Novel Routine Assay of Thyroperoxidase Autoantibodies

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This radioimmunoassay was developed for specific and large-scale routine measurement of autoantibodies to thyroperoxidase (TPO), an enzyme recently identified as the thyroid microsomal antigen. Because of the scarcity of purified thyroperoxidase, we did not base the assay on the antigen-coated method but rather on autoantibody inhibition of the binding of labeled TPO to a solid-phase-bound monoclonal antibody to TPO. This assay design ensured highly specific measurements without interference from irrelevant thyroid antigens and autoantibodies. When we used affinity-purified autoantibodies to TPO as standards, the range of the curve extended over 10³-fold differences in the autoantibodies' concentrations, which allowed us to assay most sera without dilution. Within- and between-assay coefficients of variation (CVs) ranged from 6.1% to 11.5% and from 6.6% to 12.0%, respectively. The correlation between anti-TPO and antimicrosomal autoantibodies, as assessed by hemagglutination test, was highly significant (r = 0.90, P < 0.0001). This assay is sensitive, easy to perform, and requires only trace amounts of purified TPO.

Additional Keyphrases: radioimmunoassay • thyroid microsomal antigen • monoclonal antibodies • autoimmune disorders

Autoantibodies (aAb) directed to the thyroid microsomal antigen (MIC) are found in most patients presenting with autoimmune disorders of the thyroid gland (1). Accordingly, anti-MIC aAb are measured in surveys for autoimmune thyroid diseases. Different methods—such as immunofluorescence of tissue sections, passive hemagglutination, radioimmunoassay, and enzyme-linked immunosorbent assay—have been proposed for detecting anti-MIC aAb (2–9). Because the nature of MIC remained unknown, these techniques were based on the interaction of serum immunoglobulins with thyroid preparations containing irrelevant thyroid antigens, including thyroglobulin (TG), which is also involved in autoimmune thyroid diseases (1).

Recently, we identified MIC as thyroid peroxidase (TPO) (10–12), a finding later confirmed independently by other groups (13–15). The availability of monoclonal antibodies (mAb) to TPO allowed us to immunopurify TPO and, subsequently, anti-TPO aAb (16). We have now used these reagents to study various procedures for measuring anti-TPO aAb with different assay designs. Previously (17), we used two different assays to demonstrate that anti-TPO and anti-MIC aAb were virtually identical. Despite the relevance of these methods for measuring anti-TPO mAb, both methods presented several drawbacks for routine clinical testing: the competitive radioassay showed low specific signal and high nonspecific interference from serum components, whereas the immunoradiometric assay consumed far too much TPO (17). To circumvent these problems, we devised a new assay based on the inhibition by serum anti-TPO aAb of labeled TPO binding to solid phase coated with anti-TPO mAb. An obvious advantage of this assay design is that only trace amounts of TPO are used. A prerequisite for setting up such an assay is to obtain a mAb that competes for TPO binding with virtually all anti-TPO aAb present in patients' sera. After carefully studying the cross-reactivity for the TPO binding between aAb from numerous patients and 41 anti-TPO mAb (16), we selected one mAb that we found suitable. We describe here this new assay for measuring anti-TPO aAb in sera and present the results obtained for normal subjects and for patients with various diseases.

Materials and Methods

Preparation of human TG and TPO. From human thyroid tissue obtained by surgery, we prepared TG according to the procedure of Marrig et al. (18). The protein concentration of the TG was determined by measuring its absorbance at 280 nm.

TPO was purified from human thyroid microsomes by immunoaffinity chromatography as previously described (11). The protein concentration of the TPO was determined by the micromethod of Bradford (19), with bovine IgG as the standard. Purified TPO has a specific activity (gastoic oxidation assay) of 11.9 kU/g, a A_{411nm}/A_{280nm} Ratio of 0.23, and an apparent molecular mass of 100 kDa.

Production of the monoclonal antibody. The mAb we used was derived from spleen cells of BALB/cByJICo mice after immunization with purified TPO and fused with the nonsecreting mouse myeloma X63-Ag8.653 (20) according to a protocol described previously (21). The producing cells were injected intraperitoneally into pristane-treated BALB/c mice. The resulting ascitic fluids were collected and the corresponding mAb was purified by DEAE chromatography on prepacked and ready-for-use Zeta Prep Disk (LKB, Bromma, Sweden) according to the manufacturer's instructions. The protein concentration of the mAb was determined by measuring its absorbance at 280 nm.

Production of anti-TPO antiserum in rabbits. Five-month-old male Fauve de Bourgogne rabbits (purchased from Eric Ceba, Blanquefort, France) were immunized subcutaneously with 50 µg of TPO emulsified in complete Freund's adjuvant. An intramuscular injection of 100 µg of TPO, emulsified in incomplete Freund's adjuvant, was given one month later. Subsequently, blood was sampled from rabbits weekly, and the sera were stored at -20 °C until use.

Serum samples. Human sera were obtained from 107 unselected adult patients thought to have thyroid disorders. The serum samples were used individually or pooled according to their anti-TG and anti-MIC antibody titers, as assessed with a passive hemagglutination test (Wellcome, Dartford, U.K.). The control group consisted of 28 normal adults whose sera did not contain detectable amounts of anti-TG and anti-MIC antibodies. To test the specificity of the assay, we analyzed 31 sera from patients who had...
various diseases but who showed no clinical or biochemical evidence of thyroid disorders.

**Human IgG preparation.** The total IgG fraction of pooled sera from patients with increased titers of anti-MIC antibody but no detectable anti-TG were purified on Zeta Prep DEAE Disk. From this total IgG fraction we obtained a purified preparation of human anti-TPO aAb by affinity chromatography, with TPO coupled to Affi-gel 10 (Bio-Rad Labs., Richmond, CA). The human IgG protein concentration was determined by absorbance at 280 nm.

**Labeling the TPO.** We labeled 10 μg of TPO with 250 μCi of carrier-free Na^{125}I and 2.5 μg of Chloramine-T in 30 μL of sodium phosphate buffer (0.2 mol/L, pH 7.0). The reaction was stopped after 60 s by adding 5 μL of sodium metabisulfite solution, 1 g/L in the sodium phosphate buffer. The volume of the mixture was adjusted to 0.5 mL by adding phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.3) containing, per liter, 10 g of bovine serum albumin (BSA), 1 mL of Tween 20, and 0.2 g of sodium azide. The labeled TPO was separated from 125I by gel filtration on Ultrogel ACA 34 (IBF, Villeneuve-la-Garenne, France) equilibrated in the same buffer; we adjusted the concentration of this preparation to 1 μCi/mL and stored it in 10-mL aliquots at 4 °C.

**Anti-TPO RIA.** We measured anti-TPO antibodies by a competitive binding radioassay with anti-TPO mAb coated to a solid surface and with radiolabeled TPO. We coated polystyrene tubes (Startubes; Nunc, Roskilde, Denmark) overnight at 4 °C with 200 μL of PBS containing anti-TPO mAb, 10 mg/L. Any unbound mAb was aspirated and the tubes were washed with 2.5 mL of PBS solution containing 1 mL of Tween 20 per liter, then filled with 500 μL of PBS solution containing 10 g of BSA per liter and incubated for 1 h at 37 °C. The saturating solution was removed and, without delay, we added to the coated tubes 100 μL of the test specimens (generally sera diluted 40-fold with PBS containing 2 g of BSA and 1 mL of Tween 20 per liter) and 100 μL of radiolabeled TPO (≈100 000 counts/min) and incubated for 2 h at 37 °C. To evaluate the maximal TPO binding and the nonspecific binding, we also filled a coated tube with an uncoated tube with dilution buffer alone. After removing unbound material by aspiration, we washed the tubes twice with 2.5 mL of PBS containing Tween 20 (1 mL/L), then measured the radioactivity bound to each individual tube with a gamma counter.

**Results**

The anti-TPO mAb used in the assay was first selected from a set of 41 mAb because of its high binding capacity for radiiodinated TPO. The ratio for specific bound/total radioactivity was 35 to 40% of the labeled TPO, which consistently displayed <1% nonspecific binding to uncoated tubes. The binding of 125I-labeled TPO to the coated mAb was inhibited in a dose-dependent manner by TPO but not by TG at concentrations up to 0.5 g/L (Figure 1A). The specificity of inhibition of TPO binding to coated mAb by anti-TPO aAb is shown in Figure 1B. The total IgG fraction from pooled sera was applied onto a TPO affinity column. As little as 4 ng of anti-TPO aAb, specifically eluted from the column, significantly inhibited the binding of 125I-labeled TPO; the inhibition was almost complete with about 10 μg of anti-TPO aAb per tube. At the same IgG concentration, the anti-TPO aAb-free filtrate only slightly inhibited the binding of 125I-labeled TPO to the coated mAb. Furthermore, inhibiting activity of the original IgG fraction was 20- to 30-fold less than that observed with affinity-purified anti-TPO aAb (Figure 1B). We also studied the specificity of the assay with regard to anti-TG and anti-MIC aAb. As shown in Figure 1C, only the pool of sera that contained anti-MIC aAb inhibited the binding of TPO to the coated mAb. The pool of normal sera (negative for anti-TG and anti-MIC aAb) and the pool of sera containing anti-TG but not anti-MIC aAb inhibited the mAb–TPO interaction only slightly and only in high volumes of serum, so these effects could be attributable to nonspecific interference of some serum components. Such a nonspecific effect of serum was also observed when we compared the effect of serum samples from rabbits, before and six weeks after immunization with affinity-purified TPO (Figure 1D).

We established the standard curve of the assay by assaying graded amounts of affinity-purified anti-TPO aAb diluted in buffer containing normal human serum (25 mL/L). As shown in Figure 2, the range of the assay in serum extended from 4.5 ng to 10 μg of anti-TPO aAb per tube. The limit of detection of the assay was obtained by evaluating data from the standard curve, as performed four times at weekly intervals. The least detectable dose ranged between 2.3 and 4.2 ng per tube. We therefore consider 4.5 ng of anti-TPO aAb per tube as a reliable limit of detection of the assay. In terms of anti-TPO aAb concentration in serum, the effective range of the assay extended from 2 to 1000 mg/L.

In examining parallelism between the standard curve and dilution of serum samples, we serially diluted patients' sera presenting various titers of anti-MIC aAb (from 1:400 to 1:102 400 by passive hemagglutination test) in the same normal human serum matrix, to be standards. Assayed under the same conditions (2.5 μL of serum per tube), the curves obtained with either patients' sera or affinity-purified anti-TPO appeared to parallel each other within the

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Fig. 1. Specificity of the binding of the radiolabeled TPO to mAb-coated tubes

Radiolabeled TPO was incubated with: (A) TPO and TG; (B) total IgG fraction from a pool of sera containing anti-MIC aAb and the corresponding anti-TPO aAb fraction (eluate) and anti-TPO aAb-free fraction (filtrate) after chromatography on a TPO affinity column; (C) three pools of sera differing in anti-TG and anti-MIC aAb content; and (D) normal rabbit serum (NRS) and rabbit anti-TPO antisera (RAS). Results are expressed as percent bound to total radioactivity ratio (mean of triplicate determinations).
limits of the assay range (Figure 2). Computation of the data obtained with three sera differing in aAb content indicated that dilution of sample in normal human serum did not affect determination of anti-TPO aAb concentration (Table 1).

We also evaluated the precision of the assay. The coefficients of variation of the within-assay reproducibility ranged from 6.11% to 11.49%, depending on the dose assayed (Table 2). Between-assay CVs ranged from 6.60% to 12.01% (Table 2).

We assayed anti-TPO aAb in 28 normal subjects. The anti-TPO aAb concentration in serum ranged from undetectable (<2 mg/L) to 2.3 mg/L. In 31 patients presenting with various diseases but showing no clinical or biochemical evidence of thyroid disorder, the anti-TPO aAb content in serum ranged from undetectable to 2.5 mg/L. Thus, an anti-TPO aAb concentration <2.5 mg/L appeared indicative of normal anti-TPO aAb values. The presence of rheumatoid factor (serum titers of 40 to 640 kilo-units/L), anti-nuclear factor (dilutions of 1:40 to 1:80), and aAb to the acetylcholine receptor (8–10 nmol/L) did not appear to interfere with the assay.

We measured anti-TPO aAb in 107 patients thought to have autoimmune disorders of the thyroid gland. In 38 cases, the results for anti-TPO aAb were negative (<2.5 mg/L). In the 69 remaining patients, the concentration of anti-TPO aAb in serum ranged from 4.2 to >1000 mg/L. We compared the results for anti-TPO and anti-MIC aAb in this series of patients. Two sera negative for anti-MIC aAb by the hemagglutination test displayed detectable amounts of anti-TPO aAb. Conversely, four serum samples were negative for anti-TPO aAb but were slightly positive (titers of 1:100 to 1:400) for anti-MIC aAb. Interestingly, addition of 0.5 g of TG per liter to serum samples inhibited the agglutination of erythrocytes by aAb. In spite of these minor discrepancies, the correlation between hemagglutination titers and anti-TPO assay measurements was significant (Figure 3). In calculating the correlation coefficient, we assigned a value of 0 to all samples with undetectable aAb and excluded all samples with concentrations of anti-TPO aAb >1000 mg/L.

### Table 1. Quantitative Assay of Anti-TPO aAb in Three Serum Samples

<table>
<thead>
<tr>
<th>Titer, by hemaggl.</th>
<th>Patient's serum, µL/tube</th>
<th>Anti-TPO aAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:102 400</td>
<td>2.5</td>
<td>1660</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>851</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>427</td>
</tr>
<tr>
<td>Sample 2</td>
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<td></td>
</tr>
<tr>
<td>1:8400</td>
<td>2.5</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>74.1</td>
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<tr>
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<td>35.5</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>1.25</td>
<td>5.3</td>
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<tr>
<td></td>
<td>0.625</td>
<td>&lt;4.5</td>
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### Table 2. Reproducibility of the Anti-TPO aAb Assay

<table>
<thead>
<tr>
<th>Range</th>
<th>Pool A (29.9–36.5)</th>
<th>Pool B (138.9–169.8)</th>
<th>Pool C (612.4–892.8)</th>
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</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>135.5 (9.50)</td>
<td>155.5 (9.50)</td>
<td>809.8 (93.04)</td>
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<tr>
<td>CV, %</td>
<td>6.10</td>
<td>6.11</td>
<td>11.49</td>
</tr>
</tbody>
</table>

\*Results (mg/L) of 10 tests for each pooled sera, assayed in a single run.

\*Results (mg/L) of three tests for each pooled sera, assayed in four runs.

### Discussion

This new assay for measuring anti-TPO/MIC aAb is based on competitive inhibition of the binding of radiiodinated TPO to a carefully selected anti-TPO mAb coated onto plastic tubes. The assay is rapid and easy to perform, and is well adapted to routine clinical testing. Only one 2-h incubation is involved. Within- and between-assay CVs are sufficiently low (6.1–11.5% and 6.6–12.0%, respectively) to ensure acceptable precision and reproducibility, and they compare well with those of previously published assays for anti-TPO/MIC aAb (12, 17). The design of the test ensures its specificity. We showed effectively that neither TG nor aAb to other antigens than TPO interfere with the assay, in contrast to the anti-MIC tests, which provide false-positive results because of anti-TG aAb.

We evaluated the limit of detection of the assay to 4.5 ng of affinity-purified anti-TPO aAb per tube. The use of 2.5 µL of serum sample in the assay allows us to measure, with acceptable precision, anti-TPO aAb concentrations ranging between 2 and 1000 mg/L. Under these conditions, we detected anti-TPO aAb in 69 of the 107 tested patients thought to have autoimmune thyroid diseases. The increase in the amount of the negative sera in the assay was ineffective in determining the presence of anti-TPO aAb (data not shown).

A good correlation was found between anti-MIC aAb titers by passive hemagglutination test and anti-TPO aAb
concentrations as measured with our anti-TPO assay (P <0.0001). Anti-TPO and anti-MIC aAb were detected in 64.5 and 66.4%, respectively, of the 107 tested sera. Discrepancies only occurred in a minority of sera with negative or low anti-MIC aAb titers, which may be explained by the lack of sensitivity and (or) specificity of the hemagglutination test. Conversely, sera with high anti-MIC aAb titers (>1:102400) by hemagglutination test were consistently found to contain high concentrations of anti-TPO aAb.

Considering that our assay is more sensitive and specific than conventional tests, it may be anticipated that the clinical significance of the anti-TPO aAb titration in patient's sera would be greater than that of anti-MIC aAb. Work is currently in progress to demonstrate this possibility.

Assays for aAb generally are based on the use of antigens coated to various solid phases. A prerequisite for routine clinical testing is the availability of antigenic preparations in sufficient amount for large-scale routine determination and of a high degree of purity to provide for the specificity of the test. These preparations will be easily obtained for anti-TG aAb, and commercial assay kits are already available. In contrast, this is not the case for anti-TPO aAb, because TPO is a membrane protein that can be obtained free of contaminant only in small quantity. Previous works carried out by our (17) and another group (14) indicate that 0.4 mg and 2 mg, respectively, of highly purified TPO is needed for a single determination of anti-TPO aAb. The amount of TPO is much lower for the assay presented here. Considering TPO radiiodinated to a specific activity of 20 Ci/mg and obtained with a yield half of the initial preparation, it may be calculated that when about 100000 counts/min of labeled TPO is used per tube, only 10 ng of TPO is needed for a single determination.

In conclusion, we developed the very first anti-TPO aAb assay that is suitable for routine clinical testing.

References