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Comparison of the Influence of Thyroglobulin Antibodies on Serum Thyroglobulin Values from Two Different Immunoassays in Post Surgical Differentiated Thyroid Carcinoma Patients

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Measurement of serum thyroglobulin (Tg) is a highly specific test in the management of patients with differentiated thyroid cancer (DTC) after surgical treatment. The aim of our study was to evaluate and compare Tg levels in these patients found by radioimmunoassay (RIA) and immunoradiometric assay (IRMA) and to assess the influence of Tg antibodies (TgAbs) on the values obtained for Tg concentration. Both Tg and TgAb were determined postoperatively in the serum of 71 DTC patients using RIA Tg-PEG (INEP) and Tg IRMA (CIS) for Tg, together with TgAb (CIS) for circulating endogenous anti-TgAbs. The obtained concentrations were evaluated statistically. We found a significant difference of Tg concentrations between paired samples from the IRMA and RIA, although the intermethod comparison yielded satisfactory concordance of the two assays (Spearman correlation coefficient −0.792). Positive TgAb was found in 28.2% of the serum samples analyzed. Spearman rank correlation analysis revealed a significant negative relationship between serum TgAb and Tg level measured by IRMA ($P = 0.02$), but not by RIA ($P = 0.417$). On the other hand, our clinical data revealed that 1/18 and 3/18 patients with proven lymph node metastasis had Tg values below the detection limit by RIA and IRMA assay, respectively. Their sera were TgAb positive. We concluded that RIA was less prone to influence of TgAb than IRMA. As the presence of TgAbs may interfere in Tg measurement irrespective of the method selected for determination, this should be considered during the clinical management of these patients. J. Clin. Lab. Anal. 23:341–346, 2009.

Key words: thyroglobulin; antithyroglobulin autoantibodies; radioimmunoassay; immunoradiometric assay; differentiated thyroid cancer

INTRODUCTION

Thyroglobulin (Tg) is an iodinated glycoprotein, which is produced only by thyroid follicular cells, and is present in the serum of most normal individuals in a low concentration. Under physiological conditions one gram of normal thyroid tissue is associated with serum Tg of approximately 1 μg/l (1). Serum Tg levels relate not only to thyroid mass but also to the thyrotropin (TSH) status of the patient (2). In patients with thyroid carcinomas (papillary and follicular) Tg is a useful tumor marker after total thyroid ablation by surgery and ^{131}I therapy for detecting thyroid remnant or metastatic thyroid tissue (3,4). Thus, determination of serum Tg in the follow-up of thyroid cancer patients is clinically important, but the precise measurement of Tg in serum is methodologically challenging (5,6) due to a number of technical problems (7,8). There are many commercial diagnostic sets, which can be divided into two main groups according to the detection principle: radioimmunoassay (RIA) and immunoradiometric assay (IRMA). The assay principle (9–12), as well as the potential presence of thyroglobulin antibodies (TgAb) in

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the examinee's serum (13–15), could influence the result obtained for Tg concentration.

In this study we measured Tg levels in serum samples from patients with differentiated thyroid carcinoma (DTC) before ablative therapy by ^{131}I using two different radioimmunological methods in order to evaluate the influence of TgAb concentration on the Tg values obtained.

MATERIAL AND METHODS

Material

Blood samples for measurement of Tg and TgAb concentrations were collected in the Department for In Vitro Diagnostics, Centre for Nuclear Medicine, The Clinical Centre Kragujevac. The total number of specimens was 71. All serum samples were collected postoperatively from patients with DTC before application of an ablative dose of ^{131}I (off levothyroxine therapy). Before ablative radioiodine therapy, thyroid remnants were found in 27 patients, while 18 patients had lymph node metastasis.

Reports from the histological examination were available for all patients as follows: 63 (88.7%) patients had papillary carcinoma, 7 (9.9%) patients had follicular carcinoma, and one (1.4%) had Hürthle cell carcinoma.

METHODS

Assays for Tg

The concentration of Tg was measured by RIA (Tg-PEG, INEP, Serbia) and IRMA (THYROGLOBULINE, CIS Bio-international, France).

RIA Tg-PEG

The principle of RIA is based on the competitive binding of serum Tg and a fixed amount of radioactively labeled Tg (^{125}I-Tg) for a limited number of determinants on specific TgAb (polyclonal). The formed immune complexes are precipitated with immunoadsorbent (secondary antibodies in buffer solution) and separated out. The amount of radioactivity, measured in a gamma scintillation counter, is inversely proportional to the concentration of Tg in the examined specimen. According to the manufacturer, the lower limit of detection was 4 μg/l and the calibration range up to 320 μg/l. Intra-assay coefficient of variation (CV) was $<6.2\%$ at 16 μg/l and interassay CV $<8.4\%$ at 39 μg/l. The assay was standardized against Certified Reference Material for human Tg (CRM 457) in compliance with the Community Bureau of Reference of the European Commission (1 μg/l CRM 457 = 1 μg/Tg in RIA Tg-PEG). The interval for 98 healthy volunteers ranged between 0 and 48 μg/l.

IRMA Tg

The principle of IRMA is based on binding serum Tg to an excess of TgAbs fixed to the test tube wall (four specific monoclonal TgAbs selected for their properties of avidity and complementarity). A fifth monoclonal antibody labeled with ^{125}I and specific for a different part of the Tg molecule then binds with Tg on the test tube wall forming a "sandwich" of Tg between the unlabeled and labeled Ab. After aspiration and rinsing, the amount of radioactivity, measured in a gamma scintillation counter, is directly proportional to the concentration of Tg in the examined specimen. According to the manufacturer, the lower limit of detection was 0.2 μg/l and the calibration range was up to 500 μg/l. The functional sensitivity was 0.7 μg/l. Intra-assay CV was $<7.7\%$ at 1.2 μg/l and interassay CV $<16.7\%$ at 0.8 μg/l. The assay was standardized against CRM 457. One milligram of Tg in the standard is equal to 1 mg of CRM 457 Tg. The reference values obtained in presumably healthy subjects ($n = 149$) of both sexes ranged between 0 and 50 μg/l. A Hook effect occurs with an excessively elevated serum Tg concentration above 800,000 μg/l.

Assay for TgAb

Concentrations of antiTg autoantibodies in human sera were measured by RIA (TGAB ONE STEP, CIS Bio-international, France). The assay is based on the competitive binding of a fixed amount of monoclonal TgAb and TgAbs from the examined sample with a particular, limited number of specific points on ^{125}I-labeled Tg molecules. Unbound molecules of labeled Tg are removed by aspiration and lavage. Radioactivity is measured in a gamma scintillation counter. The extent of binding is inversely proportional to the concentration of TgAb in the examined specimen. According to the manufacturer, the intra- and interassay precisions were less than 8.3% at 72.6 IU/ml and 12.8% at 390 IU/ml, respectively. The method was calibrated against the WHO First International Reference Preparation CRM 65/93 and had an analytical detection limit of 6.0 IU/ml. The cut off for negativity for anti-Tg autoantibodies was lower than 30 IU/ml according to the manufacturer.

Statistical Methods

All numerical data in our study were expressed as the median and mean ± SD. The results were analyzed by nonparametric methods that were chosen after evaluating the data for normal distribution by the

Kolmogorov–Smirnov test. The Wilcoxon-signed ranks test was used to compare differences between paired samples of Tg. The relationship between individual differences in Tg concentrations measured by IRMA and RIA was analyzed using Spearman's rank correlation. The same test was used to analyze the interrelationship between serial Tg (measured by IRMA or RIA) and TgAb values. The value $P<0.05$ was considered statistically significant.

RESULTS

In all serum samples serum Tg and TgAb were measured as described in detail in the Material and methods section. Figure 1 gives a comparative display of the Tg values obtained by RIA and IRMA and the concentrations of TgAbs in 71 specimens of serum (51 samples with negative TgAbs and 20 with positive TgAbs).

Relatively high Tg levels (above 10 μg/l) was found in 23/71 (32.39%) sera samples. In this subgroup 9 patients had lymph node metastasis. In the remaining 14 patients, Tg was produced from the thyroid remnant but not due to insufficient time being left from surgery to sera sampling (this was usually more than 6 weeks from surgery with a minimum period of 4 weeks).

Out of the 18/71 (25.35%) patients with proven lymph node metastasis, the majority had increased Tg concentrations, but one (5.5%) and three patients (16.6%) had unexpectedly low Tg values (below the detection limit) by RIA and IRMA assays, respectively. Their sera were TgAb positive.

Statistical Analysis of the Obtained Results

Basic statistical analysis describing the pattern of values obtained for Tg and TgAb in each assay is given in Table 1. The minimal Tg concentration measured by IRMA was 0.2 μg/l, while the maximal was 72.4 μg/l (median 16.4 μg/l). For RIA the median value was 17.2 μg/l, and minimal and maximal concentrations were 0 and 74.0 μg/l, respectively.

The concentration of TgAb ranged from 0.5 to 435 U/ml, with a median of 58.6 U/ml. A positive result for TgAb (above 30 U/ml) was found in 20 (28.2%) serum samples (mean value 112.9 U/ml).

Correlation Between Serial Tg Measured by IRMA and RIA

A highly significant correlation was found between the two diagnostic methods for measuring Tg concentrations in serum samples of DTC patients by Spearman correlation analysis ($\rho = 0.792$, $P<0.001$; Fig. 2). When we represented the interrelationship between the analyzed parameters for Tg as a percentage of patients analyzed, we observed that 62.73% of the results obtained by IRMA and RIA were in concordance ($0.792^2 = 62.73\%$ of results in concordance).

The Difference Between Paired Samples of Tg Measured by the Two Diagnostic Tests

The Wilcoxon-signed ranks test was used to compare the difference between results for Tg obtained by RIA and IRMA. Based the Z-value ($Z = -2.129$) and $P<0.1$ ($P = 0.033$), it was shown that the difference between the Tg concentrations obtained in these two assays was statistically significant.

Comparison of TgAb Influence on the Serum Tg levels Obtained by the Two Assays

Separate analysis of the results for TgAb with those obtained for Tg by IRMA in paired serum samples showed a significantly negative correlation ($\rho = -0.276$, $P = 0.02$, Table 2), i.e., TgAb influenced 7.6% of the results for Tg measured by IRMA. In contrast, there was no correlation between the results for TgAb and Tg determined by RIA ($\rho = -0.098$, $P = 0.417$, Table 2), i.e., in this study the RIA employed appeared relatively unaffected by the presence of TgAbs (Table 2).

DISCUSSION

In this study we compared Tg concentrations in serum samples from DTC patients measured by two independent diagnostic tests: IRMA (CIS Bio-international) and RIA (INEP, Serbia). The serum samples were obtained postoperatively from patients with DTC when they were off levothyroxine therapy for 4 weeks, which causes endogenous TSH stimulation.

In most of the samples Tg values obtained by the two methods were in concordance. However, Tg concentrations measured in some specimens differed and statistical analysis between paired samples of Tg values measured by the two diagnostic tests established this to be significant. There are many factors that can cause disparity in the results obtained for serum concentration of Tg: different reference materials, discrepancy between standards regarding the same reference material, specific properties of the primary and secondary antibodies for different antigenic determinants on Tg, and different binding affinities of these antibody epitopes, as well as interference by serum factors (TgAb in the first place) with the primary and secondary TgAbs from the set (16,17).

Both commercial sets, RIA Tg (PEG) and IRMA (THYROGLOBULINE), used here were calibrated

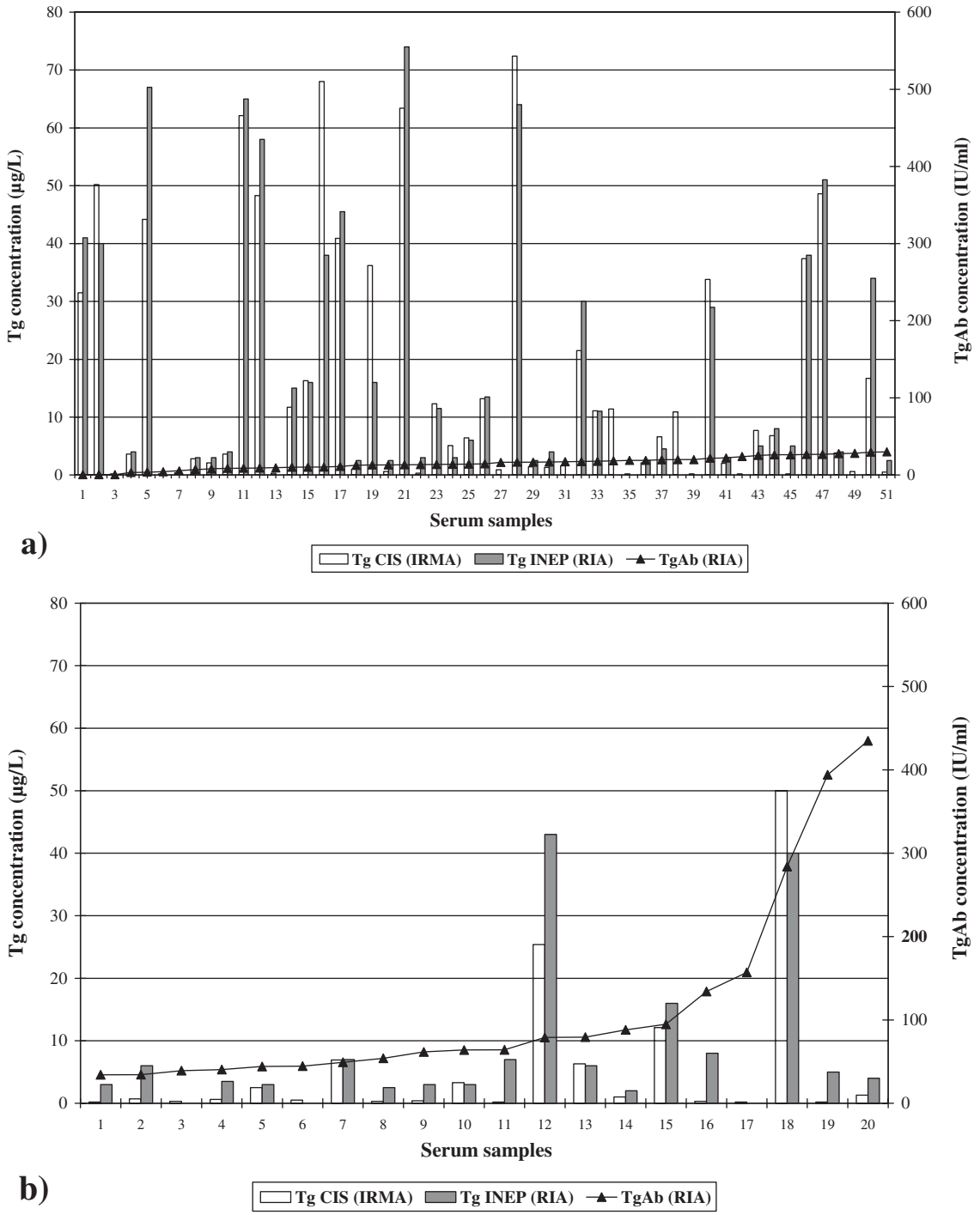

Fig. 1. Comparative display of values for TgAb and Tg in serum samples: (**a**) from 51 patients with negative TgAb and (**b**) 20 patients with positive TgAb.

according to the reference material CRM 457 in the same way, so differences in the measured values were not caused by discrepancy in standardization. Variability between these assays for Tg, despite calibration with CRM 457, could reflect differences in methodological procedure and specific properties of isoforms of circulating Tg (7,18–20), together with interference with the antibodies in the kits by diverse serum autoantibodies.

According to data in the literature, approximately 20–30% of DTC patients have TgAb in their circulation, compared with 11% of the general population (3,21–23). In this study we found TgAb in serum samples from

TABLE 1. The Values Obtained for Tg and TgAb in Each Assay

Parameters	*N*	Median	Mean	SD	Minimum	Maximum
IRMA Tg-CIS	71	16.4	13.2	19.48	0.20	72.4
RIA Tg-INEP	71	17.2	14.0	19.66	0.00	74.0
TgAb-CIS	71	58.6	43.0	76.32	0.50	435.0

N, number of serum samples, SD, standard deviation.

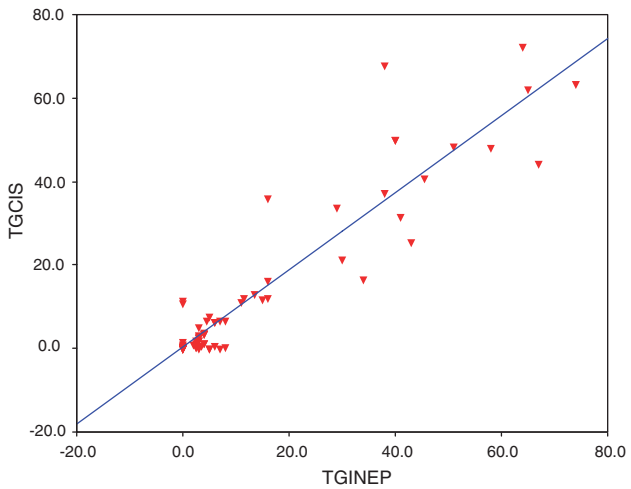

Fig. 2. Correlation between IRMA Tg (CIS) and INEP RIA Tg-PEG. The points on the curve are mean values for two replicates.

TABLE 2. Comparison of the Influence of TgAb on Serum Tg Values Obtained With IRMA Tg-CIS and RIA Tg-INEP

	Spearman's ρ	IRMA Tg-CIS	RIA Tg-INEP
TgAb-CIS	Correlation coefficient	−0.276*	−0.098
	p	0.020	0.417
	n	71	71

*Correlation is significant at the 0.05 level (2-tailed); p, significance, n, number of samples analyzed.

28.2% of our DTC patients. We showed that TgAb values above the cut off established by the manufacturer, i.e., 30 IU/ml interfered with the measurement of Tg by IRMA in some serum samples (7.6%). On the other hand, RIA was relatively unaffected by TgAb, as indicated by the absence of significant correlation between the results for Tg and TgAb. However, it should be stressed that no RIA method is immune to TgAb interference in all TgAb-positive sera and the influence of TgAb on different RIA methods is quite variable (3). Indeed, our clinical data revealed that one patient with proven lymph node metastasis and an unexpectedly low level of Tg in both assays was TgAb positive, suggesting the negative interference of such antibodies on the Tg value obtained in both assays. The possibility of an influence of TgAbs on the serum Tg values in some serum samples giving measurable levels of antibody in subjects thought to be TgAb negative (<30 IU/ml by the assay employed) should be considered. Namely, there are published data suggesting that low concentrations of TgAb below the cut off established by manufacturers can interfere with Tg measurement (3,15,24–26).

Considering that circulating TgAb may interfere with serum Tg measurement qualitatively, quantitatively, and in a method-dependent way (6,14,27–31), and that we did not obtain discordance between the two assays only for serum samples with high TgAb concentrations, we conclude that the measured Tg values probably depend not only on the concentration of TgAb present but also on their affinity and specificity. These serum autoantibodies may interfere with different epitopes on the Tg molecule forming Tg-anti Tg immunocomplexes, which may prevent binding of serum Tg to TgAb present in the diagnostic tests.

As our group of examinees included patients with DTCs, the interpretation of undetectable or low values of Tg in the presence of TgAbs must be very careful (32), especially as TgAbs are considered to be an additional tumor marker indicating the presence of thyroid tissue (14,33). Persistence of Tg-specific autoantibodies (14,33), and particularly an increase of TgAb concentration in patients with previously low values during long-term follow-up may indicate persistent or recurrent disease.

The most important application of Tg measurement in clinical practice is in the postsurgical management of differentiated thyroid cancer patients. As the presence of TgAbs may interfere in Tg measurement irrespective of the method selected for determination, this should be considered during the clinical management of these patients.

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