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Current status and performance goals for serum thyroglobulin assays

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Serum thyroglobulin (Tg) measurements are used as a tumor marker for monitoring patients with differentiated thyroid carcinoma. The clinical utility of six different Tg methods [RIA or immunometric assay (IMA)] currently used in Europe and the US was evaluated, with focus on methodologic standardization, sensitivity, interassay precision across the typical clinical monitoring interval (6 to 12 months), "hook" effects (IMA methods), and Tg autoantibody interference. The methods evaluated were: DYNOtest Tg (Henning), OptiQuant Tg (Kronus), SELco Tg (Medipan), Thyroglobulin IRMA (Pasteur), Nichols Chemiluminescent ICMA (Corning Nichols), and an RIA developed by us (USC Endocrine Services Laboratory). The clinical impact of the current methodologic problems on the use of serum Tg measurements is reviewed. Optimal performance goals are recommended for manufacturers developing and laboratories and physicians selecting a serum Tg method to use for serial long-term monitoring of thyroid cancer patients.

INDEXING TERMS: thyroid carcinoma • thyrotropin • radioimmunoassay • immunometric assay • immunochemiluminometric assay

Current Status of Tg Assays

The measurement of thyroglobulin (Tg) in serum is technically challenging.[1] The serum Tg concentration reflects primarily three factors: (*a*) the mass of differentiated thyroid tissue present; (*b*) any physical damage to or inflammation of the thyroid gland; and (*c*) the magnitude of thyrotropin (TSH)-receptor stimulation. Currently, immunometric assay (IMA) techniques are gaining popularity over RIA methods, since IMAs have the practical advantages of shorter incubation times, a wider working range, and a more stable labeled antibody reagent that is less prone to labeling damage *[1–3]*. Most IMAs are isotopic (IRMAs), although nonisotopic immunochemiluminometric assays (ICMAs) with longer reagent shelf life and the potential for automation are now becoming available.

This review evaluates the current methodologic status of serum Tg measurement as judged from studies of six different Tg methods: DYNOtest Tg (1995 formulation; Henning, Berlin, Germany) (IRMA-1); OptiQuant Tg (Kronus, San Clemente, CA) (IRMA-2); SELco Tg (Medipan Diagnostica, Selchow, Germany) (IRMA-3); Thyroglobulin IRMA (Pasteur, Marnes La Coquette, France) (IRMA-4); Nichols Chemiluminescent ICMA (Corning Nichols, San Juan Capistrano, CA); and an RIA used by us (Endocrine Services Laboratory, University of Southern California, Los Angeles, CA) that includes a high-affinity rabbit antibody and extensive tracer purification *[2–5]*.

Methodologic Problems

Currently, the clinical value of serum Tg measurements (made by either RIA or IMA) is limited by the following problems: (*a*) lack of universal method standardization, leading to significant intermethod variability *[6]*; (*b*) suboptimal assay functional sensitivity, which limits the detection of small amounts of thyroid tissue, especially when TSH is suppressed; (*c*) poor interassay precision across the typical clinical interval used for monitoring patients with differentiated thyroid carcinoma (6 to 12 months); (*d*) "hook" effects, which primarily affect IMA methods and can lead to inappropriately low- or euthyroid-range Tg values in sera with very high serum Tg concentrations *[7]*; and (*e*) Tg autoantibody (TgAb) interference, which can lead to under- or overestimation of the serum total Tg concentration, regardless of the type of method used *[8]*, and Spencer, manuscript in preparation).

METHOD STANDARDIZATION

The last international collaborative study showed that serum Tg values by RIA or IMA vary as much as 65% between methods *[6]*. A recent collaborative effort, sponsored by the Community Bureau of Reference (CBR) of the Commission of the European Communities, has now produced a Tg standard of glandular origin that is beginning to be adopted *[9]*. As shown in Fig. 1, the use of the CBR reference preparation significantly reduces,

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[1] Nonstandard abbreviations: Tg, thyroglobulin; TSH, thyrotropin; IMA, immunometric assay; ICMA, immunochemiluminometric assay; CBR, Community Bureau of Reference; rhTSH, recombinant human TSH; rlu, relative light unit; TgAb, thyroglobulin autoantibody; and TPO, thyroid peroxidase.

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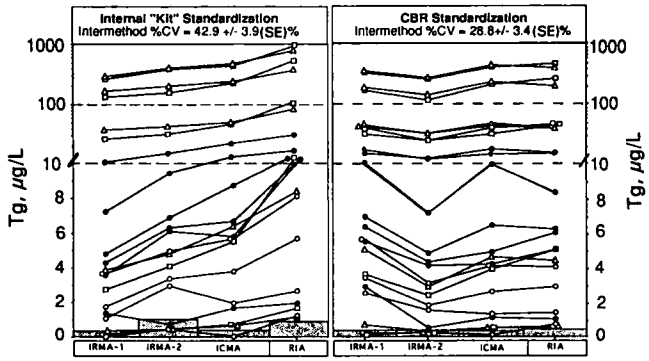


Fig. 1. Serum Tg values measured by six different Tg methods: DYNOtest Tg (IRMA-1, 1995 formulation); OptiQuant Tg (IRMA-2); SELco Tg (IRMA-3); Thyroglobulin IRMA-Pasteur (IRMA-4); Nichols Chemiluminescent ICMA; and an RIA developed by the USC Endocrine Services Laboratory.

The USC RIA involves extensive ^{125}I tracer purification *[2, 3]* and uses a high-affinity rabbit antibody that resists interference by TgAb *[4, 46]*. Four categories of patients' sera were measured in each assay, with either the internal kit standard or CBR standardization. (●), thyroxine-suppressed (TSH <0.1 mIU/L) healthy subjects; (○), thyroxine-suppressed thyroid lobectomized patients; (△), papillary carcinoma; (□), follicular carcinoma.

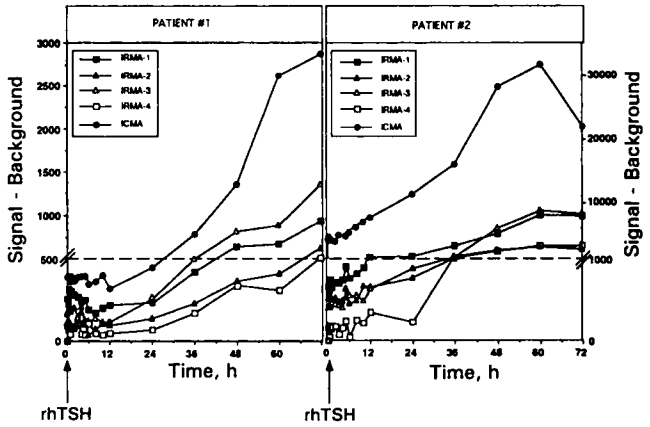


Fig. 2. Relative sensitivities of five different Tg methods as judged by the response in signal (cpm for IRMA and rlu for ICMA) after subtraction of background noise, at various times after intramuscular administration of rhTSH into two different patients.

Assays as defined in Fig. 1.

but does not eliminate, all intermethod variability (CV) [42.9% ± 3.9% (SE) vs 28.8% ± 3.4%, kit vs CBR standardization, respectively (P <0.01)]. The differences that remain after CBR standardization presumably reflect the differing specificities of the Tg antibody(s) used in the various methods.

Tg antibodies are conformational, i.e., are directed against discontinuous regions of the protein *[10]*. Tg in serum is heterogeneous, a result of differential splicing of Tg mRNA as well as carbohydrate and (or) iodide heterogeneity *[11, 12]*. This heterogeneity can lead to the exposure or masking of epitopes, resulting in antibody-dependent differences in Tg immunoactivity *[13–15]*. The conformational character of the antigen–antibody binding reaction of a Tg assay suggests that IMAs, with a single or restricted number of monoclonal "capture" antibodies, will be more likely to detect immunoactivity differences between different serum Tg isoforms, especially abnormal forms of Tg secreted by some thyroid neoplasms *[16]*. Also, immunoactivity differences between serum Tg and the Tg isoforms in the glandular extracts used for standardization have been reported *[13]*.

Although standardization of the antibodies used in the various Tg assays would probably further reduce method-to-method variability, this is probably not only impractical but may also limit the diagnostic utility of serum Tg measurement for patients with thyroid tumors that secrete abnormal forms of Tg not recognized by certain monoclonal antibodies *[12, 15]*.

FUNCTIONAL SENSITIVITY

Low-end precision determines an assay's functional sensitivity *[17]*. A tolerable interassay imprecision (CV) of 20% can be rationalized on both a methodologic and biological basis for serum TSH measurement *[18]*. It is more difficult to rationalize what level of interassay imprecision is appropriate for serum Tg measurement. The within-person biological variability (CV) in serum Tg concentrations in fasting specimens drawn with minimum hemostasis from euthyroid subjects over a 2-month period is ~14% *[19]*. This biological variability may not apply to patients with differentiated thyroid cancer, who may have unstable TSH status, have subnormal or increased serum Tg concentrations, and be evaluated at intervals of 6 to 12 months. In light of these special considerations, it is not unreasonable to use the 20% interassay CV as the determinant of Tg assay functional sensitivity, as was rationalized for serum TSH. Additional important constraints on determining a clinically relevant functional sensitivity would be the use of nonprocessed TgAb-negative human sera, and making the precision across a much longer clinical interval (6 to 12 months) than was recommended for serum TSH *[20]*.

Since current Tg methods still lack uniform standardization, the numeric values for sensitivity reported for different methods cannot be compared. As shown in Fig. 2, the serum Tg responses to recombinant human TSH (rhTSH) stimulation [signal after background subtraction, expressed as counts per minute (cpm) for IRMA and relative light units (rlu) for ICMA] have been used to assess the relative sensitivity of five different Tg IMA methods independently of their differing standardization *[21]*. This study revealed that different methods have inherently different sensitivities as judged by the generation of a signal in response to the provocative biological stimulus (rhTSH). As with TSH assays, the nonisotopic ICMA appeared to be more sensitive than the isotopic IRMAs.

Many current Tg methods have suboptimal sensitivity, as judged by an inability to measure the lower limit of the euthyroid range. This is evident from the data shown in Fig. 1, in which some methods failed to detect Tg in TSH-suppressed sera from healthy subjects or from lobectomized patients who had significant amounts of thyroid tissue present. Tg is no longer considered a secluded antigen and should be detectable in sera from all euthyroid subjects, even during TSH suppression when serum Tg concentrations are reduced by only ~50–60% *[22]*. When rhTSH becomes available (estimated to be 1996), thyroid hormone withdrawal will no longer be necessary, and

patients will be scanned with ^{131}I in the TSH-suppressed state. This change in protocol will necessitate that Tg assays have maximum sensitivity for detecting low serum Tg concentrations secreted by small amounts of thyroid tissue in the absence of TSH stimulation.

Serum Tg values are log-distributed in euthyroid subjects *[23]*. Individuals chosen for establishing the reference range should be selected carefully after excluding those with a family history of thyroid disease, palpable or visible thyroid glands, thyroid autoantibodies [anti-thyroid peroxidase (anti-TPO) or TgAb] detected by IMA, or L-thyroxine medication for any reason. Although a reference range is an important characteristic of most biochemical tests, this is less so for serum Tg measurements in patients who have received treatment for differentiated thyroid carcinoma. There is no appropriate serum Tg reference range for such a patient, in whom the serum Tg concentration is affected by such clinical factors as the completeness of surgery, radioiodine therapy, and serum TSH status. In clinical practice, the temporal serial serum Tg pattern (during TSH suppression) is a more important clinical indicator than a single serum Tg value. The only postsurgical serum Tg reference value for a patient treated for differentiated thyroid carcinoma is zero, since the tissue-specific origin of Tg biosynthesis dictates that Tg in serum will be absent (even when endogenous TSH is stimulated) when a patient has been successfully rendered athyreotic. Assays that provide the greatest distinction between the lower limit of the euthyroid reference range and the functional sensitivity limit of the assay offer the most clinical sensitivity for detecting small amounts of thyroid tissue in the TSH-suppressed state.

PRECISION

Good interassay precision across the entire Tg assay working range (estimated across a clinically realistic timespan of 6–12 months) is critical for distinguishing disease-free patients from those with residual tumor who may require diagnostic scanning or radioiodine treatment *[24]*. Good precision for measuring high serum Tg concentrations is also essential for monitoring the progression of tumor or response to therapy. A minority of patients have highly increased serum Tg concentrations (10 to 1000 times greater than the upper limit of measurement) when they have extensive disease metastatic to bone *[25, 26]*. Measurements of serum Tg concentrations requiring extensive dilution are associated with a greater overall variance (interassay + dilutional errors) than is typical of measurements made within the assay range.

Progression and recurrence of differentiated thyroid carcinoma can manifest acutely as a rapidly enlarging mass or be relatively indolent, occurring over months or years after initial treatment. The interassay precision of serial Tg measurements over 6–12 months determines the clinical significance of any change in serum Tg concentration. Poor interassay precision may delay the detection of recurrence or progression of disease. As shown in Fig. 3, clinically significant changes in serum Tg concentrations can be detected far earlier by using intraassay remeasurement of Tg in the unused stored (−20 °C) previous serum sample each time a new specimen from the patient is

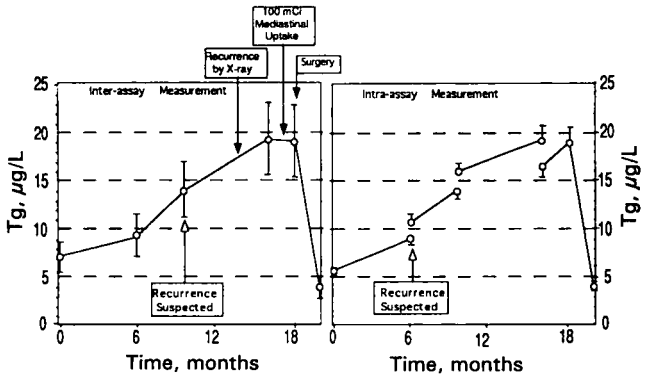


Fig. 3. Serial serum Tg values (±2 SD) obtained with either an inter- or intraassay mode of measurement during course of a recurrence of papillary carcinoma.

The larger error associated with the interassay mode prevented early detection of the clinically significant increase in the serum Tg concentration. In contrast, when the previous specimen (stored at −20 °C) was remeasured with the current specimen in the intraassay mode, which eliminates interassay variability, the clinically significant increase in serum Tg concentration was detected earlier.

received. A concurrent redilution/remeasurement approach is particularly valuable for minimizing the cumulative errors associated with measuring high Tg concentrations that require dilution. Whereas most laboratories may consider the concurrent remeasurement approach impractical for routine use, physicians should be able to make special arrangements with laboratories for storing or returning unused specimens from high-risk patients, since reliable serial serum Tg measurements can favorably influence management decisions that may involve costly imaging procedures. Methods must demonstrate freeze–thaw stability if the concurrent remeasurement approach is to be a feasible option.

"HOOK" EFFECTS

IMA (but less often RIA) methods are subject to "hook" effects *[7]*. This serious problem leads to the reporting of inappropriately normal or even low serum Tg values in sera with very high serum Tg concentrations, which require dilution. The hook effect appears to result when a massive excess of antigen (10 to 10 000 times the upper limit of the assay range) exhausts the binding capacity of the Tg capture antibody on the solid support. Although sera from patients with extensive disease and very high serum Tg concentrations account for a minority of test requests received by the laboratory, an inappropriately low serum Tg report may have major clinical and medicolegal consequences. An analysis of 2731 thyroid cancer serum specimens analyzed by our laboratory showed that 2.9% of specimens had serum Tg values >500 μg/L, 1.3% of which were >1000 μg/L; 0.1% were >10 000 μg/L. As shown in Fig. 4, all three IMA methods evaluated showed a hook effect to some extent. Since the problem is only loosely correlated with the absolute degree of increase of serum Tg concentration, every specimen should be treated as having a potential to "hook." Laboratories using IMA methods should run every serum specimen at two dilutions (e.g., undiluted and 1:10) or use other measures such as batching to detect hook problems before reporting the serum Tg concentration.

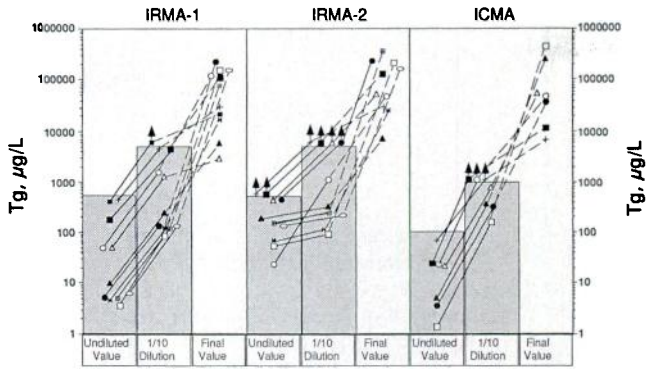


Fig. 4. Hook effects observed when measuring sera with very high (>1000 μg/L) serum Tg concentrations with three different IMA methods (as defined in Fig. 1).

Serum Tg concentrations were measured undiluted and at 1:10, 1:100, 1:1000, and 1:10 000 dilutions to establish the final Tg value, which was based on the dilutions that produced parallelism within the assay working range.

TG ANTIBODIES

Specificity differences between Tg methods result from both Tg-related and Tg-unrelated influences on the antibody–antigen interaction. The Tg-related specificity of a method is dictated by the epitope specificity of the antibody chosen for that method. Non-Tg factors also affect the assay; these include the Tg-free zero matrix used for diluting standards as well as poorly defined serum components and heterophile antibodies *[27–30]*. These non-Tg-related factors are difficult to detect or study.

TgAb interference is the most serious specificity problem affecting serum Tg measurement. Autoantibodies against Tg are present at high concentrations in sera from patients with autoimmune thyroid disorders and low concentrations in healthy individuals *[31–33]*. Epitope mapping of human thyroglobulin has revealed six different antigenic domains (regions I–VI) *[31]*. The specificities of natural and pathologic TgAbs differ for these regions *[32]*. Monospecific (region II) high-affinity antibodies appear to be characteristic of autoimmune thyroid pathologies such as Hashimoto thyroiditis and Graves disease, whereas a low-affinity polyclonal response against evolutionarily conserved hormonogenetic regions (III, IV, and V) appears characteristic of the "natural" TgAbs seen in healthy individuals *[32]*. No Tg method is free from TgAb interference from all TgAb-positive sera, although some methods appear more resistant than others, as judged from concordance between serum Tg values and clinical status. The finding that even low concentrations of TgAb can interfere with serum Tg measurement (*[34]*, and Spencer, manuscript in preparation) raises the following questions:

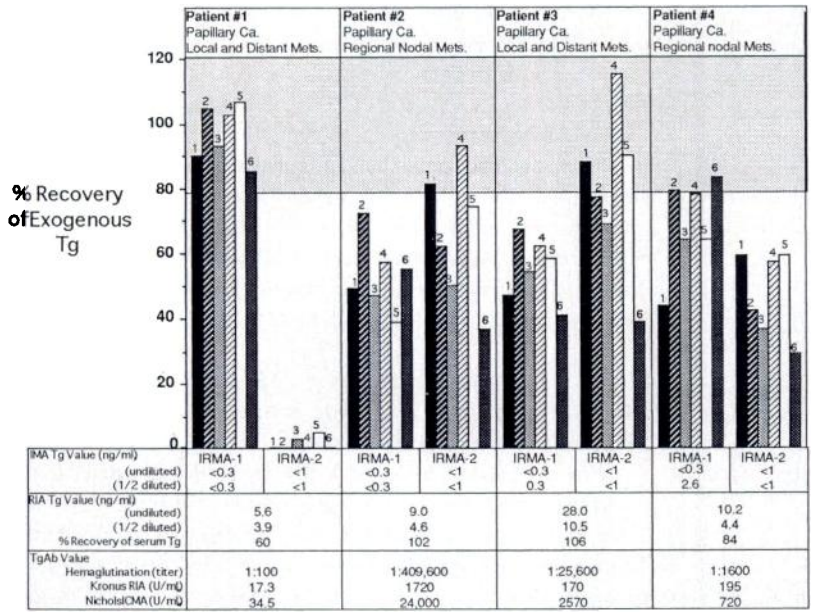
What is the incidence of serum TgAb in differentiated thyroid carcinoma? TgAb immunoassays are more sensitive than the earlier passive hemagglutination techniques *[35, 36]*. When sensitive TgAb immunoassay methods are used in preference to hemagglutination, TgAb is detected in the serum of 4–27% of healthy individuals; 51% and 97% of patients with Graves disease or Hashimoto thyroiditis, respectively; and between 15% and 30% of patients with thyroid carcinoma *[21, 35–37]*.

The incidence of both thyroid autoantibodies (TgAb and anti-TPO) appears to be increased in thyroid carcinoma as compared with the general population [26.4% vs 9.9% (TgAb) and 23.1% vs 10.7% (anti-TPO)] *[19]*. These findings are in agreement with other reports showing that the overall incidence of TgAb and (or) anti-TPO relates to the degree of tumor differentiation (papillary = follicular >anaplastic >medullary); is greater in females than in males; and unlike the general population, displays no age-related relation *[38]*. These observations suggest that thyroid carcinoma per se can produce an antigenic stimulus for some patients.

What is the prognostic significance of TgAb in serum? Autoantibodies generally decrease over time in the absence of continued antigenic stimulation (e.g., decreased titers of islet cell antibodies with progression of type 1 diabetes). Preoperative serum TgAb concentrations have no prognostic or diagnostic significance, since TgAb concentrations do not relate to tumor stage at diagnosis or subsequent clinical outcome *[39]*. Serial postoperative serum TgAb measurements do, however, appear to give some prognostic information on the long-term clinical outcome of TgAb-positive patients. Several retrospective studies show that serum TgAb concentrations generally decline or disappear over time (months to years) following initial surgical treatment for differentiated thyroid carcinoma in patients who are judged clinically disease-free after long-term follow-up. In contrast, serum TgAb concentrations remained relatively unchanged or increased when patients had persistent or progressive disease (*[38–40]*, and Spencer, manuscript in preparation). Although the release of thyroid antigen secondary to radiation damage might contribute to the persistent TgAb detected in patients with persistent disease, TgAb-positive patients are typically positive from the time of their initial surgery, and few if any patients develop detectable TgAb de novo after the diagnosis of differentiated thyroid carcinoma has been made (*[38, 41]*, and Spencer, manuscript in preparation).

What mechanisms produce TgAb interference with Tg measurement? Analytical validity tests, involving parallel dilutions of TgAb-positive sera or recovery of exogenous Tg from TgAb-positive sera, have been used to screen for TgAb interference with serum Tg measurement *[26, 34, 42–44]*. These in vitro tests have suggested that TgAb interference produces either an over- or underestimation of the serum Tg concentration in a manner that is both serum and method dependent (*[8, 44]*, and Spencer, manuscript in preparation).

Overestimation is characteristic of TgAb interference with serum Tg measurements made by many double-antibody RIA methods; however, underestimation may also occur *[34]*. The magnitude and direction of interference relates to the affinity of the first antibody, the specificity of the second antibody, the volume of serum used, and the characteristics of the TgAb present, all of which affect the partitioning of endogenous serum and exogenous ^{125}I-labeled Tg molecules between the endogenous (human) and exogenous (animal) antibody-bound fractions *[34]*. Few studies have correlated the Tg values in TgAb-positive sera with the clinical status of patients. Black et al. have reported



IMA Tg Value (ng/ml)	IRMA-1	IRMA-2	IRMA-1	IRMA-2	IRMA-1	IRMA-2	IRMA-1	IRMA-2
(undiluted)	<0.3	<1	<0.3	<1	<0.3	<1	<0.3	<1
(1/2 diluted)	<0.3	<1	<0.3	<1	0.3	<1	2.6	<1
RIA Tg Value (ng/ml)								
(undiluted)	5.6		9.0		28.0		10.2	
(1/2 diluted)	3.9		4.6		10.5		4.4	
% Recovery of serum Tg	60		102		106		84	
TgAb Value								
Hemaglutination (titer)	1:100		1:409,600		1:25,600		1:1600	
Kronus RIA (U/ml)	17.3		1720		170		195	
NicholsICMA (U/ml)	34.5		24,000		2570		720	

Fig. 5. Percent recovery of different exogenous Tg sources from sera from TgAb-positive patients with clinical or scan evidence of residual or metastatic papillary thyroid carcinoma, with two different Tg IRMA methods (as defined in Fig. 1).

Lane 1, percent recovery of serum Tg (10 μg/L); *lane 2*, percent recovery of kit standard Tg (10 μg/L); *lane 3*, percent recovery of kit standard Tg (100 μg/L); *lane 4*, percent recovery of a low-iodine glandular Tg preparation (10 μg/L); *lane 5*, percent recovery of a low-iodine glandular Tg preparation (100 μg/L); *lane 6*, same as *lane 2* but with overnight preincubation of the Tg-containing serum supplement before assay. The *shaded area* denotes the percent recovery (±SD) of serum Tg from TgAb-negative sera. In addition, sera were run at two doubling dilutions to check for parallelism. Serum Tg values were typically lower or undetectable in the IRMA methods as compared with the RIA.

a 97% clinical concordance when total (free +TgAb-bound) serum Tg was measured by an RIA that included a high-affinity first antibody together with a species-specific second antibody *[4, 45–47]*. Clinical concordance has not been generally found with RIAs that involve low-affinity first antibodies.

Recovery studies have suggested that underestimation is characteristic of the interference seen with most IMA methods, in which endogenous serum Tg complexed with TgAb appears to be prevented from fully participating in the two-site "sandwich" reaction *[27]*. Minimal TgAb interference has been reported for an IMA that includes a monoclonal capture antibody with specificity for Tg epitopes that are only minimally involved in the polyclonal immune response *[48]*. Although this approach appears scientifically attractive, a recent study suggested that TgAb interference was still present to a comparable extent because the IMA did not have a restricted capture antibody *[8]*. The inability to eliminate TgAb interference by monoclonal antibody selection might reflect the broader-based TgAb specificity characteristic of thyroid carcinoma, as compared with the more limited epitope specificity characteristics of autoimmune thyroid disease *[49]*.

How can TgAb interference be detected? Unfortunately, in vitro tests for TgAb interference are not routinely correlated with the in vivo disease status of the patient (Spencer, manuscript in preparation). Further, poor recovery of exogenous Tg is seen with some TgAb-negative sera, suggesting that there may be interference by non-TgAb serum factors, or that TgAb concentrations below the detectability limit of the measurement may interfere *[44]*. The recent study by Marriotti et al., which showed discordance between in vitro recovery and in vivo clinical status in a TSH-stimulated state, necessitates a reevaluation of whether the recovery approach to screening for TgAb interference has any utility for validating serum Tg measurements of TgAb-positive sera *[8]*. Three limitations may compromise the recovery approach:

1) There may be differences in recovery of the exogenous Tg added and the endogenous serum Tg isoforms present *[13]*. The exogenous Tg is usually a standard Tg preparation of nonneoplastic glandular origin with higher iodine content than Tg secreted by thyroid tumors *[50]*. Any difference in the molecular conformation of the exogenous vs the endogenous Tg isoforms has the potential to produce exogenous Tg–TgAb complexes with differing affinity from the endogenous Tg–TgAb complexes and thereby invalidate the recovery estimate.

2) Recovery may be influenced by the relation between the endogenous Tg concentration and the amount of exogenous Tg added for recovery *([51]*, and Spencer, manuscript in preparation). Usually the concentration of exogenous Tg used is high (50–200 μg/L) relative to the endogenous Tg present *[8]*. This discrepancy in mass may give rise to an overestimation of recovery *[43]*. The recovery of more physiological concentrations of serum Tg may give a more realistic estimate *[4]*.

3) It takes time for immune complexes involving a large molecule such as Tg to reach equilibrium. The practice of adding exogenous Tg to TgAb-positive sera immediately before adding the assay components increases the risk of producing disequilibria between the exogenous and endogenous Tg–TgAb complexes and the potential to distort recovery (Spencer, manuscript in preparation).

Figure 5 shows an evaluation of the recovery of different exogenous Tg sources, two different concentrations of which were added to four different TgAb-positive sera before the serum Tg concentration was measured by two different IRMAs (Spencer, manuscript in preparation). The TgAb-positive sera were all drawn from patients with scanning or clinical evidence of metastatic differentiated thyroid carcinoma who were expected to have detectable serum Tg concentrations. All patients had nonsuppressed serum TSH concentrations. The percent recovery of six different supplements of exogenous Tg, diluted in the appropriate assay matrix, was calculated for each serum as follows: lane 1, serum Tg (~10 μg/L) derived from a TgAb-

negative thyroid cancer patient, with the serum Tg concentration measured immediately after supplementation; lanes 2, 3, and 6, two different concentrations of the internal recovery or "kit" standard were supplemented, such that lanes 2 and 3 included supplements of ~10 and ~100 μg/L, respectively, followed immediately by assay, and lane 6 was identical to lane 2 but allowed equilibration of the supplement (overnight at ambient temperature) with the serum components before assay; lanes 4 and 5, two different concentrations of a low-iodine glandular Tg preparation (kindly provided by Alvin Taurog, Southwestern Medical Center, Dallas TX) diluted in the appropriate assay zero matrix and supplemented immediately before assay, with lane 4 containing ~10 μg/L and lane 5 containing ~100 μg/L.

The studies shown in Fig. 5 suggest that: (*a*) IMA methods typically underestimate the total (free + TgAb-bound) Tg concentration in TgAb-positive sera, as judged by the finding of undetectable serum Tg values in patients with clinical evidence of metastatic thyroid carcinoma and nonsuppressed serum TSH concentrations; (*b*) neither recovery nor dilution studies appeared to reliably detect TgAb interference, as judged from the discordance between the serum Tg concentrations reported and the clinical status of the patients; (*c*) TgAb interference was characterized by discordance between IMA and RIA values and low recovery of some exogenous Tg sources; (*d*) the percent recovery of exogenous Tg was method dependent (i.e., IRMA-1 vs IRMA-2 for patients 1 and 3); (*e*) overnight equilibration of the added exogenous Tg led to significantly lower recoveries, suggesting that time is needed to equilibrate exogenous Tg with serum components [75.3% ± 11.3% vs 86.2% ± 9.2% (SE), $P < 0.01$; and 50.2% ± 10.7% vs 62.4% ± 11.7%, $P < 0.01$; lane 6 vs lane 2; IRMA-1 vs IRMA-2, respectively (Spencer, manuscript in preparation)]; (*f*) higher doses of exogenous Tg tend to produce lower recoveries (lanes 2 vs 3 and 4 vs 5); (*g*) the percent recovery is dependent on the source of exogenous Tg, i.e., the percent recovery of serum Tg may be different from the recovery of a glandular Tg extract (lane 1 vs lanes 2 and 4); and (*h*) the percent recovery does not correlate with the concentration of TgAb (i.e., patient 1 had the lowest TgAb concentration and near-zero recovery with IRMA-2).

What is the relation between TgAb concentrations and interference? It is difficult to predict which TgAb-positive serum samples will interfere with serum Tg measurement, because the TgAb concentrations do not correlate with the degree of interference *[8, 44]*. Any TgAb detected by sensitive immunoassay appears to have the potential to interfere! Most laboratories still use insensitive hemagglutination techniques to detect TgAb in sera despite several reports showing that TgAb concentrations too low to be detected by hemagglutination can interfere with serum Tg measurements *[6]*, and Spencer, manuscript in preparation). As shown in Fig. 5, patient 1, with the lowest TgAb concentration by three different methods, had the most profound suppression of recovery with IRMA-2, suggesting that the "quality" of the TgAb present may be as important as the TgAb concentration needed to produce interference. There is currently no way to assess TgAb quality in this regard.

What are the clinical consequences of TgAb interference? Any change in the capacity or affinity of circulating TgAb has the potential to change the total Tg value independently of any change in tumor mass. Potential mechanism(s) may involve a change in total Tg binding capacity (analogous to the effect of thyroxine-binding globulin concentration on total serum thyroxine concentrations) or a change in the clearance of Tg–TgAb complexes *[52]*. The pattern of serial serum TgAb measurements may provide the physician with an additional indicator to use in therapeutic decision-making when patients are TgAb-positive *[38]*, and Spencer, manuscript in preparation). Even if the Tg method can give a valid measure of the total serum Tg concentration (as judged by clinical correlations), it is important for the physician to interpret that Tg value with caution in relation to the patient's prognostic factors *[53]*, changes in TgAb concentrations *[8, 38–40*, and Spencer, manuscript in preparation), and independent clinical and radiographic studies.

Different clinical consequences result from a failure to detect TgAb interference that produces under- or overestimation of serum Tg concentrations. The underestimation typical of IMA methods potentially leads to clinical inaction in patients with recurrence or metastases. In contrast, overestimation, more common with RIA methods, produces false-positive values, which may prompt unnecessary imaging studies and promote unnecessary patient concern. Since any Tg method has the potential for interference by some TgAb-positive sera, and since there is currently no reliable way to check which sera interfere, serum Tg measurements made in TgAb-positive patients should be interpreted with caution.

Performance Goals for Tg Methods

Standardization. Ideally, all methods would be standardized on the new CBR standard (obtained from Christos Profilis, BCR, Rue del la Loi 200, B 1049 Brussels, Belgium) to minimize intermethod variability, as shown in Fig. 1 *[9]*. Although universal standardization would facilitate the comparison of methods in scientific publications, restandardizing existing methods has the potential to disrupt the value of serial measurements made in patients with differentiated thyroid carcinoma. Physicians must be informed before a laboratory changes its Tg method or restandardizes an existing method, so that a new baseline value for each patient may be established.

Sensitivity/precision. Since sensitivity is critical when using serum Tg measurement to detect small amounts of thyroid tissue, functional assessments should be made with the same constraints as developed for TSH methods *[54]*. Functional sensitivity should be assessed from the 20% CV point of the interassay precision profile established from measuring TgAb-negative human sera over 6–12 months. The most sensitive serum Tg methods are capable of discriminating thyroid hormone-suppressed serum Tg concentrations in healthy controls from the assay functional sensitivity limit.

Serum Tg reference range. Euthyroid subjects used to establish the serum Tg reference range should be selected with care.

Exclusions should include individuals with (*a*) a family history of thyroid disease; (*b*) palpable or visible thyroid gland; (*c*) thyroid autoantibodies (anti-TPO or TgAb detected by sensitive immunoassay); (*d*) L-thyroxine medication for any reason; or (*e*) a history of subacute thyroiditis.

Note: The reference range for serum Tg measurement is an assay indicator that has little relevance for a patient who has been treated for differentiated thyroid cancer and in whom serum Tg values are influenced by the degree of surgical treatment, radioiodine therapy, and serum TSH concentration.

Hook effect (IMA methods only). Although very high serum Tg concentrations are encountered in a minority of patients (2.9% of patients have serum Tg values >500 μg/L), all sera must be screened for the hook problem either as a batch *[7]* or individually by dilution (we suggest undiluted and 1:10 dilution). The reporting of an inappropriately low or normal serum Tg value as a result of the hook problem can have major medical and legal consequences.

Serum TgAb screening. Sensitive TgAb immunoassays should be used in preference to hemagglutination techniques for prescreening sera for TgAb before, or coincident with, serum Tg measurement. Patients who are identified as TgAb negative require infrequent TgAb screening, since these patients rarely become TgAb-positive. In contrast, serial serum TgAb measurements should be reported for all patients who have detectable serum TgAb concentrations, since serial TgAb measurements provide additional clinical information *([38]*, and Spencer, manuscript in preparation).

Serum Tg measurement of TgAb-positive sera. No method is totally free from interference by all TgAb-positive sera. In vitro validations [recovery and (or) dilutions] do not always reliably detect interference. The only "gold standard" for validating serum Tg measurements in TgAb-positive sera is the demonstration of concordance between the serum total (free + TgAb-bound) Tg concentrations and the clinical status of the patients. When methods report a total serum Tg concentration in a TgAb-positive patient, this value should be interpreted with caution and with consideration of: the direction of interference typical of the type of method used; any change in TgAb concentration; and other clinical, diagnostic, and prognostic factors pertaining to that individual patient. Laboratories that choose to report serum Tg concentrations in TgAb-positive patients should identify the type of method used and provide a warning relating to the direction of interference (under- or overestimation) typical of that method, on the report.

Recommendations for Laboratories

It is the laboratory's responsibility to the physician, and ultimately to the patient, to report a serum Tg concentration that generates an appropriate clinical response. This responsibility requires that laboratories minimize sources of random error, establish relevant reference ranges, and realistically identify the upper and lower limits of the measurement on the basis of confidence limits. The characteristics of the Tg method selected by a laboratory have a significant impact on the cost-effectiveness of management as well as potentially the morbidity and mortality of patients with differentiated thyroid carcinomas. The laboratory should not change the Tg method or the method's standardization without first consulting the physician users. If a change in method is to be made, it is the laboratory's responsibility to inform the physicians how the change will affect patient values and to provide relevant data on the new method's sensitivity, precision, and TgAb interference characteristics.

Sensitivity/precision. Laboratories should establish Tg assay functional sensitivity from the 20% CV point of the interassay precision profile made from measuring TgAb-negative sera assessed over a 6–12-month interval. The functional sensitivity limit should be used as the lower reporting limit on patients' reports. The physiologic sensitivity of an assay (as judged by the discrimination between thyroid-hormone-suppressed serum Tg concentrations in healthy controls and the assay functional sensitivity limit) is an important criterion for selecting a sensitive method.

Hook problems. Users of RIA methods should periodically validate the upper assay limit by diluting specimens having concentrations close to the upper limit with patients' specimens that have undetectable serum Tg concentrations (this approach negates any matrix effect). Users of IMA methods should either measure each specimen at two dilutions (undiluted and 1:10 diluted are recommended) and check for concordance between dilutions or analyze with each run a pool made from a batch of specimens, as suggested by Cole et al. *[7]*.

TgAb screening. The TgAb status of every patient should be assessed by a sensitive TgAb immunoassay (not hemagglutination), and a positive TgAb concentration should always be reported. When patients are TgAb negative, it is necessary to check TgAb concentrations only infrequently (every 1–2 years), since TgAb-negative patients with differentiated thyroid carcinoma rarely become TgAb positive (personal observations). In contrast, when a patient is TgAb positive, it is necessary to measure and report the serum TgAb concentration of every specimen, since serum TgAb temporal patterns may provide additional clinical information.

TgAb interference with serum Tg measurements. All methods (either RIA or IMA) have the potential for TgAb interference by some TgAb-positive sera. It is the manufacturer's responsibility to establish whether their Tg method provides appropriate serum Tg measurements in TgAb-positive sera by demonstrating a clinical correlation in TSH-suppressed and nonsuppressed patients. Recovery studies are not a reliable way to detect interference in all TgAb-positive sera and should not be used to determine the validity of a serum Tg measurement. If the laboratory chooses to report serum Tg values in TgAb-positive sera, each report should include a warning to the physician to interpret the Tg value with caution. Further, the typical direc-

tion of interference (over- or underestimation) should be stated as part of that report.

Recommendations for Manufacturers

The manufacturer's responsibility is to realistically define the performance characteristics of the method, using a clinically realistic protocol as outlined above. It is also the manufacturer's responsibility to ensure that any performance claimed can be reproduced across a wide range of clinical laboratories. The kit package insert should accurately and realistically define the method standardization, functional sensitivity, reference ranges, and TgAb interference characteristics.

IMA methods. The use of a capture antibody(s) with broad-based specificity may have advantages for detecting abnormal forms of Tg secreted by some thyroid tumors. The use of a capture antibody with specificity for epitopes not involved in autoantibody formation does not appear to minimize the interference by TgAb *[8]*. Epitope mapping does not appear to offer any practical advantages. A recommended approach for detecting "hook" problems should be part of the procedure as suggested above.

RIA methods. RIA methods should be optimized for maximal sensitivity. It Is difficult to balance the clinical need for sensitivity (requiring preincubation with loss of range) with expediency (short incubations) when developing these methods. Use of a high-affinity polyclonal antibody together with a species-specific second antibody is favored *[4]*.

Recommendations for Physicians

The reliability and validity of serum Tg measurements has a major impact on the clinical management of patients with differentiated thyroid carcinoma. Serum Tg measurement is one of the most difficult biochemical tests for a laboratory to maintain at a high level of precision and reliability over the long intervals typically involved in monitoring these patients (6 to 12 months). Physicians should be aware that the diagnostic value of serum Tg measurement is influenced by the laboratory chosen to perform this test. Physician–laboratory communication is key for maximizing the clinical utility of serum Tg testing and minimizing unnecessary costly imaging studies. Serum Tg values obtained by different methods are not interchangeable, even when methods are standardized with the new CBR reference preparation. This intermethod variability dictates that serial serum Tg measurements should be made by the same method, preferably performed by the same laboratory.

Physicians need to consider several factors before selecting a laboratory to perform serum Tg measurements, including the degree of client/technical support and the characteristics of the Tg method offered. Optimal Tg methods: (*a*) should have sufficient sensitivity to detect small amounts of thyroid tissue when serum TSH is low (as judged by the discrimination between thyroid hormone-suppressed serum Tg concentrations in healthy controls and the assay functional sensitivity limit); (*b*) IMA methods used should have a protocol to screen for the hook effects; (*c*) all sera should be screened for TgAb with a sensitive TgAb immunoassay (not hemagglutination); (*d*) when detected, the serum TgAb concentration should be reported; (*e*) laboratories should state the typical direction of TgAb interference experienced with that method; (*f*) laboratories should be willing to arrange to return or store unused sera from high-risk patients for subsequent remeasurement with a later specimen. (The laboratory must, however, demonstrate freeze–thaw stability of the Tg measurement for the concurrent remeasurement approach to be reliable.)

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