A Circulating Thyroid Peroxidase-Like Substance in Healthy Human Peripheral Blood*

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Abstract

Circulating thyroid peroxidase (TPO)-like substance in healthy human peripheral blood was studied to clarify the immunological role of TPO. By using a highly sensitive radioimmunoassay system combined with murine monoclonal antibodies, TPO-like substance was measurable in 6 out of 84 sera. Characterization of this circulating substance by gel filtration revealed that the molecular weight of a major peak corresponded to that of trypsinized TPO. From these findings it is possible that peripheral blood lymphocytes are exposed to a low level of TPO as well as to thyroglobulin.

Thyroid peroxidase (TPO) is a major thyroid microsomal antigen (Czarnocka et al., 1985; Portman et al., 1985; Kotani et al., 1986) and the presence of autoantibodies (aAbs) to microsomes correlates well with histological changes in Hashimoto's thyroiditis (Hall and Evered, 1979). In order to understand the exact immunological role of TPO, it is of critical importance to study whether TPO circulates in peripheral blood. Thyroglobulin (Tg) as a normally secretory thyroid product circulates in healthy human peripheral blood in the form of undegraded protein at a very low concentration (Roitt and Torrigiani, 1968). It is thought, therefore, that this situation induces T-cell immune unresponsiveness to Tg.

In the present series of experiments, we attempted to detect circulating TPO in healthy human peripheral blood. A circulating TPO-like substance was measurable in healthy human peripheral blood and it was partially characterized.

Materials and Methods

Serum samples

Eighty four sera were obtained from healthy subjects—23 women and 61 men, mean ages being 27 yr (range, 22-37) and 34 yr (range, 24-53), respectively. Sera were stocked at −70°C until use.

Monoclonal antibodies (mAbs)

Three murine mAbs to human TPO (hTPO) were used to measure circulating TPO-like substance in peripheral blood. mAb 38E was previously described in details (Kotani et al., 1986; Ohtaki et al., 1986). The other 2 mAbs, 38D and 38G, were further established by the procedure described previously (Nakagawa et al., 1985a, b; Ohtaki et al., 1986), and these subclasses of IgG were IgG_{2b} and IgG_{1}, respectively. Both mAbs had a κ-type of light chain.
Three mAbs recognized epitopes distinct from one another when determined by the competitive binding inhibition test using \(^{125}\text{I}\)-labeled and unlabeled mAbs. Purification of mAb IgG has been described elsewhere (Nakagawa et al., 1985a, b).

**TPO preparation**

hTPO was purified from human thyroid microsomes solubilized with deoxycholate using an immunoaffinity column containing mAb 38E as described previously (Kotani et al., 1986; Ohtaki et al., 1986). The enzyme preparation thus purified (termed dm-hTPO) had a guaiacol oxidation activity of 101 U/mg protein and a ratio of absorbance at 413 nm to that at 280 nm (RZ value) of 0.24. It was further digested with trypsin and rechromatographed on the immunoaffinity column. The final preparation (termed dmtm-hTPO) had a guaiacol oxidation activity of 245 U/mg protein and a RZ value of 0.58. The protein concentration was determined by the method described by Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. hTPO activity was measured as described previously (Ohtaki et al., 1982).

**Radioimmunoassay (RIA)**

Solid-phase RIA was employed to measure the circulating TPO-like substance in serum. A flexible polystyrene plate was coated with 60 μl of mAb solution for 2 h at 25°C. After blocking unreacted sites on the surface of the plate for 2 h at 25°C with 0.5% BSA in phosphate buffered saline (PBS, pH 7.2) containing 20 mM sodium phosphate, 0.15 M NaCl and 0.05% NaN\(_3\) (RIA buffer), 40 μl samples of serum diluted to 1/5, 1/10 and 1/20 were each added to a well and incubated for 2 h at 25°C. After washing 3 times, 40 μl of \(^{125}\text{I}\)-labeled mAb (Kotani et al, 1985) (about 20 to 30 ng IgG, 60,000 to 80,000 cpm) was introduced to the well, followed by 2 h incubation. The plate was washed 4 times and cut into pieces to measure radioactivity. The concentration of the circulating TPO-like substance was standardized with dmtm-hTPO solution on the assumption that the amount of A\(_{280}\) nmo =1.0 of dmtm-hTPO solution was one U/ml. Washing of the plate and dilution of sera and standard dmtm-hTPO were done with RIA buffer. All experiments were carried out in duplicate or triplicate.

**High performance liquid chromatography (HPLC)**

Fractionation of serum was done with a gel filtration HPLC equipped with a TSK G3000SW column 7.5×600 mm, Toyo Soda Co., Tokyo) using 0.1 M phosphate buffer (pH 7.4) as a solvent at a flow rate of 0.5 ml/min. Protein elution was monitored at 280 nm. Catalase, aldolase, BSA, ovalbumin and ribonuclease A were used as molecular weight standard proteins.

**Other methods**

Anti-thyroid aAbs to Tg and microsomes were measured by passive hemagglutination tests using commercially available kits (Seroclit-TG and Seroclit-MC, Sanko Junyaku Ltd., Tokyo). Sera with titers of more than 100 were regarded as aAbs-positive sera. Serum Tg was also measured with a Thyroglobulin \(^{125}\text{I}\) KIT (CIS France, Paris).

**Results**

**RIA for measurement of circulating TPO-like substance**

In order to construct a sensitive RIA system to measure hTPO, we investigated various conditions using \(^{125}\text{I}\)-labeled and unlabeled mAbs. When mAbs 38E and 38G were combined to use as a ‘catcher’ at 5 μg/ml of individual concentration and \(^{125}\text{I}\)-labeled mAb 38D was employed as an ‘indicator’, the most sensitive assay system was obtained. To determine a detectable limit of dmtm-hTPO concentration, statistical analyses using Student’s t-test were made between counts of the background and a certain concentration of dmtm-hTPO. Consequently, the detectable limit was 1.6 μU/ml of dmtm-hTPO concentration (n=5, \(P<0.001\)).

**TPO-like substance in healthy human sera**

Eighty-four sera from healthy subjects were measured for their circulating TPO-like substance. Some sera (T-1 and XF-98) showed higher radioactivity than the detect-
able limit, while the serum of T-15 exhibited lower radioactivity (Fig. 1). There was a tendency for a higher concentration of serum to accompany lower concentration of TPO-like substance. As summarized in Table 1, 6 sera contained a TPO-like substance. All these sera were derived only from males and had a normal Tg concentration but no anti-thyroid aAbs except for T-1 serum.

Five out of six subjects were further examined to determine their concentration of circulating TPO-like substance during the subsequent 6 months. As shown in Table 2, the circulating TPO-like substance disappeared one or two months later in all except T-1 subjects.

**Gel filtration profile of circulating TPO-like substance**

To characterize the circulating TPO-like substance, T-1 serum was fractionated by gel filtration chromatography and then measured for TPO-like substance. Fig. 2a shows the gel filtration profiles of a TPO-like substance and serum proteins monitored at 280 nm. A major peak of TPO-like substance was detected at the fractions during 30 to 34.5 min of retention time and another minor peak appeared during 59.5 to 60 min. Two other sera, XF-98 and XF-140, were also fractionated by the same procedure, and the peaks of a TPO-like substance were detected at the same positions where T-1 serum showed the major peak. The minor peak from 59.5 to 60.5 min

![Graph showing gel filtration profile](image-url)

**Table 1. List of sera containing a circulating TPO-like substance**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Donor</th>
<th>Anti-thyroid aAbs*</th>
<th>Serum Tg (ng/ml)</th>
<th>TPO-like substance (Mean ± SD, μU/ml)²</th>
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<tbody>
<tr>
<td></td>
<td>Age</td>
<td>Sex</td>
<td>TGHA</td>
<td>MCHA</td>
</tr>
<tr>
<td>T-1</td>
<td>40</td>
<td>M**</td>
<td>200</td>
<td>&lt;100</td>
</tr>
<tr>
<td>T-9</td>
<td>40</td>
<td>M</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>XF-98</td>
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<td>&lt;100</td>
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<tr>
<td>XF-110</td>
<td>37</td>
<td>M</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>XF-124</td>
<td>25</td>
<td>M</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>XF-140</td>
<td>38</td>
<td>M</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
</tbody>
</table>

* autoantibodies. TGHA, thyroglobulin hemagglutination test; MCHC, microsomal hemagglutination test.
** male.
† normal range is less than 30 ng/ml.
‡ mean of 3 determinations.
Fig. 2. Elution profiles for a circulating TPO-like substance and serum proteins. Seventy-five μl of T-1 serum was applied to a TSK G3000SW column and fractionated every 30 sec. The concentration of TPO-like substance in each fraction was measured. Conditions for the gel filtration HPLC equipped with a TSK G3000SW column are described in Materials and Methods.

- a: T-1 serum containing TPO-like substance.
- b: T-15 serum in which TPO-like substance was not detected.
- c: T-15 serum with 400 μU of dmtm-hTPO added.
Table 2. Disappearance of circulating TPO-like substance in peripheral blood

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>T-1</td>
<td>183*</td>
<td>39</td>
<td>38</td>
<td>11</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>T-9</td>
<td>9</td>
<td>ND**</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NT</td>
</tr>
<tr>
<td>XF-98</td>
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<td>40</td>
<td>ND</td>
<td>NT</td>
<td>ND</td>
<td>NT</td>
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<tr>
<td>XF-124</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NT</td>
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<tr>
<td>XF-140</td>
<td>47</td>
<td>21</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NT</td>
</tr>
</tbody>
</table>

Each figure is the mean concentration in two or three determinations.
* , μU/ml
** , not detectable
#, not tested

was detected as well in these two sera. XF-149 exhibited another minor peak from 40 to 40.5 min. The serum of T-15, in which no circulating TPO-like substance was detected, was also fractionated by gel filtration, and only a faint minor peak was detected from 60 to 60.5 min (Fig. 2b). To this T-15 serum was added 400 μU dmtm-hTPO and it was then fractionated to examine what gel filtration profile dmtm-hTPO had. dmtm-hTPO was detected in fractions from 30.5 to 35 min (Fig. 2c). The gel filtration profile of dmtm-hTPO was very similar to that of the TPO-like substance in T-1 serum. Calculating the molecular weight of dmtm-hTPO from the gel filtration profiles of marker proteins, 80,000 and 82,000 were obtained (Fig. 3).

Discussion

In the RIA system used in the present study, mAbs recognizing different epitopes were used as a 'catcher' or an 'indicator'. Therefore the circulating TPO-like substance...
detected in this RIA system was certain to be a hTPO-associated substance in its epitopes. Furthermore, when the serum sample containing TPO-like substance was preincubated with mAbs, 38E and 38G, the final counts of the RIA system decreased reasonably in a dose-dependent manner. Nevertheless, anti-TPO aAbs immunoglobulins purified from pooled sera with an immunoaffinity column did not disturb the RIA system. Even if a serum sample contained anti-TPO aAbs, the measurement of circulating TPO-like substance would be possible.

The gel filtration profile of the circulating TPO-like substance showed that the molecular weight of its major peak corresponded closely to that of dmtm-hTPO. The minor peak from 40 to 40.5 min retention time might represent peptides derived from the proteolytic cleavage of hTPO. Another minor peak detected from 59.5 to 60.5 min was probably unrelated to hTPO, since 2 sera in which circulating TPO-like substance was not detected showed this minor peak.

dm-hTPO seems to be a native form of hTPO and its digestion with trypsin yields dmtm-hPTO (Nakagawa et al., 1985a; Kotani et al., 1986; Ohtaki et al., 1986). The molecular weight of dm-hTPO was also studied by gel filtration but its peak appeared in the void volume of the column. Although dm-hTPO migrates to 100,000 and 107,000 dalton regions on sodium dodecyl sulfate polyacrylamide gel electrophoresis (Kotani et al., 1986), it seemed to be eluted as an oligomer on gel filtration as well as porcine TPO (dm-pTPO) (Nakagawa et al., 1985a). dmtm-hPTO obtained by trypsin treatment is a hydrophilic portion of the enzyme containing an active site. It is conceivable that membranous TPO is attacked by a protease to yield a circulating TPO-like substance whose molecular weight is very close to that of dmtm-hTPO.

If the circulating TPO-like substance is really a TPO, peripheral blood lymphocytes can be exposed to a low level of TPO as well as Tg. This is very important when one thinks of the immunological significance of TPO.

The TPO-like substance gradually disappeared one or two months later except in T-1. Although this transient appearance of TPO-like substance in the circulation is quite interesting, we do not yet have a satisfactory explanation for this phenomenon.

T-1 serum contained a low titer of anti-thyroglobulin aAbs. The concentrations of triiodothyronine, thyroxine and thyroid-stimulating hormone in this serum and the clinical symptoms of this subject were examined, but there was no evidence to suspect an autoimmune thyroid disease. This subject, however, might suffer from subclinical thyroiditis, although it has been reported that some healthy persons have a low titer of anti-Tg aAbs (DeGroot et al., 1984.)

Since TPO-like substance was characterized only by its molecular weight, it is necessary to elaborate its enzymatic activity or spectrophotometric properties as TPO to conclude that this substance is really a TPO. For this purpose, 7 ml of T-1 serum was loaded on the immunoaffinity column coupled with mAb 38E to examine its eluate for the TPO activity, but no activity was detected. Further work will be required to clarify this point, taking the complementary DNA of TPO (Kimura et al., 1987; Seto et al., 1987; Libert et al., 1987) into consideration.

References


