (greater than CV at the respective concentration) in 50 cases and constant values in 8 cases (Fig. 6). Samples with Tg values below the functional assay sensitivity (FAS) should be considered undetectable.

Discussion

The determination of Tg in serum is an important diagnostic tool in the follow-up of patients with DTC. Assay requirements include a low detection limit and low imprecision at low Tg concentrations (14). The new assays described here demonstrate the lowest detection limits reported to date. Furthermore, the interassay CV (also taking into account different laboratories) was <10% in the clinically important range of 0.5–1 µg/L. T4 withdrawal increased the detectable Tg in most thyroid cancer patients for whom low Tg values were measured, indicating that this low Tg was indeed secreted by residual thyroid tissue and that the result was not an assay artifact. The long-term precision for Tg <1 µg/L allows prolonged clinical evaluation of patients with such low Tg concentrations. Although some physicians do not draw clinical conclusions from Tg values <1–2 µg/L (15), the assay precision is excellent throughout the clinically relevant measuring range, potentially allowing improved monitoring.

Some physicians would prefer “less sensitive” assays because it is sometimes difficult to verify the source of very low Tg concentrations (<0.5 µg/L) by additional imaging diagnostics, and confusion for the physician and the patient may occur (15). Others, however, argue in favor of a complete evaluation of patients with detectable serum Tg, irrespective of the concentration (4). It is possible that very low Tg concentrations do not require immediate treatment. The changes in serum Tg over a certain period or after T4 withdrawal are more informative than a single Tg determination (14). This is of special clinical relevance when recombinant TSH is used for exogenous stimulation of thyroid tissue, which is not as intense as stimulation after T4 withdrawal. Highly sensitive Tg assays that can distinguish a Tg increase from 0.2 to 0.8 µg/L in one patient and confirm a constant low Tg of 0.8 µg/L in another patient may be clinically valuable because only the first patient might need to undergo additional diagnoses or treatment. As increasingly sensitive assays become available and ultrasensitive methods such as PCR of Tg mRNA are considered (16, 17), the Tg threshold or Tg increase at which clinical intervention should occur must be reevaluated in clinical studies.

Many IRMAs and ILMAs have the limitation of a high-dose hook effect (5), which is the result of “neutralization” of the tracer antibody by excess Tg, so that no sandwich complex is generated on the solid phase. An important improvement of the assays described here was the lack of such a high-dose hook effect in all samples with very high Tg concentrations.

Other Tg assays have tried to overcome the limitation of Tg determination in the presence of Tg-Abs, for example, by use of capture antibodies specific for certain Tg epitopes (18). However, in clinical evaluations, interfe-
ence by Tg-Abs was still reported (19). In the two assays studied here, the frequencies of disturbed recovery in Tg-Ab-positive samples were considerably lower (12% for the ILMA, 9% for the IRMA) than have been reported in other systems [50–68% (19)].

The use of recovery to assess any interference in the Tg assay is supported by several groups (7–10) who have found that the detection of Tg-Abs may not be sufficient for this purpose. Others favor Tg-Ab detection and consider recovery data in the presence of Tg-Abs invalid (4, 11). Furthermore, the ratio between recovery Tg and sample Tg is an important factor. The use of 50 μg/L Tg in recovery studies might not allow the detection of slight interferences by autoantibodies because the autoantibody concentration does not allow any conclusions on immunoreactivity with serum Tg. Thus, serum Tg values <5 μg/L might not be detected, even if the recovery of 50 μg/L is undisturbed (>70%). As a consequence, relapse or spreading of thyroid cancer could occur.

The conditions of the two-step assays (overnight incubation and 1.5 mol/L NaCl) increased the binding of Tg to the polyclonal capture antibodies, whereas nonspecific interference that might influence the assays was reduced. Furthermore, under these stringent conditions, complexes of Tg with anti-Tg antibodies are disrupted. As suggested by Mariotti et al. (19), however, the problem of Tg detection in the presence of Tg-Abs may not be limited to assay problems. It may also reflect a faster clearance of the antigen-antibody complex from the patient in vivo, in which case the negative result in vitro may be analytically correct but is unable to exclude residual Tg-producing DTC tissue in the patient. Another point of criticism is the difference between serum Tg in patients and the Tg from thyroid glands used for recovery studies (12). Finally, the difference between serum Tg isoforms in healthy individuals and patients with thyroid cancer may also be relevant (20). We are currently evaluating whether a low-dose recovery consisting of 1 μg/L serum Tg is able to alleviate
some of these problems because it excludes structural and immunologic differences between serum Tg and colloidal Tg. Ongoing studies in a European multicenter trial will clarify whether this approach has a clinical benefit over the conventional recovery regime.

The excellent correlation of the new TG plus assay to the established and broadly used DYNOtest TG-S justifies the use of earlier Tg determinations in the same patient and makes additional measurements with stored serum samples less important. Despite the introduction of CRM 457, a comparison of Tg values measured with different Tg assays is still difficult because no assay is calibrated 1:1 to the CRM. Calibration of the DYNOtest Tg-plus with the CRM 457 produced 50.1% activity. Thus, 1 ng of CRM 457 is equivalent to 0.5 ng in this assay.

In conclusion, we established IRMA and ILMA assays for Tg determination with low detection limits and excellent precision at Tg concentrations as low as 0.2 (or 0.06) μg/L. Both assays are free of hook effects to the very high concentrations tested and appear to be less influenced by Tg-Abs. The clinical benefits of these new assays are currently under investigation in a European multi center trial.

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