Low Serum Thyroxine due to Anti-Thyroxine Antibody

KATSUJI IKEKUBO1, JUNJI KONISHI1, KOTOKO NAKAJIMA1, KEIGO ENDO1, TORU MORI2 AND KANJI TORIZUKA1

1Department of Radiology, Kyoto University School of Medicine, Kyoto 606 and 2Department of Internal Medicine, Kobe Central Municipal Hospital, Kobe 650, Japan

Synopsis

In one case of untreated Hashimoto's thyroiditis, the serum thyroxine (T₄) value, obtained by radioimmunoassay (RIA) employing resin to separate bound and free T₄, was significantly lower than that obtained by competitive protein binding analysis (CPBA). The discrepancy was found to be due to the presence of anti-thyroxine autoantibody in the serum. This phenomenon is considered to be of practical importance in interpreting the T₄ value by RIA in cases with autoimmune thyroid diseases.

The binding of serum thyroxine (T₄) to gammaglobulin has been reported in patients with thyroid cancer (Robbins et al., 1956) and Hashimoto's thyroiditis (Premachandra and Blumenthal, 1967; Ochi et al., 1972). Recently Staeheli et al. (1975) demonstrated the presence of anti-T₄ and anti-triiodothyronine (T₃) antibodies in different thyroid conditions by disrupting the antigen-antibody complex, and predicted the possibility of spuriously high or low serum T₄ and T₃ values by radioimmunoassay (RIA) due to these antibodies.

In fact, one case of euthyroid Graves' disease with ophthalmopathy has been reported to have undetectable T₃ by RIA, while large amounts of T₃ could be detected from an ethanol extract of the serum (Sterling et al., 1973). In addition, a patient with abnormal T₃-binding immunoglobulins has also been described (Wu and Green, 1976). In this case, using a single-antibody technique, serum T₃ (RIA) was reported as being indeterminate due to this abnormal T₃-binding protein. However, when a double-antibody method was used a high T₃ value was obtained.

We have recently studied a patient with Hashimoto's thyroiditis whose serum T₄ value measured by a single-antibody RIA was much lower than that measured by competitive protein binding analysis (CPBA). It is suggested that this discrepancy is caused by the presence of the anti-T₄ autoantibody in the serum.

Materials and Methods

The patient was a 59-year-old woman with 30-year history of goiter. A diagnosis of Hashimoto's thyroiditis had been established by open biopsy of the thyroid gland ten years previously. Since then she had not taken any drugs. In April, 1975 she was examined again because of further enlargement of the thyroid, and was judged to be euthyroid on the basis of clinical and laboratory findings (mean serum T₄ by CPBA, 5.0 µg/100 ml; serum T₃, 165 ng/100 ml; T₃ resin uptake, 31.8%; and serum TSH, 2.0 µU/ml). Microscopic examination of the thyroid gland, obtained by needle biopsy at this time, showed degeneration of the follicular epithelium and widespread lymphocytic infiltration. This finding was

Received October 25, 1977.
not markedly different from that of the open biopsy specimen obtained ten years previously. The T₄-binding capacity of TBG in her serum was 24 μg/100 ml. Anti-thyroglobulin antibody (anti-Tg) by the tanned red-cell agglutination test was negative. The serum T₄ was measured by the following kits, T₄ RIA (Abbott Lab., North Chicago), RIA-Mat T₄ (Mallinckrodt, Inc., St. Louis), Tetrasorb (Dainabot Co., Tokyo) and Tetra-Tab (Nuclear medical Lab., Inc., Taxas). T₄ values by RIA (using T₄ RIA and RIA-Mat T₄ kits) in sera from normal subjects and from patients with various thyroid diseases were found to be in good correlation with those obtained by a CPBA kit, Tetrasorb. Values obtained by two CPBA kits, Tetrasorb and Tetra-Tab, also showed close correlation.

The 19S and 7S protein fractions of the serum were obtained by gel filtration through a Sephadex G-200 column (1.5×90 cm) in phosphate buffered saline (PBS) 0.02 M, pH 7.6. The IgG fraction of the serum was prepared by a DEAE cellulose chromatography with a 0.01 M phosphate buffer, pH 7.6. ¹²⁵I labelled T₄ (¹²⁵I-T₄) was obtained from Dainabot Laboratories, Tokyo; the specific activity was 620 pCi/μg.

The binding of ¹²⁵I-T₄ to the serum or fractions of the serum was studied by the following methods:

1) Using RIA kit systems
   The binding of ¹²⁵I-T₄ to the serum was studied using two kinds of RIA kits without the addition of the provided anti-T₄ serum. After incubation of the serum with ¹²⁵I-T₄, free ¹²⁵I-T₄ was separated from the bound by the provided anionic exchange resin.

2) Polyethylene glycol (PEG) method
   Fifty μl of the serum were incubated with ¹²⁵I-T₄ (30,000 cpm) in 50 μl of 0.075 M barbital buffer, pH 8.6 containing 1,200 μg/ml of 8-anilino-1-naphthalene-sulfonic acid (ANS), for 20 hr at 4°C. In the cases of 19S, 7S and IgG fractions (about 500 μg each), and anti-T₄ rabbit serum of the RIA-Mat T₄ kit, 50 μl of each were incubated in the same manner for 20 hr at 4°C without the addition of ANS. The tubes were then centrifuged at 3,000 rpm for 20 min at 4°C. The supernate was decanted and the radioactivity of the precipitate was counted.

3) Double antibody method
   Five μl of the serum were incubated with ¹²⁵I-T₄ in a 0.075 M barbital buffer, pH 8.6 containing 600 μg/ml of ANS, for 20 hr at 4°C. Purified IgG fraction (about 10-50 μg) was incubated in the same manner without ANS. After the addition of an excess of anti-human IgG goat serum, the mixture (200 μl) was incubated for another 20 hr in the same condition and centrifuged at 10,000×g for 10 min.

4) Column chromatography
   (a) The patient’s serum, a control serum and the anti-T₄ rabbit serum (from RIA-Mat T₄) were incubated with ¹²⁵I-T₄ under the presence of ANS (600 μg/ml) at room temperature for one hr. Each of the mixtures was then applied to a Sephadex G-200 column (1.0×30 cm) and the column was eluted with barbital buffer, 0.075 M, pH 8.6 containing the same concentration of ANS. Fifteen drop aliquots were collected and radioactivity was counted.
   (b) The serum was incubated with ¹²⁵I-T₄ after thermal inactivation (60°C, 2 hr) of TBG (Sterling and Milch, 1974). The incubation mixture was applied to a Sephadex G-200 column (1.0×90 cm), and was eluted with PBS 0.02 M, pH 7.6. Three ml fractions were collected and both radioactivity and optical density at 280 nm were measured.

Results

The serum T₄ values measured by RIA and CPBA.

The mean serum T₄ measured twice in duplicate by T₄ RIA, RIA-Mat T₄, Tetrasorb and Tetra-Tab kits were 1.70±0.90 (SD), 2.9±0.37 (SD), 5.4±0.34 (SD) and 4.6±0.26 (SD) μg/100 ml, respectively. The mean recoveries of 0.5 to 10 μg nonradioactive T₄ added to the patient’s serum, evaluated by T₄ RIA and RIA-Mat T₄ kits, were 74.2% and 71.2%, respectively. While the mean recoveries of T₄ obtained in three control sera were 110 and 108%, respectively.

Binding of ¹²⁵I-T₄ to the patient’s serum.

The significant binding of ¹²⁵I-T₄ to the patient’s serum was observed by using the T₄ RIA and RIA-Mat T₄ systems without anti-T₄ serum (Table 1).

Fig. 1 shows the result of Sephadex G-200 column chromatography of ¹²⁵I-T₄ preincubated with the test sera or with the rabbit anti-T₄ antibody under the presence of ANS. An early radioactive peak was observed by using the patient’s serum, as in the case of the anti-T₄ antibody, whereas such radioactivity was not detected by using a control serum.
Fig. 2 compares the elution patterns from a Sephadex G-200 column of $^{125}$I-T$_4$ added to heat-treated patient’s and normal control sera. Only with the patient’s serum was an early radioactive peak observed in the area where the 7S protein fraction was eluted. The binding of $^{125}$I-T$_4$ to the 7S and IgG fractions determined by the PEG method were 34.0% and 51.0%, respectively, whereas binding to the 19S fraction was negligible (Table 2). By using the double antibody method, the binding of $^{125}$I-T$_4$ to the patient’s serum and its IgG fraction were 15.4% and 9.0%, respectively, while the binding to a control serum and its IgG fraction were 1.8% and 0.5% (Table 3). However, when the untreated patient’s serum was incubated with $^{125}$I-T$_4$ without addition of ANS, no significant radioactivity was observed in the γ-globulin or 7S region on paper electrophoresis or Sephadex G-200 gel filtration.

![Graph](image)

**Fig. 1.** Sephadex G-200 column chromatography of $^{125}$I-T$_4$, preincubated with the sera from a patient and a control, and the rabbit anti-T$_4$ serum in the presence of ANS.

![Graph](image)

**Fig. 2.** Sephadex G-200 column chromatography of $^{125}$I-T$_4$ preincubated with the heat-treated serum; protein peaks (OD 280) are shown as dotted lines and radioactivity as solid lines.

### Table 1. Binding of $^{125}$I-T$_4$ to the serum detected by using the T$_4$ RIA and RIA-Mat T$_4$ systems without anti-T$_4$ serum. $^{125}$I-T$_4$ binding to the anti-T$_4$ rabbit serum of each kit was shown for comparison.

<table>
<thead>
<tr>
<th></th>
<th>T$_4$ RIA</th>
<th>RIA-Mat T$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. (n=2)</td>
<td>31.7±1.70 (SD) %</td>
<td>16.8±1.27 (SD) %</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>17.4±1.82 (SD) %</td>
<td>6.8±0.34 (SD) %</td>
</tr>
<tr>
<td>Anti-T$_4$ rabbit serum (n=5)</td>
<td>80.6±1.14 (SD) %</td>
<td>60.6±3.09 (SD) %</td>
</tr>
</tbody>
</table>

### Table 2. Binding of $^{125}$I-T$_4$ to the serum and its fractions detected by precipitation at 12.5% PEG.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>19S</th>
<th>7S</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. (n=2)</td>
<td>30.1%</td>
<td>0.5%</td>
<td>34.0%</td>
<td>51.0%</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>8.5%</td>
<td>0.2%</td>
<td>6.2%</td>
<td>6.0%</td>
</tr>
</tbody>
</table>

### Table 3. Binding of $^{125}$I-T$_4$ to the serum and its IgG fraction detected by using double antibody method.

<table>
<thead>
<tr>
<th></th>
<th>$^{125}$I-T$_4$ precipitated by anti-IgG (% bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>IgG</td>
</tr>
<tr>
<td>Pt. (n=2)</td>
<td>15.4%</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>1.8%</td>
</tr>
</tbody>
</table>

In the case of the serum, ANS was added in a final concentration of 600 μg/ml.
Specificity of the $T_4$ binding.

Fig. 3 shows the dose-dependent displacement of binding of $^{125}$I-$T_4$ to the patient's IgG by the addition of unlabeled $T_4$. The assay was performed by PEG method. The cross reaction with $T_3$ was approximately 8.3%. MIT and DIT did not displace the labeled $T_4$ when tested in amounts varying from 0.1 to 100 ng per assay. No binding to the serum of other radioactive peptide hormones such as TSH, HGH or insulin could be detected.

Association constant and binding capacity for $T_4$ of the patient's IgG and the anti-$T_4$ rabbit serum of RIA-Mat $T_4$.

The Scatchard plots (Scatchard, 1949) of the $T_4$ binding to the patient's IgG and the anti-$T_4$ serum of the RIA-Mat $T_4$ are shown in Fig. 4.

The association constants for $T_4$ were calculated to be $1.9 \times 10^8$ l/mol and $1.2 \times 10^9$ l/mol, and the binding capacities were 0.8 µg/100 ml serum and 0.3 µg/100 ml serum, respectively. As 10 µl of serum sample and 100 µl of anti-$T_4$ serum were used in RIA-Mat $T_4$, the binding capacities of the patient's IgG and the anti-$T_4$ serum for $T_4$ in this system were 0.08 ng and 0.3 ng, respectively.

Discussion

By using single antibody RIA systems, significantly lower serum $T_4$ values than those by CPBA were obtained in the present case with Hashimoto's thyroiditis. Possible under-estimation of the serum $T_4$ by RIA was suspected because the patient was clinically euthyroid and no elevation of serum TSH was present. In experiments where nonradioactive $T_4$ was added to the patient's serum, the recovery of $T_4$ determined by RIA was significantly low. The binding of $^{125}$I-$T_4$ to the patients serum was confirmed by several methods. And the

![Fig. 3. Inhibition of the binding of $^{125}$I-$T_4$ by $T_4$ or $T_3$.](image)

![Fig. 4. Scatchard plot of $T_4$ binding to; (A) Patient's IgG (B) Anti-$T_4$ rabbit serum of RIA-Mat $T_4$.](image)
binding activity was present in the IgG fraction of the serum. All these data should be reasonably understood assuming a concept that the presence of the T₄ binding IgG could produce spuriously low T₄ value in the RIA system used. In fact the methods employed are based on absorption of free T₄ by resin. Therefore, the presence of the anti-T₄ antibody increased the bound fraction, resulting in a decrease of the T₄ values obtained.

Recently Wu and Green (1976) reported a case with T₃-binding immunoglobulin, which evoked artefactually high or low T₃ measurements depending on the method employed.

The association constant of the patient's IgG against T₄ was 1.9×10⁸ l/mol, and this was somewhat lower than that of the anti-T₄ rabbit serum of RIA-Mat T₄, 1.2×10⁹ l/mol. Its binding capacity was about 1/4 of that of the latter under the assay condition. This limited affinity and capacity of this IgG may explain the reason why we could not see the binding of ¹²⁵I-T₄ to the γ-globulin or IgG without the presence of the binding inhibitor to TBG.

Therefore, the presence of this anti-T₄ antibody in the serum was considered to have quite a limited effect on the T₄ distribution in the serum. However, the fact that even such a low affinity immunoglobulin could significantly affect T₄ value in RIA was considered to be of practical importance.

As the binding of T₄ to the patient's IgG was demonstrated to be specific, this T₄-binding IgG was considered to be an anti-T₄ autoantibody. Although the binding of T₃ to the IgG was also observed, the binding affinity was much lower than that with T₄ and this binding was considered to be the cross-reaction of T₃ with the anti-T₄ antibody.

Although the possibility that thyroid medication might evoke an antibody formation against the thyroid hormone was suggested by Staeheli et al. (1975), the patients in the present study had received no treatment for 10 years. Her serum anti-T₄, which had been positive 10 years ago was negative at the time of the present study. Staeheli et al. (1975) have also reported cases with anti-T₄ antibodies without anti-Tg. Further accumulation of case studies is required for the understanding of these antibodies.

The existence of such a case should be kept in mind to avoid possible erroneous clinical interpretation of the T₄ value by RIA. As was shown by Wu and Green (1976) for T₃, if the double antibody method is used for the assay, the value would be high in this case. T₄ assay after extraction should be performed when the data on unextracted serum are discordant with the clinical state, especially in cases with autoimmune thyroid diseases.

Acknowledgements

We are grateful to Dr. Shunyo Yakura for his help in studying the case. We wish to thank Miss Chisako Furumatsu for the preparation of the manuscript.

References


