False negative results observed in anti-thyroid peroxidase autoantibody determination by competitive radioimmunoassays using monoclonal antibodies

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Objective: Anti-thyroid peroxidase autoantibody (anti-TPO) and anti-thyroid microsomal antibody (anti-M) are strictly related, but discrepancies are sometimes observed. The aim of this study was to assess the incidence and to identify the causes of these discrepancies. Design and antibody measurements: Anti-M by passive hemagglutination and anti-TPO by two competitive monoclonal antibody-assisted radioimmunoassays (RIA-1 and RIA-2) were measured in 10,103 sera from 4,232 subjects (663 male, 3,569 female) screened for thyroid disease. Results: Anti-TPO and anti-M correlated quite well (r = 0.7 and p < 0.0001 by RIA-1; r = 0.74 and p < 0.0001 by RIA-2), with discrepancies mostly limited to sera with low antibody titres. After exclusion of the latter samples, anti-TPO were detected in only 79 (1.4%) out of 5,317 anti-M negative sera, but were undetectable in a more consistent proportion (130/2,880 = 4.5%) of sera from patients with autoimmune thyroid disease and positive anti-M. In 61 sera of the latter group, anti-TPO was measured by a non-competitive RIA (RIA-3). Forty-one (67.7%) were positive by RIA-3, suggesting the presence of anti-TPO not competing with the monoclonal antibodies of RIA-1 and RIA-2. The remaining 20 sera had undetectable anti-TPO also by RIA-3. Nineteen (95%) of these sera had positive anti-thyroglobulin (anti-Tg) autoantibody and preincubation with thyroglobulin inhibited the agglutination reaction of anti-M tests. Conclusion: Anti-TPO by competitive monoclonal antibody-assisted RIA is negative in a minority of sera of patients with autoimmune thyroid disease and positive anti-M. This could be accounted for by anti-Tg producing false positives in the anti-M assay and by a subset of anti-TPO not competing with the monoclonal antibodies in the RIA. When autoimmune thyroid disease is suspected on clinical grounds, a negative anti-TPO test with a competitive RIA should be confirmed always by a non-competitive assay.

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Thyroid peroxidase (TPO) is the main component of the formerly called “thyroid microsomal antigen” (M) (1, 2). The availability of purified TPO allowed the development of radioimmunoassays (RIA) (3–5) and enzyme-linked immunosorbent assay (ELISA) (6) methods for anti-TPO autoantibody (anti-TPO), which are employed with increasing frequency in the clinical routine. Some years ago we (7) and others (4) developed a solid-phase RIA for anti-TPO based on competitive inhibition of the binding of radiiodinated TPO to an anti-TPO monoclonal antibody coated on plastic wells or tubes. The results obtained with this assay in selected groups of patients with different thyroid diseases showed that it was more sensitive and specific than the classic anti-thyroid microsomal antibody (anti-M) by passive hemagglutination (7). Since then, several commercial variants of this technique became available, and gained a wide diffusion, confirming the validity of the method. Few other studies (8–10), however, are available on the clinical validation of this assay, with particular regard to the comparison with other differently designed RIAs. This prompted us to re-evaluate the relationship between anti-TPO detected by commercial RIA and anti-M by passive hemagglutination in a very large series of unscreened sera with the purpose of studying the incidence and identifying the causes of discrepancies.

Materials and methods

Sera

A total of 10,103 sera obtained from 4,232 subjects (663 males, 3,569 females) attending the out-patients thyroid clinic of our Institutions were included in this
study. Assays always were carried out in fresh samples as part of routine thyroid function assessment. When needed, repeated tests were carried out on aliquots kept frozen at −20°C.

Thyroid autoantibody assays by passive hemagglutination

Anti-M and anti-thyroglobulin antibody (anti-Tg) were assayed by passive hemagglutination (PH) using commercially available kits (Thyroglobulin Test Kit and Microsome Test Kit, Fujizoki Pharmaceutical Co., Tokyo, Japan, respectively). According to the manufacturer's instructions, antibody titres of ≥1/100 were considered as positive results.

In some experiments anti-Tg and anti-M tests were carried out after addition of excess Tg (50 µg) to the first sample dilution, as described previously (11). Thyroglobulin was prepared from surgical samples of Graves' glands by Sepharose 4B gel chromatography (12).

Anti-TPO assays

Anti-TPO was measured in all sera using either of two commercially available monoclonal antibody-assisted RIAs (Anti-TPO RIA, Sorin Biomedica S.p.A., Saluggia, Italy [RIA-1]; Dyno test Anti-TPO, Henning GmBH, Berlin, Germany [RIA-2]). In total, 1333 sera (from 558 patients) were assayed by RIA-1 and 8770 sera (from 3674 patients) were assayed by RIA-2. As mentioned previously, both RIA-1 and RIA-2 are based on a method developed in this (7) and in another laboratory (4). Briefly, the serum prediluted 1 : 51 (RIA-1) or 1 : 21 (RIA-2) is incubated with 125I-labeled TPO (50 µl corresponding to 30 000–50 000 cpm) for 18–22 h (RIA-1) or 2 h (RIA-2) at room temperature in tubes coated with a monoclonal antibody directed against a major autoepitope of TPO. After incubation, the procedure for the two assays is the same: the radioactive mixture is aspirated and the tubes carefully washed and counted. Results are expressed as a percentage of the maximal binding (i.e. the radioactivity bound in the absence of anti-TPO). Quantitation of anti-TPO antibody is obtained by adding a standard curve made by progressive amounts of a positive serum.

The standard serum employed by RIA-1 and RIA-2 was reportedly calibrated in International Units (IU) by comparison with a positive sample (MRC standard 66/387) from the National Institute for Biological Standards and Control (Hampstead, London, UK). According to the manufacturer's instructions, anti-TPO levels of >10 IU/ml and >100 IU/ml are considered positive results by RIA-1 and by RIA-2, respectively. The reason for such a discrepancy in the cut-off levels for positive results is unclear, but already reported by others (10). For the purpose of the present study, we decided to analyze the results according to the cut-off levels for positivity suggested by the respective manufacturer. We also selected higher cut-offs (>20 IU/ml for RIA-1 and >200 IU/ml for RIA-2) in order to exclude from the analysis those samples with low anti-M and anti-TPO titers (see Results, for details).

Anti-TPO was measured also in a subgroup of 61 sera with undetectable anti-TPO by RIA-1 or RIA-2 and anti-M titers of ≥1/1600 by PH using a non-competitive co-precipitation RIA (Immune test anti-TPO, Henning GmBH [RIA-3]). Similarly to RIA-1 and RIA-2, RIA-3 is a commercially available version of a technique reported previously (5). Briefly, 50 µl of serum diluted 1 : 21 is incubated with 50 µl of [125I]TPO and 50 µl of a solution of Protein A bound to Staphylococcus aureus cells for 1 h at room temperature. The complex [125I]TPO–anti-TPO is then diluted with 1 ml of buffered solution and precipitated by centrifugation at 2000 g for 15 min. After discarding the supernatant, the radioactivity recovered within the pellet is counted. Results are expressed as a percentage of the total radioactivity recovered in the pellet; similarly to RIA-1 and RIA-2, anti-TPO are expressed in IU/l using a standard curve made of progressive dilutions of a positive serum. As in RIA-2, anti-TPO antibody levels of >100 IU/ml are suggested by the manufacturer as positive results.

Statistical analysis

General correlations were analyzed by linear regression analysis. The chi-squared method was applied in the analysis of the frequency of discrepancies by different RIAs. Statistical calculations were performed on personal computer software (Stat View®, Abacus Concepts, Inc.).
Table 1. Comparison of anti-thyroid microsomal antibody (anti-M) by passive hemagglutination and anti-thyroid peroxidase autoantibody (anti-TPO) by radioimmunoassay (RIA) in 1013 sera. Positive (+ve) results are $\geq 1/100$ for anti-M, $>10$ IU/ml for anti-TPO by RIA-1 and $>100$ IU/ml by RIA-2.$^a$

<table>
<thead>
<tr>
<th></th>
<th>Anti-TPO (RIA-1)</th>
<th>Anti-TPO (RIA-2)</th>
<th>Anti-TPO (Total)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+ve (N = 4248)</td>
<td>-ve (N = 5855)</td>
<td></td>
</tr>
<tr>
<td>Anti-M +ve</td>
<td>497 (12.3)</td>
<td>70</td>
<td>3181 (13.5)</td>
</tr>
<tr>
<td>Anti-M -ve</td>
<td>32 (4.2)</td>
<td>734 (11.2)</td>
<td>569 (13.2)</td>
</tr>
<tr>
<td></td>
<td>500 (13.5)</td>
<td>4520 (10.2)</td>
<td>601 (10.2)</td>
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<tr>
<td></td>
<td>3678 (13.4)</td>
<td>5254 (10.2)</td>
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</table>

$^a$Numbers in parentheses indicate the percentual fraction of sera giving discrepant results between anti-M and anti-TPO by RIA-1 or RIA-2.

Table 2. Comparison of anti-thyroid microsomal antibody (anti-M) by passive hemagglutination and anti-thyroid peroxidase autoantibody (anti-TPO) by radioimmunoassay (RIA) in 8197 sera, after exclusion of samples with low antibody titers ($1/100-1/400$ by PH, 11–20 IU/ml by RIA-1 or 101–200 IU/ml by RIA-2). In contrast to Table 1, only sera giving anti-M titers of $>1/1600$ and anti-TPO levels of $>20$ IU/ml (by RIA-1) or $>200$ IU/ml (by RIA-2) were included as positive (+ve) results.$^a$

<table>
<thead>
<tr>
<th></th>
<th>Anti-TPO (RIA-1)</th>
<th>Anti-TPO (RIA-2)</th>
<th>Anti-TPO (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve (N = 2880)</td>
<td>-ve (N = 5317)</td>
<td></td>
</tr>
<tr>
<td>Anti-M +ve</td>
<td>369 (3.1)</td>
<td>12 (1.3)</td>
<td>2381 (4.7)</td>
</tr>
<tr>
<td>Anti-M -ve</td>
<td>10 (1.3)</td>
<td>732 (1.5)</td>
<td>69 (1.5)</td>
</tr>
<tr>
<td></td>
<td>118 (4.7)</td>
<td>4506 (1.4)</td>
<td>79 (1.4)</td>
</tr>
<tr>
<td></td>
<td>2750 (4.5)</td>
<td>5238 (1.4)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Numbers in parentheses indicate the percentual fraction of sera giving discrepant results between anti-M and anti-TPO by RIA-1 or RIA-2.

Results

Comparison between anti-TPO by monoclonal antibody-assisted RIA and anti-M by PH

As shown in Fig. 1, a good general correlation ($r = 0.7$ and $p < 0.0001$ by RIA-1; $r = 0.74$ and $p < 0.0001$ by RIA-2) between anti-TPO and anti-M was observed in all sera studied, but several discrepant results also were found, as detailed in Table 1. Positive anti-TPO levels ($>10$ IU/ml by RIA-1, $>100$ IU/ml by RIA-2) were detected in 601 (10.2%) out of 5855 sera with anti-M negative by PH. The prevalence of positive anti-TPO tests in sera with undetectable anti-M antibody was

Fig. 2. Absolute number of sera giving discrepant results between anti-thyroid microsomal antibody (anti-M) and anti-thyroid peroxidase autoantibody (anti-TPO) tests in the whole series of sera examined, subdivided according to the anti-M or anti-TPO titer. Panel A shows the distribution of sera with undetectable anti-TPO ($<10$ IU/ml by RIA-1 or $<100$ IU/ml by RIA-2) and positive anti-M tests: sera with negative (<1/100) anti-M and positive anti-TPO tests are reported in panel B.
Table 3. Clinical diagnoses of the 38 patients whose sera were analyzed by RIA-3.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
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<tbody>
<tr>
<td>Graves’ disease</td>
<td>13</td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td>12</td>
</tr>
<tr>
<td>Thyroid-differentiated carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>(with histologically proven)</td>
<td></td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td></td>
</tr>
<tr>
<td>Non-toxic nodular goiter</td>
<td>7</td>
</tr>
<tr>
<td>Toxic adenoma</td>
<td>1</td>
</tr>
</tbody>
</table>

significantly higher using RIA-2 (11.2%) when compared to RIA-1 (4.2%) \( \chi^2 = 25.55, p < 0.0001 \). The reverse pattern (i.e. sera with anti-M antibody detectable by passive hemagglutination and anti-TPO \( \leq 10 \) IU/ml by RIA-1 or \( \leq 100 \) IU/ml by RIA-2) was observed in a slightly higher number of samples (570 of 4248 = 13.4%), without any significant difference between RIA-1 and RIA-2. As reported in Fig. 2, discrepancies between anti-TPO and anti-M were confined mostly to sera with low antibody titers (1/100–1/400 for anti-M, 11–20 IU/ml for RIA-1 and 101–200 IU/ml for RIA-2).

To focus on more significant discrepancies between the two tests, the comparison was performed after excluding sera with low anti-M or anti-TPO antibody levels. To this purpose we considered as “positive” tests only samples showing anti-M titers of \( \geq 1/1600 \) by PH or \( > 20 \) IU/ml and \( > 200 \) IU/ml by RIA-1 and RIA-2, respectively. The results obtained are reported in Table 2, where 1906 sera with low anti-M and anti-TPO titers were excluded. As expected, the prevalence of discrepant results was much lower than that reported in Table 1. Only a minimal percentage (1.4%) of sera with undetectable anti-M antibody showed positive anti-TPO by RIA, without any significant difference between RIA-1 and RIA-2. Unexpectedly, the prevalence of sera with undetectable anti-TPO and anti-M antibody titers of \( \geq 1/1600 \) was higher (130/2880 = 4.5%), without a significant difference between RIA-1 (12/381 = 3.1%) and RIA-2 (118/2499 = 4.7%). Further studies then were carried out on a subgroup of sera with undetectable anti-TPO and anti-M titers of \( \geq 1/1600 \), as specified in the following paragraphs.

Anti-TPO assay by RIA-3 in anti-M-positive sera with negative anti-TPO by RIA-1 or RIA-2

Anti-TPO was assayed by RIA-3 in 61 anti-M-positive sera with negative anti-TPO by RIA-1 (\( N = 13 \)) or RIA-2 (\( N = 48 \)). These sera were selected because they came from 38 patients of whom a complete clinical evaluation was available. As shown in Table 3, in most of these patients a diagnosis of autoimmune thyroid disease could be established. Anti-TPO antibody measurements were repeated by RIA-2 in all 61 samples to allow a more precise comparison with RIA-3 which employs the same reference standard. Anti-TPO was confirmed

![Fig. 3. Comparison of anti-thyroid peroxidase autoantibody (anti-TPO) levels by RIA-2 and RIA-3 in 61 sera with high (> 1/400) anti-M and anti-TPO undetectable by either RIA-1 or RIA-2: sera with positive (●) and sera with negative (○) anti-thyroglobulin autoantibodies.](image1)

![Fig. 4. Anti-thyroid microsomal antibody (anti-M) titers of 20 sera with positive (\( \geq 1/1600 \)) anti-thyroglobulin (anti-Tg) by passive hemagglutination and undetectable anti-thyroid peroxidase autoantibody (anti-TPO) by RIA-1 or RIA-2 preincubated with (Tg+) or without (Tg−) 50 µg of purified Tg: (●) sera with undetectable anti-TPO by RIA-3: (○) sera with positive anti-TPO by RIA-3.](image2)
to be negative (<100 IU/ml) by RIA-2 in all cases. As shown in Fig. 3, anti-TPO antibody was clearly positive (range 240–>10 000 IU/ml) in 41 (67.2%) of the discrepant sera when assessed by RIA-3, while in the remaining 20 sera anti-TPO was undetectable in 18 and borderline positive in two, also by RIA-3. As reported also in Fig. 3, 18 (90%) of these 20 sera showed positive anti-Tg antibody by PH (with titers of \( \geq 1/1600 \)), while anti-Tg was negative in all but four (4.1%) of the 41 sera with high anti-TPO titers by RIA-3 (\( \chi^2 = 34.4, \ p < 0.001 \)). This finding strongly suggested the possibility that in some discrepant sera false-positive anti-M tests could be produced by the interference of anti-Tg antibody reacting with Tg present on the thyroid microsomal antigen preparations.

**Effect of preincubation with Tg on anti-M titers in sera with positive anti-Tg tests**

The results of this experiment are reported in Fig. 4. Preincubation with Tg completely abolished in 12/20 (60%) and substantially reduced in 6/20 (30%) the anti-M titers in the 20 sera with anti-TPO of <200 IU/ml by RIA-2 and RIA-3. In contrast, anti-M titers did not change after addition of Tg in the two sera with anti-Tg and anti-M by PH and anti-TPO of >200 IU/ml by RIA-3.

**Discussion**

In the present investigation anti-TPO and anti-M were measured in a very large series of sera (>10 000) obtained from subjects undergoing a routine thyroid function test evaluation at two Endocrinological Institutions. Anti-TPO was measured by two different commercial kits based on the same assay design, i.e., competition by serum autoantibodies with a solid-phase anti-TPO monoclonal antibody. The results obtained confirmed the good correlation between anti-TPO and anti-M antibodies found in smaller series of patients with different autoimmune and non-autoimmune thyroid and non-thyroid diseases (3–5, 7, 8). As reported previously in most of the above studies, discrepant results also were observed; owing to the wide series of samples tested, we were able to carry out a detailed analysis of a large number of sera showing marked inconsistencies between anti-TPO and anti-M tests. Sera with positive anti-TPO and negative anti-M were observed in about 10% of cases. This finding is in keeping with the higher analytical sensitivity of the anti-TPO RIAs suggested by previous reports (5, 7, 9). Unexpectedly, however, several sera (13.4%) also were found with negative anti-TPO antibodies and positive anti-M tests; of these, a consistent proportion (4.5%) was represented by samples with high (\( \geq 1/1600 \)) anti-M titers. This finding strongly suggests that this discrepancy cannot be attributed simply to different sensitivities of the two techniques. When anti-TPO antibody was assayed in these discrepant sera by a non-competitive co-precipitation RIA (RIA-3), anti-TPO was clearly detected in the majority (67.2%) of samples. It is unlikely that this finding might be the consequence of a higher RIA-3 sensitivity, because anti-TPO concentrations detected by RIA-3 were in the range easily detected by RIA-2; furthermore, preliminary experiments carried out in our laboratory using sera with different levels of anti-TPO antibody provided evidence for a good parallelism and a similar analytical sensitivity between RIA-2 and RIA-3 (data not shown).

Both RIA-1 and RIA-2 detect anti-TPO autoantibody by its ability to compete with a monoclonal anti-TPO antibody; thus, it is conceivable that individual sera contain anti-TPO autoantibody binding to a TPO epitope distinct from that recognized by the monoclonal antibody(ies) of RIA-1 and RIA-2 (Fig. 5). Several studies (for an extensive review, see Ref. 2) strongly support the concept that many antigenic determinants (including both conformational and linear epitopes) are recognized by human anti-TPO autoantibodies. Different immunogenic regions clustering into four antigenic domains may be defined on TPO by cross-inhibition analysis of mice anti-TPO monoclonal antibodies (13). Interestingly, human polyclonal anti-TPO autoantibodies appear to recognize only two of these immunogenic regions (13). More recently, molecular cloning and expression of the human anti-TPO gene repertoire provided direct evidence that two major domains of an immunodominant region of TPO contain the binding sites of approximately 80% of circulating anti-TPO (14). Indirect evidence for heterogeneity of human anti-TPO autoantibodies also comes from the observation that some (but not all) serum autoantibodies inhibit TPO enzymatic activity (15–18), and that the degree of cross-reaction with other related peroxidases is highly variable (19).

In previous studies employing competitive monoclonal antibody-assisted RIA, only occasional sera with positive anti-M by PH and low or negative anti-TPO were observed (7, 8), suggesting that the anti-TPO monoclonal antibodies react with an immunodominant TPO epitope recognized by almost all human autoantibodies. The results obtained in the present investigation confirm this concept but also show, provided that a very large number of samples are analyzed, sera containing anti-TPO autoantibody unable to compete with a single monoclonal anti-TPO may be observed. We do not know what anti-TPO monoclonal antibody is employed in RIA-1 and in RIA-2; however, as all sera containing anti-TPO by RIA-3 gave negative results by RIA-1 and RIA-2, it is likely that the monoclonal antibodies of both assays recognize the same epitope or the same antigenic cluster on the TPO molecule.

Although about two-thirds of anti-M positive, anti-TPO (by competitive RIAs) negative sera did actually
show the presence of anti-TPO autoantibody when assessed by a co-precipitation RIA. anti-TPO remained undetectable by an assay in one-third of them. Interestingly, all these sera had positive anti-Tg antibody and the addition of Tg inhibited the agglutination reaction of the anti-M test. The possibility that anti-Tg antibody, when present alone or in large excess with respect to anti-M antibody, may account for false-positive results in anti-M by passive hemagglutination has been reported previously in sporadic sera (7, 11, 20) and attributed to contamination by Tg of the crude microsomal preparations used as antigen. The results obtained in the present investigation further support this concept and suggest that this phenomenon could account for approximately one-third of sera showing positive anti-M test by PH with undetectable anti-TPO.

In conclusion, anti-TPO measurement by competitive monoclonal antibody-assisted RIA may provide negative results in a small minority of sera with positive anti-M. In about one-third of the cases this phenomenon is due to an interference by anti-Tg, giving false-positive results in anti-M tests. In the remaining cases it is apparently due to the presence of a subset of anti-TPO that does not compete with the monoclonal antibodies commonly used in competitive RIA. From the practical point of view, it should always be considered that when autoimmune thyroid disease is suspected on clinical grounds, negative anti-TPO tests with a competitive RIA can be artifactual and the result should be confirmed by a non-competitive assay. If only one test of anti-TPO has to be recommended, this should be a non-competitive assay.

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