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ORIGINAL PAPER

In vitro assays to test the interference of anti-thyroglobulin antibodies on thyroglobulin measurement

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Abstract *Objective* To assess the interference of anti-thyroglobulin antibodies (TgAb) on serum thyroglobulin (Tg) measurement by in vitro experiments. *Design* Re-evaluation of Tg concentration after dilution with different TgAb-positive sera. On a first step, dilutions of the same Tg with different TgAb sera were performed and on a second step, different Tgs were diluted with the same TgAb serum. *Methods* Tg measurements were performed using an immunometric (IMA) chemiluminescence assay. TgAb measurements were performed using two methods: immunoflurimetric assay (UNICAP 100) and IMA chemiluminescent assay (IMMULITE 2000). *Results* Dilution of a known concentration of Tg with different TgAb-positive sera resulted in a variation of the final concentration of Tg ranging from −24 to −79%. A weak correlation was observed between the TgAb concentration and the percentage of the Tg deviation. Dilution of different Tgs with the same TgAb-positive serum illustrated how the same TgAb positive serum may determine a high interference or a neutral effect. *Conclusions* Present results suggest that the interference on Tg measurement observed in the presence of TgAb may result not only from the anti-thyroglobulin antibodies, but also from the thyroglobulin itself.

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Introduction

Thyroglobulin is the glycoprotein precursor to the thyroid hormones. It is the most sensitive and specific marker of differentiated thyroid carcinoma (DTC), after total thyroidectomy and radioiodine ablation [1–3]. The first method for routine measurement of serum Tg, described in 1973, was a competitive radioimmunoassay (RIA) [4]. Immunoradiometric assays (IRMAs) using monoclonal anti-Tg antibodies have been available since the mid-1980s.

The presence of serum anti-Tg antibodies has the potential to interfere with Tg measurement [5–13]. Even low concentrations of TgAb that may not be detected by some methods can interfere with Tg measurement [14, 15]. On the other hand, TgAb are detected in a higher percentage of DTC patients [16] than the general population (25% vs. 10%, respectively) [17, 18].

Under or overestimation of serum Tg levels depends on the type of method used [1, 14, 19–25]. The immunometric (IMA) methodology, used by most clinical laboratories, is associated with underestimated results. The RIA methodology is less prone to the interference of TgAb; however, bidirectional interference is possible [2, 20, 22, 24]. Underestimation can potentially lead to recurrent or persistent disease being missed, whereas overestimation may cause unnecessary interventions.

Tg heterogeneity has been reported in patients with DTC [13, 26–32]. Whether this might influence Tg measurement in patients with TgAb has not been fully addressed.

Herein, we tried to assess how the TgAb concentration and Tg heterogeneity might interplay and interfere with

serum Tg measurement by the IMA method currently in use in our laboratory.

Results

TgAb status was assigned as follows: UNICAP—14/20 samples were considered TgAb-positive, six presented results within an equivocal zone and none was negative; IMMULITE—18/20 samples were considered TgAb-positive and two negative (Fig. 1). These two specific samples were in the equivocal range by UNICAP.

Assay A

Conducted to the evidence that the dilution of a serum with a known Tg concentration (555 ng/ml) with different TgAb resulted in a variation of the final Tg concentration ranging from −24 to −79% and a mean Tg of 283.2 ± 90.2 ng/ml. Even the two samples regarded as TgAb-negative, by the IMMULITE assay, were associated with a Tg reduction of 24 and 32%, respectively (Fig. 1). A weak correlation (Fig. 1) was observed between the TgAb concentration and the percentage of Tg deviation. (UNICAP: $r = -0.508$, $r^2 = 0.258$, $p = 0.0224$. IMMULITE: $r = -0.583$, $r^2 = 0.339$, $p = 0.007$).

Assay B

Re-evaluation of the mean Tg concentration of 10 DTC patients, after dilution with four TgAb-positive sera, disclosed a significant reduction of the mean Tg concentration with all four sera although less significant with serum C2 (Table 1 and Fig. 2). Analysis sample by sample made us to conclude that dilution of different Tgs with the same TgAb might result in a highly variable reduction on the final Tg concentration (Fig. 3). This was particularly evident for serum C2 that induced a Tg reduction of almost 50%, after dilution with sample 8, whereas dilution with sample 2 resulted in a slight increment of Tg (8%).

Discussion

Measurement of serum Tg is a highly specific and sensitive test for the follow-up of patients with DTC. Unfortunately, the presence of anti-Tg antibodies limits the accuracy of Tg measurement justifying the guideline n° 46 published by the National Academy of Clinical Biochemistry (NACB) that recommends "laboratories should not report undetectable serum Tg values in the presence of TgAb if that method produces inappropriately low or undetectable serum Tg values for TgAb-positive DTC patients with documented disease" [33].

In clinical practice, the presence of TgAb alerts for the need of a complementary or additional approach prior to conclude for remission.

Failure to document the presence of TgAb cannot be regarded as a guarantee of a reliable Tg measurement. In fact, sera from all six cases considered ambiguous by the UNICAP method, among which were the two negative cases by the IMMULITE method, were shown to interfere with the Tg measurement. This observation reinforces the fact that assays for TgAb measurements have different sensitivities and even low concentrations of TgAb, which may not be detected by some methods, can interfere with Tg measurements as previously reported [1, 13, 23, 34, 35]. Thus, in doubtful cases of undetectable Tg, it might be

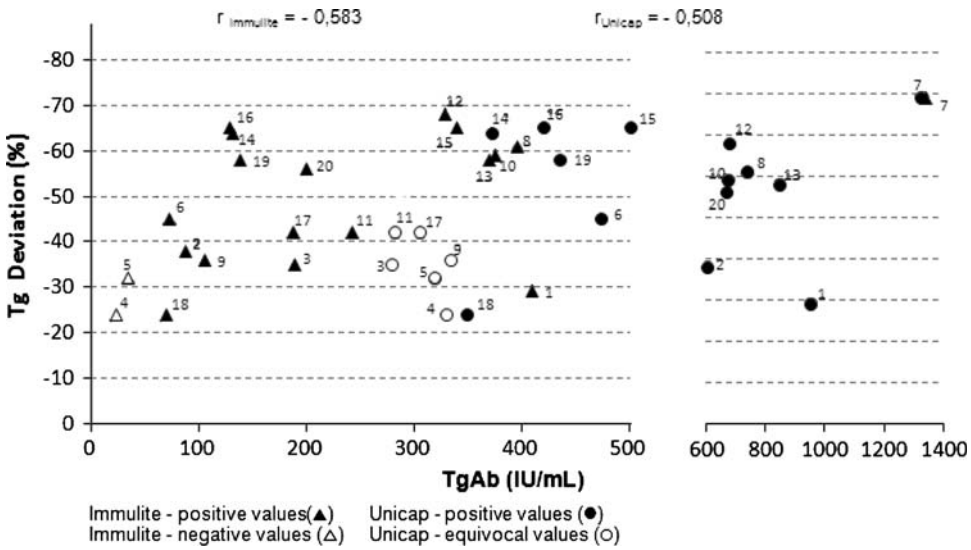

Fig. 1 Assignment of TgAb status of 20 sera samples by analysis with two different methods and correlation between the title of TgAb and the variation of Tg expressed as % deviation from baseline. % deviation = $(Tg_A - Tg)/Tg_A \times 100$

Table 1 Assay B Tg concentration after dilution of ten different Tgs with four different TgAb-positive sera (C1–C4)

	C1 305 IU/ml	C2 350 IU/ml	C3 447 IU/ml	C4 1,459 IU/ml
Tg_i mean ± SD (ng/ml)	5.4 ± 3.7			
Tg_f mean ± SD (ng/ml)	2.3 ± 1.6	3.8 ± 2.4	1.5 ± 1.0	0.9 ± 0.8
	$p = 0.0018$	$p = 0.0118$	$p = 0.0018$	$p = 0.0016$
Tg_f min/max (ng/ml)	0.8/4.7	1.6/8.8	0.6/3.3	<0.2/2.2
% deviation (mean)	−58.6	−26.3	−72.2	−83.3

% deviation = $(Tg_i - Tg_f)/Tg_i \times 100$ (Tg_i = initial Tg; Tg_f = final Tg, after dilution)

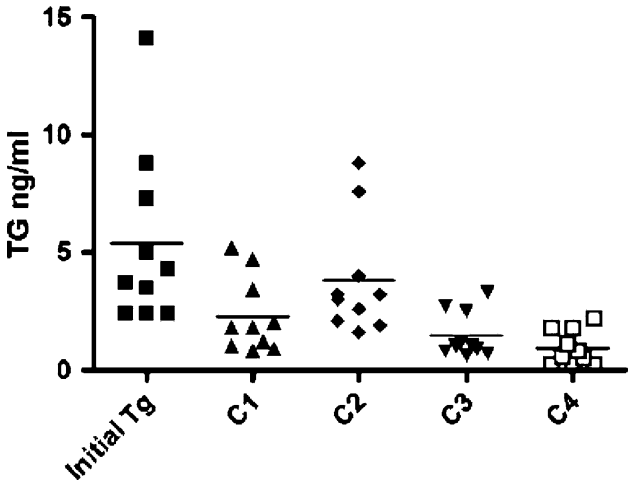

Fig. 2 Tg concentration of ten sera samples prior (initial Tg) and after dilution with four TgAb-positive sera (C1–C4)

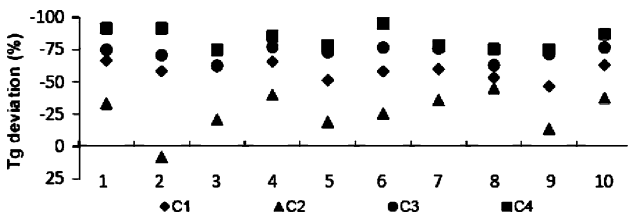

Fig. 3 Variation of Tg concentration, expressed as % deviation from baseline, observed in ten sera samples (1–10) after dilution with four TgAb-positive sera (C1–C4); % deviation = $(Tg_i - Tg_f)/Tg_i \times 100$

helpful to measure serum Tg by RIA methodology, since recovery tests do not reliably detect interfering TgAb [32]. Neck ultrasonography is also advocated.

There is a trend for a higher interference in association with a higher concentration of TgAb but this is not a rule. In a few cases, the qualitative characteristics of TgAb and not the concentration appear to determine whether the interference occurs.

Results from assay B strengthen the hypothesis that the interference depends on the type of TgAb. Moreover, the highly variable interferences after dilution of different Tgs with a particular TgAb (from a DTC patient) suggest that results are dependent on the TgAb epitope recognition.

Based on the concepts of restricted and broad specificities [25], we consider that endogenous antibodies with a restricted specificity, mainly for the analytical binding sites, are likely to be associated with a higher interference depending on a competitive reaction. On the contrary, a broad specificity, allowing binding of the endogenous antibodies with different epitopes, is expected to result in a lower interference.

By diluting different samples of Tg (deriving from different patients) with the same TgAb, we tried to mimic what happens in vivo, when there are different Tg variants resulting from alternative splicing. Identification of TgAb is currently associated with the persistence of an antigenic stimulus. It does not allow any conclusion concerning the number of Tg variants in circulation. Furthermore, the variant with the highest antigenic potential is not necessarily the one presenting the highest concentration thus explaining how variable might be the interference, determined by TgAb, on Tg measurement.

Overall, present data reinforce prior studies documenting the interference of TgAb on Tg measurements by IMA methodology. In addition, present results are likely to suggest a role for Tg in deciding the interference.

Material and methods

Tg measurements

Serum Tg measurement was performed using an IMA chemiluminescence assay (IMMULITE 2000, Diagnostic Products Corp., Los Angeles, CA) calibrated against the international reference preparation (CRM 457). The functional sensitivity described by the manufacturer is 0.9 ng/ml; Tg values < 0.2 ng/ml are regarded as undetectable.

Sera derived from 11 patients with DTC that underwent total thyroidectomy followed by immediate ^{131}I remnant ablation and currently under L-thyroxine (L-T4) treatment.

TgAb measurements

Two different methods were used: (i) immunoflurimetric assay (UNICAP 100, Pharmacia Diagnostic, Germany). The intra and interassay precisions were 2.1 and 3.4%, at 1,443 IU/ml, respectively. In accordance with the manufacturer's instructions, values below 280 IU/ml were considered negative and values above 344 IU/ml were considered positive, whereas values between 280 and 344 IU/ml were regarded as equivocal. (ii) IMA chemiluminescent assay (IMMULITE 2000, Diagnostic Products Corp., Los Angeles, CA). The intra and interassay precisions were 3.9 and 5.7% at 736 and 1,644 IU/ml, respectively. Values below or equal to 40 IU/ml were regarded as negative. Both methods were calibrated against the WHO First International Reference Preparation (IRP) 65/93.

In vitro evaluation of the TgAb interference on the Tg measurement

Assay A (Table 2). To assess how different TgAb interfere on Tg measurement: aliquots from one serum (Tg_A) with a Tg concentration of 555 ng/ml and TgAb-negative were diluted (1:1) with 20 different sera selected for being TgAb-positive or equivocal, by at least one method, and Tg undetectable.

Assay B (Table 2). To assess whether the TgAb interference might depend on the Tg itself: aliquots from ten samples with Tg concentrations ranging from 2.4 to 14.1 ng/ml (Tg_i) and TgAb-negative were diluted (1:1) with four (C1–C4) different TgAb-positive sera without measurable Tg. Sera C1 and C2 were obtained from disease-free DTC patients and sera C3 and C4 from patients with lymphocytic thyroiditis.

For assays A and B, the diluted sera were incubated for 4 h at room temperature.

Statistics. Two-tailed paired *t* tests were used to examine changes in Tg concentration (baseline and after dilution). Simple regression was used to examine relationships between Tg and TgAb measurements and Pearson's correlation coefficients were reported to indicate the strength of the relationships. $p < 0.05$ was considered significant.

Table 2 Conditions of assays A and B

Assay A	Assay B
1 sample (Tg_A)	10 samples
Tg_A = 555 ng/ml	Tg_i:
	mean ± SD = 5.4 ± 3.7 ng/ml
	Min/Max (2.4–14.1)
TgAb-negative	TgAb-negative
+	+
20 samples	4 samples (C1–C4)
Tg undetectable	Tg undetectable
$TgAb_1$ (method 1):	TgAb:
mean ± SD = 544 ± 271 IU/ml	mean ± SD = 647.8 ± 546 IU/ml
Min/Max (280–1324)	Min/Max (305–1459)
Tg Ab_2 (method 2):	
mean ± SD = 259 ± 284.5 IU/ml	
Min/Max (23.1–1340)	

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