

Thyroglobulin Antibodies in Serum of Patients with Differentiated Thyroid Cancer: Relationship between Epitope Specificities and Thyroglobulin Recovery

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Background: Serum antibodies against thyroglobulin (TgAbs) are common in patients with differentiated thyroid cancer (DTC) and can interfere in thyroglobulin (Tg) assays. We identified the epitopes on Tg recognized by TgAb-positive sera from patients with DTC and examined the association between epitope specificity patterns and Tg recovery.

Methods: We tested 50 DTC sera for Tg epitope specificity, TgAbs, and Tg recovery. Epitope recognition was determined by use of a panel of 10 well-characterized Tg monoclonal antibodies directed against 6 Tg antigenic clusters (I–VI) in competitive reactions with test sera. Tg was measured by the Thyroglobuline IRMA (CIS bio international). Recovery of added Tg (TgREC) was determined by an in-house assay.

Results: Epitope recognition was restricted to immunodominant clusters in 58% of patients, whereas the rest were either broadly heterogeneous (16%) or nonreactive (26%). Median Tg recovery did not differ between sera with restricted and unrestricted specificities (69% vs 80%; $P > 0.05$). TgREC was inversely correlated with the total number of epitopes recognized by sera ($r = -0.66$; $P < 0.001$).

Conclusions: TgAbs with both restricted and broad specificities are present in patients with DTC. TgAb

interference is related to the number of epitopes recognized by sera rather than the pattern of epitope recognition.

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Thyroglobulin (Tg)⁴ is a useful serum marker in the postoperative monitoring of patients with differentiated thyroid cancer (DTC) (1). Antibodies to thyroglobulin (TgAbs) may, however, interfere with Tg measurements in serum. TgAb interference may lead to underestimation of serum Tg as measured by IRMA methods (2). With RIA methods, TgAbs may cause under- or overestimation, depending on the method used (3,4). Underestimation could potentially lead to recurrent or persistent disease being missed, whereas overestimation may cause needless investigation and intervention (4). This is a limitation to the value of Tg in DTC management given that 20–40% of DTC patients have TgAbs in serum (5) and no method has been found that completely eliminates interference.

With IRMA methods, TgAbs bind Tg, preventing it from reacting with analytical antibodies (2). The determinants of these antigen–antibody interactions are unclear. Interference is not seen in all patients with TgAbs and cannot be reliably predicted from TgAb concentrations because samples from some patients with very high TgAb activities do not show interference, whereas others with low antibody activities are subject to interference (4). The determinants of interference might therefore involve TgAb characteristics other than the concentration in serum. High-avidity TgAbs are more likely to interfere with

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⁴ Nonstandard abbreviations: Tg, thyroglobulin; DTC, differentiated thyroid cancer; TgAb, thyroglobulin antibody; AITD, autoimmune thyroid disease; Mab, monoclonal antibody; TPO, thyroid peroxidase; TgREC, thyroglobulin recovery; and AP, alkaline phosphatase.

Tg, suggesting that qualitative properties of TgAbs play a role in deciding interference (6).

Studies on TgAb epitope recognition patterns have provided one method of defining qualitative differences in the TgAb repertoire seen in various autoimmune and nonautoimmune thyroid disorders as well as in the healthy state. TgAbs in sera from patients with autoimmune thyroid disease (AITD) recognize a restricted number of epitopes, whereas healthy sera exhibit an unrestricted epitope-binding pattern (7). In sera from patients with thyroid cancer, epitope reactivity studies have given conflicting results. Using monoclonal antibodies against Tg (Tg-Mabs) in competitive reactions with test sera, some workers have described a highly heterogeneous epitope recognition pattern (8), whereas others have reported a more restricted pattern similar to that seen in AITD sera (9). However, design of Tg IRMAs that use epitope-specific Mabs has not eliminated the problem of interference; thus, it is unlikely that the TgAb specificity in thyroid cancer is wholly restricted (4).

TgAb epitope recognition in DTC serum is therefore unclear, and it is not known how these epitope-binding patterns relate to interference in Tg assays. The current study therefore aimed to clarify the Tg epitope recognition pattern in TgAb-positive DTC sera and to ascertain whether there is an association between epitope specificity and interference with Tg IRMA measurements, with Tg recovery scores used as an indication of interference. This knowledge could advance our understanding of the Tg-TgAb interactions that influence interference.

Materials and Methods

SERA

We selected 50 sera with high TgAb titers from serum samples from patients being monitored for DTC (8 males and 42 females; age range, 18–84 years; mean age, 56.2 years). All of the patients had histologically confirmed DTC, were postoperative, and had received radioiodine treatment. To compare Tg epitope recognition of DTC sera with the typical pattern seen in health and AITD, serum was also obtained from typical Hashimoto thyroiditis patients ($n = 20$) attending the thyroid clinic at the University Hospital of Wales, Cardiff, and TgAb-positive healthy blood donors at the University Hospital of Wales Cardiff ($n = 16$). Hashimoto thyroiditis was diagnosed based on the presence of goiter, TgAbs and/or anti-thyroid peroxidase antibodies (TPOAbs), thyroid lymphocytic infiltration on cytology, and where available, thyroid ultrasound findings suggestive of chronic thyroiditis.

BIOCHEMICAL EXAMINATIONS

Thyroid antibodies. TgAbs (reference values <98 kIU/L) and TPOAbs (reference values <19.4 kIU/L) were measured by an ELISA technique standardized against National Institute for Biological Standardisation reference standards (10).

Tg recovery (TgREC). Samples for recovery of Tg were set up the day before the Tg assay. We incubated 20 μ L of buffer and 20 μ L of calibrator 6 (added Tg concentration, 33.3 μ g/L) with 100- μ L portions of each test sample and allowed them to equilibrate overnight at room temperature before measurement of Tg.

Tg was measured by the Thyroglobuline IRMA (CIS bio international) according to the manufacturer's instructions. This assay uses an IRMA technique based on the following principle: a mixture of four Tg-Mabs is coated on the walls of the tubes as the capture antibody, and then a fifth Tg-Mab (I^{125} -labeled) that recognizes an epitope different from those recognized by the other four is used as a tracer. All five antibodies are directed against Tg epitopes not recognized by the TgAbs present in thyroid diseases as found in previous studies (11). The interassay CV, calculated from 40 independent assays, was 6% at 5 μ g/L, 3% at 15 μ g/L, and 3% at 502 μ g/L. The functional sensitivity (lowest concentration for which the interassay CV is 20%) was 2 μ g/L. All measurements were performed in duplicate.

Alkaline phosphatase-labeled Mabs (AP-Mabs). We used a previously defined panel of murine anti-human Tg-Mabs produced and characterized at the INSERM U555 (ex-U38), Faculté de Médecine (Marseille, France). In criss-cross experiments, antigenic domains were defined by competitive inhibition of Tg binding of radiolabeled Mabs by the same or other unlabeled Mabs. Mabs were classified into six antigenic clusters (I–VI) based on cross-inhibition of one another (12). Clusters I, III, and IV define the immunodominant region on the Tg molecule, which is recognized by typical AITD sera (13).

Competitive ELISA studies. We determined epitope recognition by TgAb in sera from patients with a competitive ELISA as described previously (14). Tests were performed in duplicate as follows. Briefly, 96-well microtiter plates were coated overnight with 100 μ L of a 10 mg/L solution of Tg in carbonate–bicarbonate buffer. Plates were then washed four times with phosphate-buffered saline-Tween, after which 100 μ L of test serum at various dilutions (1:10, 1:100, 1:1000) was added to the wells and incubated in a humid box at 37 °C for 2 h. After another washing step, the plate was incubated with 100 μ L of AP-Mabs in a humid box at 37 °C for 2 h. Each AP-Mab was diluted to give an absorbance of 1–1.5 at 405 nm in the absence of an inhibitor. After additional washing, 4-nitrophenyl phosphate was added to the wells as substrate to reveal the AP-labeled Mabs. Percentage inhibition of Mab binding by serum samples was calculated as:

$$\left[\frac{(\text{Absorbance in the absence of serum}) - (\text{absorbance in the presence of serum})}{\text{Absorbance in the absence of serum}} \right] \times 100$$

Inhibition >70% was taken as complete inhibition, and 35–70% was interpreted as partial inhibition.

STATISTICAL ANALYSES

We compared differences in TgREC between groups by use of the Mann–Whitney test. Associations between variables were determined for pairs of associations by use of the Pearson coefficient of correlation. The difference in the proportion of patient sera reactive with antigenic clusters was compared between subject groups by the χ^2 test with the Yates correction applied in instances where the expected frequency was <2. The level of statistical significance at which the null hypothesis was rejected was chosen as 0.05.

Results

Tg EPIOTOPE PATTERNS

Samples from 29 patients (58%) showed restricted epitope heterogeneity, preferentially recognizing the immunodominant clusters I, IV, and III in a pattern similar to that seen with typical AITD sera (Fig. 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol51/issue4/>). Samples from the rest of the patients, however, either reacted with the epitopes in a broadly heterogeneous pattern ($n = 8$) or did not recognize any of the epitopes in our panel of Tg-Mabs ($n = 13$; Fig. 1 in the online Data Supplement). However, of those sera that recognized at least one Mab, 29 of 37 (78%) showed restricted epitope specificities. TPOAbs were found in 11 of the 50 patients. Eight of these had restricted epitope specificities, whereas three had broad specificities (Fig. 1 in the online Data Supplement). The relationship between TPOAb status and epitope specificity was insignificant ($P > 0.05$, χ^2 test). We compared recognition of the various clusters (I–VI) by DTC sera (mean TgAb activity = 770 kIU/L) with recognition by typical AITD sera (mean TgAb activity = 1100 kIU/L) and sera from TgAb-positive healthy individuals (mean TgAb activity = 680 kIU/L; Fig. 1). There was preferential recognition of the immunodominant clusters

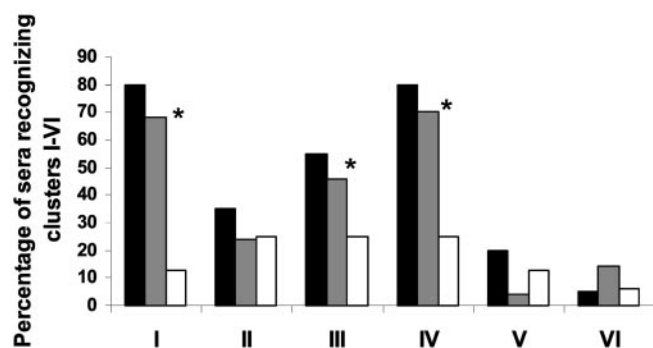


Fig. 1. Percentages of sera reacting with clusters I–VI. Sera from patients with DTC (■) and AITD (▒) differed significantly from sera from TgAb-positive healthy individuals (□) in their recognition of the immunodominant clusters I, III, and IV. *, $P < 0.05$, χ^2 test.

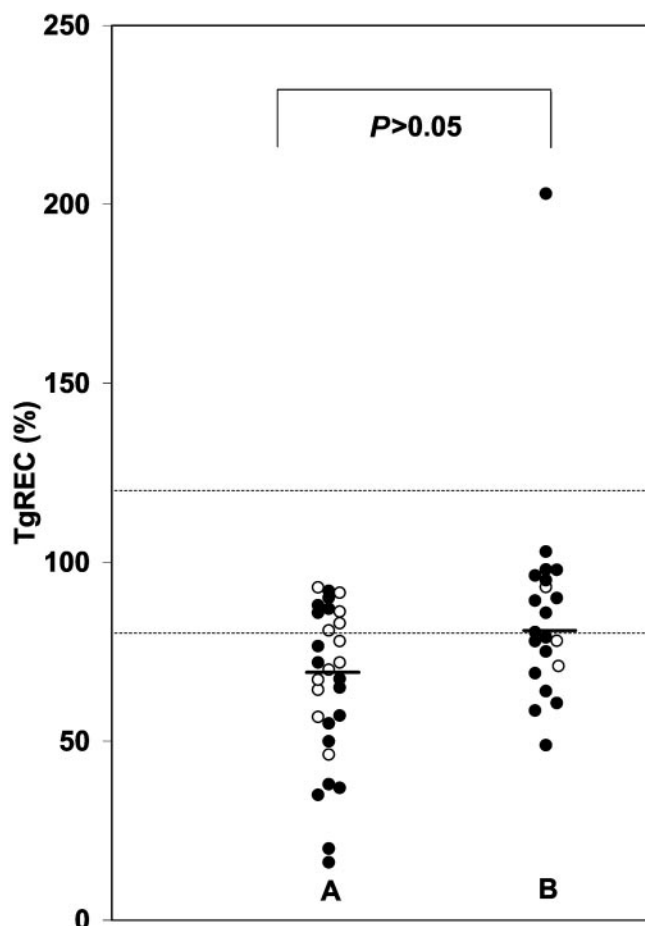


Fig. 2. Percentage TgREC in sera with different epitope reactivity patterns.

(A), sera with a reactivity pattern restricted to the immunodominant clusters I, III, and IV. (B), sera that were either nonreactive or reactive in a broadly heterogeneous pattern. Median TgREC was not significantly different in both groups. ○, Tg-positive sera; ●, Tg-negative sera. The dashed horizontal lines indicate the cutoffs for the recovery reference interval (80–120%).

I, III, and IV in AITD and DTC sera but not by sera from healthy individuals.

Tg EPIOTOPE PATTERNS AND RECOVERY

The TgREC in samples from DTC patients is shown in Fig. 2, which highlights the different patterns of epitope recognition. Sera from patients were classified into two groups based on the epitope specificity pattern. Group A consisted of patients whose sera showed a restricted epitope pattern ($n = 29$), whereas group B included sera that were either nonreactive or reactive in a broadly heterogeneous manner ($n = 21$). Sera were considered to be restricted if they recognized one or more epitopes in the immunodominant clusters III, IV, and VI without recognizing epitopes in other clusters. A few high-TgAb-activity sera strongly recognizing epitopes within the immunodominant clusters spilled over to an additional epitope in the adjacent nondominant cluster and were considered to be restricted. Although the median TgAb

activity was significantly higher in the group A sera (518 vs 218 kIU/L; $P < 0.05$, Mann-Whitney test), median TgREC did not differ significantly between the two groups (69% vs 80%; $P > 0.05$, Mann-Whitney test). Of the 50 DTC patients, 35 had Tg concentrations $< 2 \mu\text{g/L}$, whereas 15 had higher Tg concentrations (median Tg concentration, $14.9 \mu\text{g/L}$; range, $2.8\text{--}59 \mu\text{g/L}$). Twelve of these had restricted epitope patterns, whereas 3 has broad patterns or were nonreactive (Fig. 2). There was no association between Tg status and the epitope pattern ($P > 0.05$, χ^2 test with Yates correction).

NUMBER OF REACTIVE EPITOPES, TgAb ACTIVITY, AND TgREC

The relationship between TgREC and the number of epitopes recognized by sera from patients is shown in Fig. 3. Overall, the number of Mabs recognized by patient sera was inversely correlated with the Tg recovery (Pearson $r = -0.66$; $P < 0.001$; Fig. 3). This association was stronger in the subset of patients with restricted epitope specificities

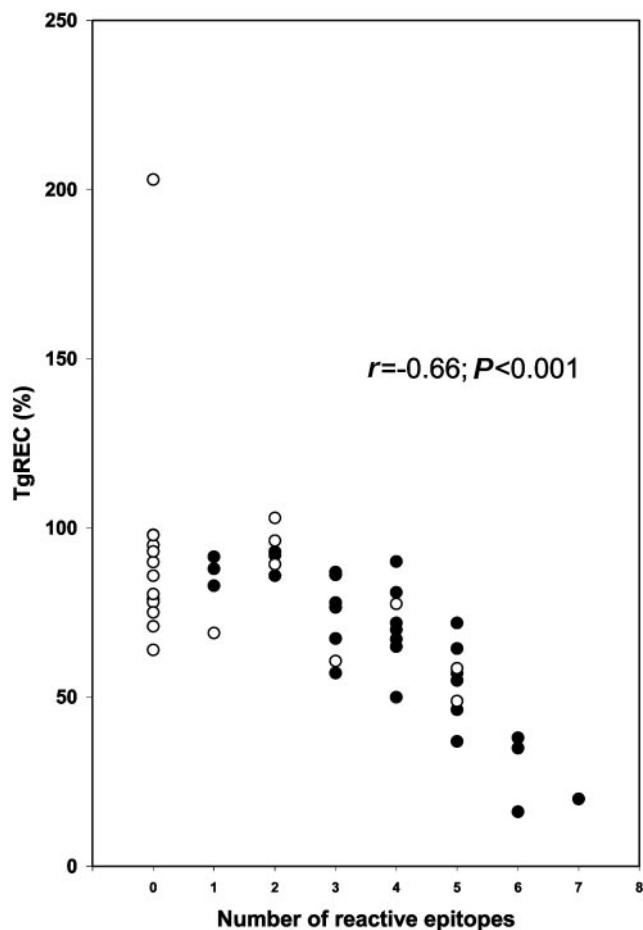


Fig. 3. Relationship between number of epitopes recognized and percentage TgREC.

There is an inverse correlation between the number of reactive epitopes and percentage TgREC (Pearson $r = -0.66$; $P < 0.001$). ○, nonreactive or broadly reacting sera; ●, sera with restricted specificities.

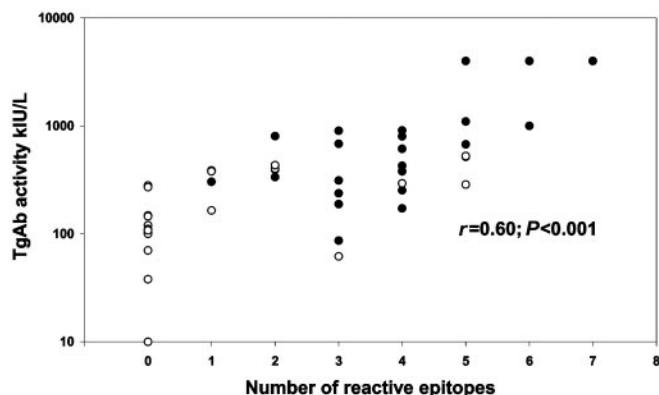


Fig. 4. Relationship between TgAb concentration and the number of Mabs recognized by individual sera.

There is a direct correlation between the TgAb concentration and the number of Mabs recognized (Pearson $r = 0.60$; $P < 0.001$). ○, nonreactive or broadly reacting sera; ●, sera with restricted specificities.

ties ($r = -0.83$; $P < 0.001$; $n = 29$) than in those with unrestricted specificities ($r = -0.37$; $P > 0.05$; $n = 21$; Fig. 3). TgAb activity was weakly inversely correlated with TgREC (Pearson $r = -0.55$; $P < 0.001$; data not shown). In addition, the number of Mabs inhibited was directly correlated with TgAb concentration (Pearson $r = 0.6$; $P < 0.001$; Fig. 4).

Discussion

The reliability of Tg measurements in the monitoring of patients with DTC is hampered by the presence of TgAbs in serum (15). The design of epitope-specific Mabs for use in Tg IRMAs has not eliminated the problem (16), necessitating assessment of the TgAb epitopes in DTC sera and the way in which they relate to interference with Tg assays.

In this study, samples from 58% of the patients had a restricted epitope recognition pattern, whereas the other patterns were either broadly heterogeneous (16%) or nonreactive (26%). Overall, the proportion of serum samples recognizing the six antigenic clusters (I–VI) was similar to that seen in a population of AITD patients but differed from the proportion in TgAb-positive healthy individuals. These findings show that the typical epitope-restricted pattern, although seen in the majority, is not present in all DTC patients. Nonetheless, the Tg epitope recognition pattern on the whole appears to be no different from that in AITD patients.

The link between thyroid cancer and thyroiditis has been debated. TgAbs are certainly more prevalent in DTC patients than in the general population (4, 5). Several reports have suggested that patients with AITD have an increased risk of developing thyroid cancer (17, 18), whereas other reports do not confirm this association (19). Nevertheless, a coexistence of thyroiditis and DTC has been documented. In one study, 6.6% of DTC patients had thyroiditis (20), whereas other workers noted an almost 50% occurrence of AITD in TgAb-positive DTC patients

(21). It remains unclear, however, whether the two conditions are related causally or coincidentally. Our confirmation of a similar Tg epitope specificity in both conditions distinct from that in healthy persons suggests that DTC and AITD may share similar immunopathogenetic pathways. On the other hand, it is possible that some of these patients had coexistent AITD and DTC. However, the lack of a significant association between TPOAb status and the epitope specificity of these patients makes it unlikely that coexistent AITD was wholly responsible for their AITD-like epitope patterns.

The second part of this study examined the association between Tg epitope specificity and TgREC. To the best of our knowledge, this relationship has not been tested previously. A point of interest was whether TgAbs with immunodominant specificities differed from broadly heterogeneous TgAbs with respect to their ability to cause interference. Such differences, if present, could be exploited as diagnostic tools in deciding whether there is interference in TgAb-positive DTC sera. However, we observed no difference in recoveries between highly restricted TgAbs and broadly heterogeneous or nonreactive TgAbs. Rather, the overall number of Tg-Mabs recognized by TgAbs in the patients' sera was related to recovery. The lowest recoveries were in individuals whose sera recognized five or more Tg-Mabs. Although TgAb concentration related only weakly to recovery, we observed a direct relationship between the number of Mabs recognized and the TgAb concentration. This correlation between TgAb concentration and the number of reactive epitopes has been observed previously, and it has been proposed that with increasing antibody activity there is overlap of reactivity to adjacent epitopes (22). Interestingly, sera with restricted epitope patterns and spread to epitopes in adjacent clusters had the lowest recoveries.

These findings have implications in the design of Mabs for use in Tg assays. The concept of exploiting Tg-Mabs specific for particular epitopes not recognized by DTC sera is appealing (11), but the success of such an approach will depend on a tightly restricted epitope pattern in patients with DTC. We observed a restricted pattern in just over one half of the patients tested. Even then, the phenomenon of epitope overlap to adjacent clusters was apparent in sera with high TgAb activity. Thus, epitope spread may in part explain the failure of epitope-specific Tg-Mabs to completely eliminate interference when used in Tg IRMAs. The interpretation of these findings must, however, take into consideration the fact that exogenous recovery tests are not entirely reliable in deciding interference and that current guidelines do not support their routine use in authenticating Tg results in TgAb-positive patients (15).

In conclusion, the TgAb properties influencing interference remain unclear. Both TgAbs with restricted and those with broad specificities are present in DTC sera, but these specificity patterns do not affect Tg recoveries.

Recoveries were lower in sera recognizing a greater number of epitopes, thus suggesting that the Tg epitopes involved in interference in TgAb-positive DTC sera are not tightly restricted.

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