Thyroglobulin Autoantibodies in Patients with Papillary Thyroid Carcinoma: Comparison of Different Assays and Evaluation of Causes of Discrepancies

Francesco Latrofa, Debora Ricci, Lucia Montanelli, Roberto Rocchi, Paolo Piaggi, Eleonora Sisti, Lucia Grasso, Fulvio Basolo, Clara Ugolini, Aldo Pinchera, and Paolo Vitti

Department of Endocrinology, World Health Organization Collaborating Center for the Diagnosis and Treatment of Thyroid Carcinoma and Other Thyroid Diseases, Centro Eccellenza AmbiSEN (F.L., D.R., L.M., R.R., P.P., E.S., L.G., A.P., P.V.); and Department of Oncology, Section of Pathology 3 (F.B., C.U.), University of Pisa, 56124 Pisa, Italy

Context: Thyroglobulin autoantibodies (TgAb) have been proposed as a surrogate marker of thyroglobulin in the follow-up of differentiated thyroid carcinoma. Commercially available TgAb assays are often discordant. We investigated the causes of discrepancy.

Design: TgAb were measured by three noncompetitive immunometric assays and three competitive RIA in 72 patients with papillary thyroid carcinoma and associated lymphocytic thyroiditis (PTC-T), 105 with papillary thyroid carcinoma and no lymphocytic thyroiditis (PTC), 160 with Hashimoto’s thyroiditis, and in 150 normal subjects. The results of the six assays were correlated. TgAb epitope pattern, evaluated by inhibition of serum TgAb binding to thyroglobulin by TgAb-Fab regions A, B, C, and D, were compared in sera which were positive in all six assays (concordant sera) and positive in only one to five assays (discordant sera) were compared. TgAb International Reference Preparation (IRP) was measured in 2007 and 2009.

Results: The correlations of the six assays ranged from 0.01 to 0.93 and were higher in PTC-T and Hashimoto’s thyroiditis than in PTC and normal subjects. Two uncorrelated components, one including the three immunometric assays, the other the three RIA, explained 40 and 37% of the total variance of the results of the six assays. The levels of inhibition were higher in concordant sera than in discordant sera by TgAb-Fab region B (27.0%, 21.2–34.0 vs. 6.0%, and 2.7–12.7%) and region C (30.5%, 21.3–37.7 vs. 4.0%, and 1.0–6.5%); thus, the epitope pattern was more homogeneous in concordant sera than in discordant sera. TgAb IRP ranged from 157 to 1088 (expected 1000) IU/ml in 2009; results in 2007 were similar in all but two assays.

Conclusions: TgAb assays are highly discordant. Discrepancy is lower when comparing assays with similar methodology. Results of TgAb from PTC-T are more concordant than those from PTC because their epitope pattern is more restricted. The internal standardization of TgAb is generally, but not completely, satisfactory. (J Clin Endocrinol Metab 97: 3974–3982, 2012)
Patients and Methods

Study group

Sera from 72 PTC-T, 105 PTC, and 160 HT patients (positive controls) and 150 NS (negative controls) were consecutively collected at the Thyroid Clinic of the Department of Endocrinology, University Hospital of Pisa, Italy, from 2007 to 2009. The study was approved by the institutional review board of the Department of Endocrinology. Subjects gave their consent for phlebotomy.

Their ages (median, 25th–75th percentiles) were 44.5 (36.5–51.0), 48.0 (38.0–56.5), 48.0 (37.5–57.0), and 42.0 (34.0–53.0) yr for PTC-T, PTC, and HT patients and NS, respectively. The female: male ratio was 6.2:1, 2.3:1, 6.0:1, and 3.2:1 for PTC-T, PTC, and HT patients and NS.

The diagnosis of PTC-T and PTC was based on the histologic specimens taken at the time of total thyroidectomy. Lymphocytes were detected with a monoclonal antibody (CD45; Ventana Medical Systems, Inc., Tucson, AZ) and counted at ×40 magnification. An associated lymphocytic thyroiditis was diagnosed when more than 10 lymphocytes per field were detected and when oxyphilic transformation and secondary follicles were present in the extratumoral tissue (PTC-T patients). Lymphocytic thyroiditis was ruled out when lymphocytic infiltration was absent (<10 lymphocytes per field) (PTC patients). Sera were collected when patients underwent thyroid remnant ablation with 131I, off L-T4 therapy, about 3 months after total thyroidectomy.

Sera from HT patients and NS were checked for free T4 (FT4), free T3 (FT3), TSH, and thyroperoxidase autoantibodies (TPOAb), and all subjects had a neck ultrasound. HT was diagnosed in the presence of elevated serum TPOAb, hypothyroidism (TSH >3.7 μU/ml), and a hypoechoic pattern at thyroid ultrasound. NS had negative TPOAb, normal thyroid hormones, and TSH values and a normoechogenic pattern at thyroid ultrasound.

TgAb assays

TgAb were measured by three noncompetitive IMA (IMA 1, AIA-Pack 2000 TgAb (Tosoh Corporation, Tokyo, Japan); IMA 2, Immulite 2000 anti-Tg Ab (Siemens Medical Solutions Diagnostics, Los Angeles, CA); and IMA 3, Access Thyroglobulin Antibody [Beckman Coulter Inc., Fullerton, CA]) and by three competitive RIA (RIA 1, SelCo anti-Tg (Medipan, GMBH, Berlin, Germany); RIA 2, BRAHMS anti-Tgn (BRAHMS Hennsdorf, Germany); and RIA 3, RADIM TgAb One Step CT (RADIM S.p.A., Rome, Italy)). In IMA, sera are incubated with solid-phase Tg, and after washing of unbound IgG, conjugated antihuman IgG (IMA 1 and IMA 2) or enzyme-labeled Tg (IMA 3) is added. In RIA, serum TgAb compete with coated human monoclonal TgAb (RIA 1), human polyclonal TgAb (RIA 2), or murine monoclonal TgAb (RIA 3) for binding to labeled Tg. The analytical sensitivities, the functional sensitivities, and the cutoffs were 6, 8, and 30 IU/ml (IMA 1); 18, 20, and 40 (IMA 2); 0.9, 1.2, and 4 (IMA 3); 20, 96, and 150 (RIA 1); 20, 40, and 60 (RIA 2); and 20, 49, and 70 (RIA 3). TgAb values above the cutoff were considered as positive, and values above the functional sensitivity were included in the comparison of the results of the assays.

TgAb-Fab expression

Cloning and characterization of human recombinant monoclonal TgAb expressed as Fab has been previously reported (23, 24). Four TgAb-Fab corresponding to different epitope regions [no. 37 (region A), no. 26 (region B), no. 6 (region C), and no. 32 (region D)] (24) were employed in the present study. TgAb-Fab were expressed in bacteria, and their binding to Tg was assayed by ELISA. All four TgAb-Fab were expressed at similar levels (OD, ~2.0).

TgAb epitope pattern

The epitope pattern of 87 (37 PTC-T, 10 PTC, and 40 HT) sera was evaluated by inhibiting serum TgAb binding to Tg by each single TgAb-Fab in ELISA, as previously reported (2, 24, 25). In preliminary experiments to determine the most appropriate serum dilution for epitope analysis, wells coated with human Tg (Calbiochem, San Diego, CA) (4 μg/ml at 4°C overnight) were incubated with serial dilutions of sera (1:100 to 1:1000). Antibody binding was detected with antihuman IgG-Fc conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). After the addition of substrate (o-phenylene diamine + H2O2) and H2SO4 to stop the reaction, the OD was read at 490 nm. Serum dilutions yielding OD of approximately 1 were used.
in later inhibition experiments. To determine inhibition, 50 μl per well of TgAb-Fab (or culture medium) was incubated with an equal volume of serum (diluted to provide a final OD of ~1.0) in Tg-coated ELISA wells (60 min at room temperature) (24). Binding of serum TgAb was detected with horseradish peroxidase-conjugated antihuman IgG-Fc, which binds to IgG but not to Fab molecules. After the addition of substrate (o-phenylene diamine + H₂O₂) and H₂SO₄ to stop the reaction, the OD was read at 490 nm. The inhibition by TgAb-Fab was calculated from the OD values for serum TgAb alone − serum TgAb + TgAb-Fab and expressed as a percentage of serum TgAb alone. Nonspecific binding of a TgAb-negative serum (6–12%) was subtracted in calculating percentage inhibition. The experiments were performed three times, each time in triplicate.

**Measurement of TgAb-Fab in RIA**

Twenty microliters of conditioned medium of each TgAb-Fab (diluted in normal human serum to obtain similar results in the ELISA TgAb assay) were measured in the three RIA. The samples were incubated and developed according to the manufacturers’ instructions.

**TgAb serum international standard**

TgAb serum IRP (International Reference Preparation) (NIBSC 65/093) was purchased from the National Institute for Biological Standards and Control (Hertfordshire, UK). IRP (neat and diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) was incubated in the six assays, and the reaction was developed according to the assay instructions.

**Statistical analysis**

Bivariate correlations among TgAb kits were assessed by Spearman’s rank correlation for non-normally distributed variables. TgAb results of all six assays were analyzed by the principal component analysis (PCA) based on data correlation matrix to identify groups of highly correlated and concordant TgAb assays (26). PCA is a nonparametric multivariate technique that seeks the best linear combinations of variables [called principal components (PCs)] that explain most of the data variance. PCs are uncorrelated to each other and are ranked in a decreased order of explained variance (PC eigenvalue). The Kaiser’s criterion (retaining only eigenvalues >1) was employed to identify the number of significant PCs (the latent assays). The contribution of each TgAb assay to the latent assays was quantified by their correlation matrix (PCA loadings matrix). The varimax rotation (27) was applied to improve the clinical interpretation of latent assays in terms of original TgAb assays.

Inhibition by TgAb-Fab in discordant and concordant sera was compared using the Mann-Whitney U test for unpaired data. Statistical significance was considered for P values <0.05. Data are presented as median and 25th-75th percentiles; Spearman’s correlation indexes (Rho) are reported along with their 95% confidence interval.

**Results**

**Proportion of positive TgAb and TgAb concentrations**

Of 72 PTC-T patients, 30 (41.7%) were negative in all assays, four (5.6%) were positive in one, four (5.6%) in...
two, nine (12.5%) in three, two (2.8%) in four, eight (11.0%) in five, and 15 (20.8%) in six assays (Fig. 1A). Of 105 PTC patients, 95 (90.7%) were negative in all assays, four (3.8%) were positive in one, one (0.9%) in two, two (1.9%) in three, one (0.9%) in four, one (0.9%) in five, and one (0.9%) in six assays. Of 160 HT patients, 32 (20.0%) were negative in all assays, eight (5.0%) were positive in one, six (3.8%) in two, 22 (13.8%) in three, 19 (11.8%) in four, 19 (11.8%) in five, and 54 (33.8%) in six assays. Of 150 NS, 139 (92.8%) were negative in all assays, six (4.0%) were positive in one, one (0.8%) in two, one (0.8%) in three, one (0.8%) in four, none (0%) in five, and one (0.8%) in six assays (Fig. 1A). Overall, 37.5% of PTC-T and 46.2% of HT were positive in one to five assays.

The highest TgAb concentrations were observed in HT, followed by PTC-T, and the lowest in PTC and NS (Fig. 1B). Because of their extremely high levels in all six assays, six sera (five HT and one PTC-T) were excluded from the subsequent statistical analysis.

Comparison of TgAb concentrations

The correlations of the six assays in the four groups of subjects were extremely variable, depending upon TgAb methodology and the clinical diagnosis (Fig. 2). They were significant in all cases, with the exception of IMA 2 compared with RIA 1 in PTC and NS (P > 0.05). Correlations were higher and significant when the three IMA were compared to each other (0.24 < Rho < 0.93). Correlations were lower and, in some cases, nonsignificant when IMA were compared to RIA (−0.01 < Rho < 0.80). Correlation values ranged from 0.67 to 0.93 in PTC-T, from 0.18 to 0.58 in PTC, from 0.50 to 0.90 in HT, and from −0.01 to 0.44 in NS.

The latent assays

To get more insight into the correspondence among the six assays, we applied PCA with varimax rotation to TgAb results. Two significantly uncorrelated components (latent assays), which explained 40 and 37% of the total variance of the six assays, respectively, were identified by the Kaiser criterion. The first latent assay included IMA 1 (PCA loading value = 0.83), IMA 2 (0.90), and IMA 3 (0.87); the second included RIA 1 (0.73), RIA 2 (0.89), and RIA 3 (0.92) (Fig. 3).

Comparison of the epitope pattern of concordant and discordant sera

The 87 sera evaluated for their epitope pattern were subdivided into two groups, according to the results obtained by the six TgAb assays: positive in six assays (concordant sera) (n = 54); and positive in one to five assays (discordant sera) (n = 33) (Fig. 4).

Inhibition by region A TgAb-Fab was similar in concordant (49.5%, 36.0–60.5%) and discordant (53.5%, 31.6–69.0%) sera. Results of inhibition by region D TgAb-Fab were also similar in concordant (8.5%, 1.7–30.0%) and discordant (10.0%, 1.0–28.9%) sera. Conversely, inhibition by region B TgAb-Fab was significantly higher in discordant (27.0%, 16.5–36.7%) than in discordant (6.0%, 1.0–17.8%) sera (P < 0.001). Similarly, inhibition by region C TgAb-Fab was higher in discordant (30.5%, 18.0–42.0%) than in discordant (4.0%, 1.0–9.3%) sera (P < 0.001).

Monoclonal TgAb-Fab in RIA

The results of measurement of region A TgAb-Fab were similar in RIA 1 (205 IU/ml) and RIA 2 (207 IU/ml) and slightly higher in RIA 3 (435 IU/ml) (Fig. 5). The results were slightly different for region B TgAb-Fab: 277 IU/ml in RIA 1, 1096 IU/ml in RIA 2, and 1624 IU/ml in RIA 3. The figures for region C TgAb-Fab were 154 IU/ml (RIA
1), 349 IU/ml (RIA 2) and 434 IU/ml (RIA 3). Region D TgAb-Fab was undetectable in all three assays.

TgAb IRP in the six TgAb assays

TgAb IRP was measured in the six assays in two sets of experiments performed in 2007 and 2009 (Fig. 6). The results of measurements of undiluted IRP in 2009 were: 948 (IMA 1), 965 (IMA 2), 1088 (IMA 3), 638 (RIA 1), 157 (RIA 2), and 299 (RIA 3) (expected, 1000) IU/ml. IRP was detectable up to 1:128 in IMA 1, 1:64 in IMA 2, 1:256 in IMA 3 and RIA 1, 1:16 in RIA 2, and 1:32 in RIA 3.

The results obtained measuring IRP in IMA 2, IMA 3, RIA 2, and RIA 3 in 2007 were similar to those obtained in 2009. IMA 1 yielded higher values (undiluted = 1454 and detectable up to 1:256), and RIA 1 yielded lower values (undiluted = 283 and detectable up to 1:16) in 2007 than in 2009.

Discussion

After their identification in HT, serum TgAb became a marker of thyroid autoimmunity, to be shortly thereafter overcome by serum TPOAb, which proved to be more sensitive for the diagnosis of AITD. However, when AITD is suspected despite negative TPOAb, TgAb are still useful for AITD diagnosis (1). TgAb are also present in 7.5–25% of DTC patients (3–8), as well as in nodular goiter (28) and NS (9). The relationship between DTC and HT is still uncertain (5, 29–34).

The presence of serum TgAb in papillary thyroid carcinoma is clinically relevant. The guidelines on DTC state that TSH-stimulated Tg testing is sensitive enough to be used alone in the follow-up of DCT patients (who are TgAb negative) after total or near-total thyroidectomy and thyroid remnant ablation with $^{131}$I, particularly in those at low risk, who are the majority of patients (10–12, 35, 36). However, Tg measurement is hampered by coexistent TgAb, and therefore measurement of TgAb is mandatory on all specimens requiring Tg testing (10–14, 33, 37, 38). All attempts to overcome the interference of serum TgAb on Tg measurement have been unsuccessful (6, 13, 14). DTC patients with undetectable Tg and coexistent serum TgAb remain a challenge because of the uncertainty of their clinical condition. A recent guideline states that no evidence-based suggestion can be proposed in this group of patients (12), whereas another recommends periodical diagnostic $^{131}$I whole body scan and neck ultrasound (11).

We have shown that, in patients with DTC and associated thyroid autoimmunity, total thyroid ablation is required to attain the disappearance of serum TgAb, TPOAb, and TSH-receptor autoantibodies (8). In addition, changes of TgAb concentrations have been correlated with the course of DTC: increasing or stationary TgAb values with the persistence of thyroid tumor, de-
creasing TgAb values with its reduction (3, 4). Moreover, changes of TgAb have been shown to be a prognostic factor for DTC (16). Based on these findings, serial TgAb measurements have been proposed as a surrogate for Tg measurements in the postsurgical follow-up of DTC patients (11, 12, 14, 15, 33). However, reliability of TgAb measurement is greatly hampered by the low degree of correlation of commercially available TgAb assays (7, 17–21, 39, 40). Indeed, it is recommended to use the same TgAb assay during the follow-up of a single DTC patient and, when a new TgAb method is introduced, to measure an old specimen of the patient with the old and the new methods in order to establish the ratio between the two methods and to re-baseline TgAb concentrations (13). Nevertheless, it has been recently confirmed that the degree of correlation of TgAb assays is low and that the conversion of results of different methods is often unreliable (21). Discrepancy of TgAb assays lessens the consistency of serial TgAb measurements. TgAb can be measured by direct, noncompetitive, and competitive assays. They employ different detection tools: immunoenzymatic, radioimmunometric, immunochromiluminescent, and immunofluorescent. Several causes of discrepancy of TgAb assays have been proposed (14), but not extensively investigated in commercial TgAb assays. In evaluating patients with papillary thyroid carcinoma after total thyroidectomy and before thyroid remnant ablation (who are expected to have detectable Tg), we have recently reported that the proportion of undetectable Tg was higher and Tg levels were lower in PTC-T than in PTC (22). The percentage of positive TgAb, as evaluated by six TgAb assays (three noncompetitive IMA and three competitive RIA), was significantly higher in PTC-T (29.2–50.0%) than in PTC patients (1.9–6.7%). Among patients with undetectable Tg, TgAb were more frequently positive by IMA (57.1–63.3%) than RIA (30.6–42.9%). In the present study, we compare the results of the six TgAb assays and investigate the reasons for their differences.

The finding that 37.5% of PTC-T and 46.2% of HT were positive in one to five assays highlights the partial correlation of the six assays. TgAb concentrations were higher in HT and PTC-T, whereas TgAb were negative in most PTC patients and NS. In comparing TgAb concentrations from the six assays performed on the four subject groups, the values of correlation were extremely variable (−0.01 < Rho < 0.93) and nonsignificant in two cases. Two factors influenced the level of correlation: the methodology of the assay and the underlying clinical condition; the correlations were higher when assays of the same methodology (noncompetitive IMA or competitive RIA) were compared to each other and were greater in PTC-T and HT than PTC and NS.

In the second part of our study, we investigated the causes of discrepancy of the TgAb results. Our first aim was to confirm that methodology (noncompetitive IMA or competitive RIA) is relevant for discrepancy of TgAb assays. Indeed, PCA revealed two uncorrelated components (latent assays) explaining 40 and 37%, respectively, of the variance of the results of the six assays. The first latent assay included the three IMA, and the second included the three RIA. This evidence confirmed that methodology plays a relevant role in discrepancy of TgAb assays.

Second, we investigated whether the intrinsic characteristics of heterogeneous, polyclonal TgAb populations contribute to discrepancy. We hypothesized that TgAb with a more heterogeneous epitope pattern yielded more discrepant results. To confirm this hypothesis, we compared the epitope pattern evaluated by inhibition of TgAb binding to Tg by human monoclonal TgAb-Fab regions A, B, C, and D (24) in PTC-T, PTC, and HT sera that were positive in all six assays (concordant sera) and positive in one to five assays (discordant sera). These TgAb-Fab rec-
The standardization induce a false modification of TgAb concentrations by the same assay due to changes of internal standards in some assays. Differences in serial results of TgAb measurements with TgAb IRP and its variations over time can be relevant for the assays, the suboptimal correlation of the internal standard used for the experiments of 2007 with those of 2009, the results showed higher values and another showed lower values. Thus, although it is not a matter of concern in most TgAb assays, the suboptimal correlation of the internal standard used for the experiments of 2007 with those of 2009, the results showed higher values and another showed lower values.

In conclusion, we compared the results of six commercial TgAb assays, that employ two different methodologies (noncompetitive IMA and competitive RIA) in patients with PTC-T, PTC, and HT and in NS. The two most relevant causes of discrepancy were the TgAb assay methodology and the underlying disease. According to the present data, when replacing a TgAb assay during the follow-up of DTC patients, influence the characteristics of TgAb and in particular their epitope pattern is an issue that deserves to be investigated.
results it is advisable to choose another with a similar methodology. TgAb of PTC-T patients yield similar results in different assays because their epitope pattern is restricted. On the other hand, TgAb of PTC are heterogeneous and yield discrepant results. Obviously, the heterogeneous epitope pattern of TgAb is an intrinsic and nonmodifiable cause of discrepancy. Fortunately, this is not a relevant issue because TgAb are common in PTC-T and rare in PTC (22). Differences of Tg and TgAb preparations used by different TgAb assays do not seem to contribute significantly to the discrepancy of the results. The internal standardization of TgAb is generally, but not completely, satisfactory. The imperfect standardization contributes to the discrepancy of different assays and lessens the reliability of TgAb measured by the same assay over time. A constant and careful assessment of the internal standard by TgAb assay manufacturers remains therefore pivotal to guarantee reliability of serial TgAb measurements.

Acknowledgments

Address all correspondence and requests for reprints to: Francesco Latrofa, Department of Endocrinology, University Hospital of Pisa, Via Cisanello 2, 56124 Pisa, Italy. E-mail: latrofa@libero.it.

This work was supported by the grant “Riento dei cervelli” no. 311 from the Ministero dell’Istruzione, dell’Università e della Ricerca and the University of Pisa (to F.L.).

Disclosure Summary: The authors declare that they have no conflicts to disclose.

References

20. Spencer CA, Berggolio GM, Kazarosyan M, Fatemi S, LoPresti JS 2005 Clinical impact of thyroglobulin (Tg) and Tg autoantibody method differences on the management of patients with differentiated thyroid carcinomas. J Clin Endocrinol Metab 90:5566–5575
21. Spencer C, Petrovic I, Fatemi S 2011 Current thyroglobulin autoantibody (TgAb) assays often fail to detect interfering TgAb that can result in the reporting of falsely low/undetectable serum Tg IMA values for patients with differentiated thyroid cancer. J Clin Endocrinol Metab 96:1283–1291
33. Spencer CA Clinical 2011 Utility of thyroglobulin antibody (TgAb) measurements for patients with differentiated thyroid cancers (DTC). J Clin Endocrinol Metab 96:3615–3627