Effect of anti-thyroid peroxidase (TPO) antibodies on TPO activity measured by chemiluminescence assay

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A chemiluminescence method was developed to measure thyroid peroxidase (TPO) activity and the inhibitory effect of anti-TPO antibodies in purified porcine TPO. The TPO preparation was characterized kinetically and controlled by Western-blotting technique. The chemiluminescence method proved to be reproducible and much more sensitive than the widely used guaiacol method, being able to detect TPO concentrations of 2.21 \times 10^{-3} \text{ g/L} vs 6.63 \times 10^{-2} \text{ g/L} with the latter. Otherwise, the determinations with the two methods correlated well ($r = 0.76$). Investigating the effect of IgGs from 23 hypothyroid patients on measured TPO activity, we detected inhibition in 19 cases with the chemiluminescence technique (15 with the guaiacol method). Anti-TPO antibodies showed competitive inhibition of TPO activity with respect to the substrate guaiacol. In both systems, the inhibition is present in the IgG F(ab')2 fragment. We conclude that the high sensitivity of chemiluminescence detection allows routine determination of the inhibition of TPO activity by anti-TPO antibodies.

INDEXING TERMS: kinetic characterization • immunoinhibition • autoimmune disease • thyroiditis

Thyroid peroxidase (TPO) is a major thyroid microsomal antigen (M/TPO) [1, 2] in autoimmune thyroiditis [3, 4]. A glycoprotein [5–7] sensitive to reductive agents [8], TPO is an integral part of thyroid membrane and plays a key role in the iodination of tyrosyl residues in thyroglobulin and other proteins [9–12]. Evaluation of the diagnostic value of an anti-TPO assay in patients with different types of thyroid disease and in controls showed increased anti-TPO antibodies in >90% patients with active autoimmune thyroiditis [13, 14]. The antigenic determinants of TPO-binding anti-TPO antibodies are heterogeneous (largely of IgG1 and IgG4 subclasses) [15]. The antibodies are produced in both thyroidal and extrathyroidal compartments [15].

The autoimmune response to TPO is known to be associated with a large number of different epitopes on TPO, including the enzymatic site [11, 16], and two methods accepted for determination of TPO are based on oxidation of iodide [17] or guaiacol [9]. However, these methods require relatively high amounts of purified TPO. The inhibition of TPO by anti-TPO antibodies varies with the method used (iodide or guaiacol), because the inhibition of TPO activities measured by the assays can be caused by autoantibodies that recognize either one of the enzyme’s binding sites [18]. Other polyclonal anti-TPO antibodies are assumed not to bind to the catalytic site of molecule because they do not modify the activity of enzyme [19]. The aims of the present study were: (a) to develop a new chemiluminescence technique for determination of TPO activity, (b) to obtain purified TPO antigen suitable for testing the effect of anti-TPO IgGs on enzyme activity, (c) to test the possible inhibitory activity of anti-TPO antibodies from patients with Hashimoto thyroiditis, and (d) to study the kinetics of the inhibition.

Materials and Methods

PREPARATION OF PORCINE TPO

This was performed essentially according to Neary et al. [9]. Porcine thyroid glands were ground and washed four times by suspending in 1.15 mol/L KCl plus 0.1 mmol/L KI and centrifuging (5000g, 40 min). The tissue was then homogenized with 0.25 mol/L sucrose plus 0.1 mmol/L KI and filtered through cheesecloth; the filtrate was centrifuged (5000g, 20 min). From the supernatant the microsome fraction was pelleted by ultracentrifugation at

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50 000 g for 180 min and washed successively with (a) 1 mol/L NaCl, 10 mmol/L Tris-HCl, and 0.1 mmol/L KI, pH 7.4, and (b) 0.1 mmol/L KI. The washed microsome fraction was treated with chymotrypsin-free, diphenylcarbamyl chloride-treated trypsin (35 kU/g protein; Merck, Darmstadt, Germany) at 37 °C for 1 h and the reaction was terminated with soybean trypsin inhibitor (2 mg/mg trypsin). The supernatant obtained after 105 000 g for 1 h was applied to an 1.6 × 22 cm anion-exchange column (DE-52; Whatman, Maidstone, Kent, UK) equilibrated with 50 mmol/L potassium phosphate plus 0.1 mmol/L KCl) in 0.1 mmol/L KI plus 15 mmol/L Tris-HCl, pH 7.4. The TPO activity was eluted by applying a linear KCl gradient (from 25 to 225 mmol/L KCl) in 0.1 mmol/L KI and pH 7.4. The pooled fractions were concentrated with a PM-30 ultrafiltration membrane (Amicon, Oosterhout, The Netherlands), applied to a 1.6 × 85 cm Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column equilibrated with 50 mmol/L potassium phosphate plus 0.1 mmol/L KI, pH 7.2, and eluted with the same buffer. The active fractions were concentrated as before and stored frozen at −70 °C.

Identification of purified TPO fragments
The activity of purified porcine TPO was measured after all steps (Table 1), and the material was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and monitored by Western blotting. SDS-PAGE was carried out by the method of Laemmli [20], with use of a 80 g/L separating gel in conjunction with a 100 g/L stacking gel. The prepared TPO fractions and prestained molecular mass markers (MW SDS 7B; Sigma, St. Louis, MO), 0.15 mL each, were boiled for 5 min in 0.3 mL of sample-reducing buffer (Reanal, Budapest, Hungary) consisting of 20 g/L SDS, 50 mL/L 2-mercaptoethanol, 100 mL/L glycerol, and 65 mmol/L Tris and were electrophoresed at 30–40 mA for 10 h. Separated gel proteins were stained with Amidoblack 10B (Sigma).

Western blotting was performed as described by Towbin et al. [21]. The purified TPO proteins were electrotransferred onto nitrocellulose filters (0.45 nm pore size; Parafilm, Budapest, Hungary) at 180 mA for 24 h at 4 °C. Filters were incubated in Cohnhe-buffer (Reanal) consisting of 1 g/L Ficoll, 1 mL/L Tween 20, 15 g/L bovine serum albumin, 0.5 g/L gelatin, 0.02 mol/L Tris, and 100 mL/L glycerol and were cut into 3-mm-wide strips. The strips, in groups of three, were incubated overnight with 3 mL of 40-fold-diluted serum samples at room temperature. After washing, the strips were incubated at room temperature for 1 h with 1000-fold-diluted goat antihuman IgG and IgA antibodies conjugated with horseradish peroxidase (Sigma). The strips were stained at room temperature with 0.2 g/L dianinobenzidine (Serva, Heidelberg, Germany) in 1 mol/L Tris-buffered saline (pH 7.4) containing 20 μL of 300 mL/L hydrogen peroxide solution.

Measurement of TPO activity
Guaiacol method. TPO activity was routinely determined in a 1.0-cm-lightpath cuvette containing 33 mmol/L guaiacol (Fluka, Buchs, Germany) in a 1-mL solution of 0.1 mol/L phosphate buffer, pH 7.4, and 0.3 mmol/L hydrogen peroxide (Merck). The reaction was initiated by adding H2O2. The rate of reaction was calculated with an absorptivity value of 5.75 L cm−1 mol−1 for oxidized guaiacol [9]; 1 G.U. was defined as the amount of enzyme that transformed 1 μmol of guaiacol per minute. For kinetic measurements the guaiacol concentrations were varied between 0.5 and 48 mmol/L and the H2O2 concentrations between 0.3 and 0.85 mmol/L.

Peroxidase activity was assayed only by the guaiacol method, because Hosoya and Morrison had found in 1967 that the purified porcine TPO could oxidize 48.9 μmol of guaiacol per min/mg protein, but only 7.35 μmol of iodide ion per min/mg protein [1].

Chemiluminescence method. This method is based on the oxidation of luminol by H2O2 as catalyzed by the peroxidase [22]. Chemiluminescence was measured with a Luminometer 1250 (LKB Wallac, Uppsala, Sweden). The 1-mL reaction mixture contained 1 mol/L glycine–NaOH buffer, pH 9, 1 mmol/L EDTA, 10 μmol/L Luminol, and aliquots of TPO. The reaction was initiated by injection of 0.1 mmol/L H2O2; the emitted signals were measured at 37 °C. The resulting light output was determined in mV with a chart recorder (LKB 2210). The luminol–peroxidase blank was simultaneously determined in every measurement.

<table>
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<th>Step</th>
<th>Total protein, mg</th>
<th>Total</th>
<th>Specific</th>
<th>Protein conc., g/L</th>
<th>Volume, mL</th>
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<tr>
<td>KI-washed “microsome”</td>
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<td>1.7005</td>
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**IGG ISOTYPE OF ANTI-TPO ANTIBODIES**

IgG was obtained from sera of patients or healthy controls by precipitation with polyethylene-glycol M_r 6000 (PEG 6000; Fluka), as reported by Tamai et al. [23]. The IgG isotype anti-TPO antibodies were separated by affinity chromatography on a Protein G–Sepharose (Pierce, Rockford, IL) column. The eluted antibodies were desalted by gel-filtration. IgG fragments were obtained by treating the IgG solution with pepsin (Pierce) and the resulting IgG Fc and IgG F(ab')2 fragments were separated on a Protein A–Sepharose column (also from Pierce) [24, 25]. Elution of the IgG was monitored at 280 nm.

**INHIBITION OF TPO ACTIVITY BY ANTIBODIES**

In the guaiacol system (kinetic method), we in general used 3.4 μmol/L IgG (in the kinetic measurements, 1.8–105 μmol/L), M_r 156,000, after separation by PEG precipitation, and 3.7 g/L IgG F(ab')2. The solution containing anti-TPO antibodies was incubated with TPO with shaking at 23 or 37 °C. Control IgGs (from healthy subjects) were also incubated the same way. The results were corrected for the absorbance of IgG alone in every measurement.

In the luminol system (non-kinetic method), we routinely preincubated TPO with 1.78 pmol/L PEG-precipitated IgG (in studies, 0.178–89.1 pmol/L IgG) and 5.4 mg/L–1.54 g/L IgG F(ab')2 used as above.

In routine measurements, the degree of inhibition is expressed as "inhibition percent" in both systems.

**Results**

Purified TPO showed immunoreactivity at 105 and 110 kDa under nonreducing conditions (data not shown). The specific activity of the final porcine TPO preparation determined with the guaiacol method was 14.18 kG.U./g protein, similar to the value reported Neary et al. [9]. The $K_m$ for guaiacol determined according to Lineweaver–Burk was 5.6 × 10^{-4} mol/L [26]; $K_m$ values of 10^{-4} mol/L, 3 × 10^{-6} mol/L, and 6 × 10^{-3} mol/L were found for protein iodination [5], Glu-Tyr-Glu iodination [6], and iodide-oxidation [7], respectively.

In a previous luminescent horseradish peroxidase assay, a relationship was demonstrated between the concentrations of the peroxidase and the intensity of light emitted [27]. Our assay, based on luminol-dependent chemiluminescence, detected TPO concentrations between 22.1 μg/L and 66.3 mg/L (the mean + 3SD signal of zero calibrator is ±22.1 μg/L) (Fig. 1).

Inhibition of TPO activity by anti-TPO antibodies was proportional to antibody concentrations in both systems. In the chemiluminescence method this inhibition was detected in the range 0.178–89.1 pmol/L for IgG (Fig. 2) and 27.7 mg/L to 1.54 g/L for IgG F(ab')2 (Fig. 3). Interassay variation (CV) was 11.3%. To characterize the type of inhibition involved, we examined various substrate and inhibitor concentrations and found a competitive inhibition of IgG isotype anti-TPO antibodies with guaiacol substrate [26]. The inhibition coefficient was determined by Dixon's diagram as 20.1 μmol/L for IgG (Fig. 4; see also our previous study [26]). The inhibition can be attributed to F(ab')2 fragment (in both systems) [26]. A few workers reported an effect of anti-TPO antibodies binding on enzyme activities, but only one part of anti-TPO antibodies was able to inhibit the TPO activity [18, 28]. In our experiment, IgGs from 15 of the 23 patients with Hashimoto disease inhibited the TPO activity in the guaiacol method; in contrast, 19 showed TPO inhibition in the chemiluminescence technique. Agreement between the determinations performed by the chemiluminescence and guaiacol methods was modest (Fig. 5).

**Discussion**

Evidence has accumulated in the last few years that anti-TPO antibodies are closely associated with the activity of autoimmune thyroiditis [29]. These antibodies are believed to be of greater pathogenetic significance than

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**Fig. 1.** Chemiluminescence assay of TPO activity: TPO dependency of chemiluminescence.

TPO concentration ranged from 66 mg/L to 22 μg/L; initial luminol concentration was kept at 10 μmol/L. Assay performed as described in Materials and Methods.

**Fig. 2.** Effect of anti-TPO antibodies on porcine TPO activity determined by chemiluminescence method.

IgG isotype anti-TPO antibodies were obtained by PEG precipitation; inhibition as described in text.
anti-thyroglobulin antibodies in thyroid cellular dysfunction and in the pathogenesis of hypothyroidism [30]. Anti-TPO antibodies fix complement, resulting in destruction of thyroid gland [31]. Measurements of anti-TPO antibodies are used for diagnosis and determination of disease activity of thyroiditis [3]. The observation concerning the capability of one type of anti-TPO antibodies to inhibit the enzyme activity has suggested a new mechanism that might be involved in thyroiditis. The functional determination of anti-thyroid antibodies, including anti-TPO, is thus potentially important in autoimmune thyroid patients. The conventional guaiacol and iodide techniques require relative large amounts of TPO and are not used routinely. The chemiluminescence method developed for measuring TPO activity has several advantages over other methods. It is more sensitive than the usual guaiacol method (detecting as little TPO as 22.1 µg/L vs only 66.3 mg/L in the guaiacol method) and requires much less TPO preparation for the detection of IgG inhibition. Also, its specificity and reproducibility make it suitable for testing a relatively large number of samples, thus making the method potentially more economical.

Although in vitro a substantial proportion of anti-TPO antibodies inhibit the activity of this enzyme, it is not clear whether in vivo these antibodies mediate thyroiditis by regulating or modifying the TPO activity. Our observations support the concept of an inhibitory effect of anti-TPO on thyroid hormone production, in that 15 or 19 IgGs from 23 patients with hypothyroidism inhibited TPO activity. A direct effect on the enzyme activity is supported by the finding that anti-TPO antibodies exert competitive inhibition with the substrate guaiacol [26]. Our prospective study using the chemiluminescence method should provide further evidence on the possible pathogenetic role of inhibitory-type anti-TPO antibodies in autoimmune thyroid disease.

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References