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 (T_4) value, obtained by radioimmunoassay (RIA) employing resin to separate bound and free T_4 , 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 T_4 value by RIA in cases with autoimmune thyroid diseases.

The binding of serum thyroxine (T_4) 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- T_4 and anti-triiodothyronine (T_3) antibodies in different thyroid conditions by disrupting the antigen-antibody complex, and predicted the possibility of spuriously high or low serum T_4 and T_3 values by radioimmunoassay (RIA) due to these antibodies.

In fact, one case of euthyroid Graves' disease with ophthalmopathy has been reported to have undetectable T_3 by RIA, while large amounts of T_3 could be detected from an ethanol extract of the serum (Sterling *et al.*, 1973). In addition, a patient with abnormal T_3 -binding immunoglobulins has also been described (Wu and Green, 1976). In this case, using a single-antibody technique, serum T_3 (RIA) was reported as being indeterminate due to this abnormal

T_3 -binding protein. However, when a double-antibody method was used a high T_3 value was obtained.

We have recently studied a patient with Hashimoto's thyroiditis whose serum T_4 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- T_4 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 T_4 by CPBA, $5.0 \mu\text{g}/100 \text{ ml}$; serum T_3 , $165 \text{ ng}/100 \text{ ml}$; T_3 resin uptake, 31.8%; and serum TSH, $2.0 \mu\text{U}/\text{ml}$). 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

not markedly different from that of the open biopsy specimen obtained ten years previously. The T_4 -binding capacity of TBG in her serum was 24 $\mu\text{g}/100\text{ ml}$. Anti-thyroglobulin antibody (anti-Tg) by the tanned red-cell agglutination test was negative. The serum T_4 was measured by the following kits, T_4 RIA (Abbott Lab., North Chicago), RIA-Mat T_4 (Mallinckrodt, Inc., St. Louis), Tetrasorb (Dainabot Co., Tokyo) and Tetra-Tab (Nuclear medical Lab., Inc., Texas). T_4 values by RIA (using T_4 RIA and RIA-Mat T_4 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 $\times$ 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 column chromatography with a 0.01 M phosphate buffer, pH 7.6. ^{125}I labelled T_4 ($^{125}\text{I}-T_4$) was obtained from Dainabot Laboratories, Tokyo; the specific activity was 620 $\mu\text{Ci}/\mu\text{g}$.

The binding of $^{125}\text{I}-T_4$ to the serum or fractions of the serum was studied by the following methods:

1) Using RIA kit systems

The binding of $^{125}\text{I}-T_4$ to the serum was studied by using two kinds of RIA kits without the addition of the provided anti- T_4 serum. After incubation of the serum with $^{125}\text{I}-T_4$, free $^{125}\text{I}-T_4$ was separated from the bound by the provided anionic exchange resin.

2) Polyethylene glycol (PEG) method

Fifty μl of the serum were incubated with $^{125}\text{I}-T_4$ (30,000 cpm) in 50 μl of 0.075 M barbital buffer, pH 8.6 with 1,200 $\mu\text{g}/\text{ml}$ 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- T_4 rabbit serum of the RIA-Mat T_4 kit, 50 μl of each were incubated in the same manner for 20 hr at 4°C without the addition of ANS. At the end of the incubation period, normal human γ -globulin was added to the same concentration as IgG. An aqueous solution of PEG (Carbowax 6,000) was added to make a final concentration of 12.5%. 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 $^{125}\text{I}-T_4$ in a 0.075 M barbital buffer, pH 8.6 containing 600 $\mu\text{g}/\text{ml}$ 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 $\times g$ for 10 min.

4) Column chromatography

(a) The patient's serum, a control serum and the anti- T_4 rabbit serum (from RIA-Mat T_4) were incubated with $^{125}\text{I}-T_4$ under the presence of ANS (600 $\mu\text{g}/\text{ml}$) at room temperature for one hr. Each of the mixtures was then applied to a Sephadex G-200 column (1.0 $\times$ 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 $^{125}\text{I}-T_4$ 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 $\times$ 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 T_4 values measured by RIA and CPBA.

The mean serum T_4 measured twice in duplicate by T_4 RIA, RIA-Mat T_4 , 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) $\mu\text{g}/100\text{ ml}$, respectively. The mean recoveries of 0.5 to 10 ng nonradioactive T_4 added to the patient's serum, evaluated by T_4 RIA and RIA-Mat T_4 kits, were 74.2% and 71.2%, respectively. While the mean recoveries of T_4 obtained in three control sera were 110 and 108%, respectively.

Binding of $^{125}\text{I}-T_4$ to the patient's serum.

The significant binding of $^{125}\text{I}-T_4$ to the patient's serum was observed by using the T_4 RIA and RIA-Mat T_4 systems without anti- T_4 serum (Table 1).

Fig. 1 shows the result of Sephadex G-200 column chromatography of $^{125}\text{I}-T_4$ preincubated with the test sera or with the rabbit anti- T_4 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- T_4 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 $^{125}\text{I-T}_4$ 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 $^{125}\text{I-T}_4$ 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 $^{125}\text{I-T}_4$ 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 $^{125}\text{I-T}_4$ 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.

Table 1. Binding of $^{125}\text{I-T}_4$ to the serum detected by using the T_4 RIA and RIA-Mat T_4 systems without anti- T_4 serum. $^{125}\text{I-T}_4$ binding to the anti- T_4 rabbit serum of each kit was shown for comparison.

	% bound with	
	T_4 RIA	RIA-Mat T_4
Pt. (n=2)	31.7 ± 1.70 (SD) %	16.8 ± 1.27 (SD) %
Control (n=5)	17.4 ± 1.82 (SD) %	6.8 ± 0.34 (SD) %
Anti- T_4 rabbit serum (n=5)	80.6 ± 1.14 (SD) %	60.6 ± 3.09 (SD) %

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

	Serum	19S	7S	IgG
Pt.	30.1%	0.5%	34.0%	51.0%
Control	8.5%	0.2%	6.2%	6.0%

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

	$^{125}\text{I-T}_4$ precipitated by anti-IgG (% bound)	
	Serum	IgG
Pt.	15.4%	9.0%
Control	1.8%	0.5%

In the case of the serum, ANS was added in a final concentration of 600 $\mu\text{g/ml}$.

Fig. 1. Sephadex G-200 column chromatography of $^{125}\text{I-T}_4$ preincubated with the sera from a patient and a control, and the rabbit anti- T_4 serum in the presence of ANS.

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

Specificity of the T₄ binding.

Fig. 3 shows the dose-dependent displacement of binding of ¹²⁵I-T₄ to the patient's IgG by the addition of unlabeled T₄. The assay was performed by PEG method. The cross reaction with T₃ was approximately 8.3%. MIT and DIT did not displace the labeled T₄ 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 T₄ of the patient's IgG and the anti-T₄ rabbit serum of RIA-Mat T₄.

The Scatchard plots (Scatchard, 1949) of the T₄ binding to the patient's IgG and the anti-T₄ serum of the RIA-Mat T₄ are shown in Fig. 4.

The association constants for T₄ were calculated to be 1.9×10^8 l/mol and 1.2×10^9 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-T₄ serum were used in RIA-Mat T₄, the binding capacities of the patient's IgG and the anti-T₄ serum for T₄ in this system were 0.08 ng and 0.3 ng, respectively.

Discussion

By using single antibody RIA systems, significantly lower serum T₄ values than those by CPBA were obtained in the present case with Hashimoto's thyroiditis. Possible under-estimation of the serum T₄ by RIA was suspected because the patient was clinically euthyroid and no elevation of serum TSH was present. In experiments where nonradioactive T₄ was added to the patient's serum, the recovery of T₄ determined by RIA was significantly low. The binding of ¹²⁵I-T₄ to the patients serum was confirmed by several methods. And the

Fig. 3. Inhibition of the binding of ¹²⁵I-T₄ by T₄ or T₃.

Fig. 4. Scatchard plot of T₄ binding to ; (A) Patient's IgG (B) Anti-T₄ rabbit serum of RIA-Mat T₄.

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 T_4 binding IgG could produce spuriously low T_4 value in the RIA system used. In fact the methods employed are based on absorption of free T_4 by resin. Therefore, the presence of the anti- T_4 antibody increased the bound fraction, resulting in a decrease of the T_4 values obtained.

Recently Wu and Green (1976) reported a case with T_3 -binding immunoglobulin, which evoked artefactually high or low T_3 measurements depending on the method employed.

The association constant of the patient's IgG against T_4 was 1.9×10^8 l/mol, and this was somewhat lower than that of the anti- T_4 rabbit serum of RIA-Mat T_4 , 1.2×10^9 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 ^{125}I - T_4 to the γ -globulin or IgG without the presence of the binding inhibitor to TBG.

Therefore, the presence of this anti- T_4 antibody in the serum was considered to have quite a limited effect on the T_4 distribution in the serum. However, the fact that even such a low affinity immunoglobulin could significantly affect T_4 value in RIA was considered to be of practical importance.

As the binding of T_4 to the patient's IgG was demonstrated to be specific, this T_4 -binding IgG was considered to be an anti- T_4 autoantibody. Although the binding of T_3 to the IgG was also observed, the binding affinity was much lower than that with T_4 and this binding was considered to be the cross-reaction of T_3 with the anti- T_4 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-Tg, 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- T_4 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 T_4 value by RIA. As was shown by Wu and Green (1976) for T_3 , if the double antibody method is used for the assay, the value would be high in this case. T_4 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

- Ochi, Y., K. Shiomi, T. Hachiya, M. Yoshimura and T. Miyazaki (1972). *J. Clin. Endocrinol. Metab.* 35, 743.
- Premachandra, B. N. and H. T. Blumenthal (1967). *ibid.* 27, 931.
- Robbins, J., J. E. Rall and R. W. Rawson (1956). *ibid.* 16, 573.
- Scatchard, G. (1949). *Ann. N. Y. Acad. Sci.* 51, 660.
- Staeheli V., M. B. Vallotton and A. Burger (1975). *J. Clin. Endocrinol. Metab.* 41, 669.
- Sterling, K., P. O. Milch, D. A. Holub and A. Burger Program 49th Ann. Meet. Amer. Thyroid Assoc., p. T II (Abstract), (1973).
- Sterling, K. and P. O. Milch (1974). *J. Clin. Endocrinol. Metab.* 38, 866.
- Wu, S. Y. and W. L. Green (1976). *ibid.* 42, 642.