NOTE

An Improved and Simplified Method for the Detection of Thyroid Hormone Autoantibodies (THAA) in Serum

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Abstract

We have developed and evaluated a new and simplified method for the detection of thyroid hormone autoantibodies (THAA) in serum. The method includes acidification of serum followed by adsorption of liberated thyroid hormones onto dextran-coated charcoal and then alkalisation of the serum in assay buffer prior to performing a binding study. Using our method, specific binding of $^{125}\text{I-T}_4$ to serum THAA in two patients with Hashimoto's thyroiditis was almost the same regardless of whether or not the sera had been preincubated with a large amount of cold T_4 . On the other hand, without the acid treatment, preincubation with cold T_4 considerably inhibited the binding of $^{125}\text{I-T}_4$ to serum THAA in both cases. These results indicate that serum THAA can be easily detected under conditions in which circulating thyroid hormones hardly affect the binding study by using our new sensitive method.

In 1956, the presence of T_4 -binding γ -globulin was first reported in a patient with papillary thyroid carcinoma who had been treated with ^{131}I (Robbins *et al.*, 1956). Since then thyroid hormone autoantibodies (THAA) have been demonstrated in patients with various thyroidal (Staeheli *et al.*, 1975; Wu and Green, 1976; Ikekubo *et al.*, 1978; Inada *et al.*, 1980; Faerch *et al.*, 1980; Pudek and McIntosh, 1981; Konishi *et al.*, 1982; Wiersinga *et al.*, 1982; Nakamura *et al.*, 1982; Sakata *et al.*, 1985) and non-thyroidal (Trimarchi *et al.*, 1982 & 1983) diseases.

In most of the previous reports, THAA has been detected by radioimmunoassay

(RIA). In RIAs, THAA is usually measured by the addition of labelled thyroid hormones to serum in the presence of a TBG-blocking agent such as 8-anilino-1-naphthalene sulfonic acid (ANS) (Staeheli *et al.*, 1975; Wu and Green, 1976; Ikekubo *et al.*, 1978; Inada *et al.*, 1980; Konishi *et al.*, 1982; Nakamura *et al.*, 1982; Sakata *et al.*, 1985) or salicylate (Faerch *et al.*, 1980; Pudek and McIntosh, 1981) followed by B/F separation with polyethylene glycol (PEG) (Ikekubo *et al.*, 1978; Inada *et al.*, 1980; Konishi *et al.*, 1982; Wiersinga *et al.*, 1982; Trimarchi *et al.*, 1983; Nakamura *et al.*, 1982; Sakata *et al.*, 1985), charcoal (Faerch *et al.*, 1980; Pudek and McIntosh, 1981), resin (Ikekubo *et al.*, 1978), ammonium sulfate (Wu and Green, 1976), or second

antibody (Ikekubo *et al.*, 1978; Nakamura *et al.*, 1982; Sakata *et al.*, 1985). In these RIA systems, however, serum thyroid hormones can interfere with the binding of labelled thyroid hormones to serum THAA. Therefore, to minimize the effect of endogenous and/or therapeutic thyroid hormones on the THAA assay, it is necessary to remove circulating thyroid hormones from the serum. Concerning this point, Staeheli *et al.* (1975) reported that thyroid hormone-THAA complex was partially disrupted by 45% ammonium sulfate precipitation. Premachandra and Walfish (1982) stated that the stripping of serum with charcoal increased the $^{125}\text{I-T}_4$ binding to serum γ -globulin in two patients with chronic autoimmune thyroiditis on T_4 therapy. Recently, labelled thyroid hormone analogues which do not bind to TBG have been introduced for the THAA assay (Allan *et al.*, 1982; Rodrigues-Espinosa *et al.*, 1984). However, a method using an acid pH buffer, in which the antigen-antibody complex is dissociated (Dixon, 1974; Gerbitz and Kemmler, 1978) has not been developed for this assay.

In this paper, we report a simplified and improved method for the detection of THAA, which includes acidification of serum followed by adsorption of liberated thyroid hormones onto dextran-coated charcoal prior to binding study.

Materials and Methods

Norit A, 8-anilino-1-naphthalene sulfonic acid, ammonium salt (ANS), and PEG 6000 were obtained from Wako Pure Chemicals, Osaka, Japan. Dextran T 70 was from Pharmacia Fine Chemicals, Uppsala, Sweden. T_4 and bovine γ -globulin (Cohn Fraction II) were purchased from Sigma Co., St. Louis, MO, and $^{125}\text{I-T}_4$ from New England Nuclear, North Billerica, MA. The specific activity of $^{125}\text{I-T}_4$ was $1250 \mu\text{Ci}/\mu\text{g}$.

ANS (400 $\mu\text{g}/\text{ml}$), bovine γ -globulin (10 mg/ml), or PEG (250 mg/ml) was dissolved in 0.06M barbital buffer (pH 8.6 at 25°C). $^{125}\text{I-T}_4$ (400

pg/ml) was dissolved in the 0.06 M barbital buffer containing bovine serum albumin (Rehelis Chemical Co., Phoenix, AZ) with a concentration of 1 mg/ml . Two point five grams of Norit A and 0.25 g of dextran T 70 were dissolved in 100 ml of 0.05 M glycine-HCl buffer ($\mu=0.16$) at different pH. The final pH of the acid-charcoal solution was between 1.50 and 4.20 at 25°C.

Serum samples were obtained from two patients with Hashimoto's thyroiditis associated with anti- T_3 and anti- T_4 autoantibodies in serum (Nakamura *et al.*, 1982). We used these sera for the THAA assay, before and after adding 0.01 ml of T_4 solution (40 μg of T_4 in 1 ml of saline) to 2 ml of the serum, followed by incubation at 37°C for 2 h and then at 4°C for 48 h. Serum from a normal euthyroid subject who had no detectable anti- T_3 or anti- T_4 antibodies served as a control.

All assays except those for intraassay variations were performed in duplicate.

Procedures

1) Conventional binding study

To 0.02 ml of serum, 0.78 ml of ANS solution, 0.1 ml of γ -globulin solution, and 0.1 ml of $^{125}\text{I-T}_4$ solution were added. The mixture was incubated at 37°C for 1 h, followed by the addition of an equal volume (1 ml) of PEG solution and then centrifuged (3000 rpm, 30 min., 4°C). The radioactivity of the pellet was counted.

2) Acid-charcoal method

Four-tenths of a milliliter of the acid-charcoal solution at different pH (1.50–4.20) was added to 0.1 ml of serum. The mixture was incubated at 4°C (1st incubation), followed by centrifugation (10000 rpm, 5 min., 25°C). To 0.1 ml of the supernate, 0.1 ml of γ -globulin solution, 0.7 ml of ANS solution with or without cold T_4 (40 ng), and 0.1 ml of $^{125}\text{I-T}_4$ solution were added. They were mixed well with a vortex mixture. The mixture was then incubated at 37°C (2nd incubation), followed by the addition of 1 ml of PEG solution and centrifugation (3000 rpm, 30 min., 4°C). The results were expressed as percentage specific binding as follows:

% Specific Binding = $\frac{\text{cpm in the pellet (without cold } \text{T}_4 \text{ in the 2nd incubation)} - \text{cpm in the pellet (with cold } \text{T}_4 \text{ in the 2nd incubation)}}{\text{total cpm}} \times 100 (\%)$.

Results

1) Conventional binding study (Fig. 1)

Bindings of $^{125}\text{I-T}_4$ to serum γ -globulin of original sera in case 1, case 2, and a normal control were 56.3, 11.3, and 5.1%, respectively. However, the $^{125}\text{I-T}_4$ bindings in T_4 -added sera from both cases were decreased to 9.4% in case 1 and 7.2% in case 2.

2) Acid-charcoal method

(a) Influence of pH of the acid-charcoal solution on the $^{125}\text{I-T}_4$ binding to patients' sera

Fig. 1. Binding of $^{125}\text{I-T}_4$ to serum γ -globulin. Binding study was performed in the sera which had been preincubated with (■) or without (□) a large amount of cold T_4 .

Fig. 2 shows the bindings of $^{125}\text{I-T}_4$ to the original and T_4 added sera in both cases and a control serum. Maximum binding was obtained at pH 2.25 in the T_4 -added sera and between pH 2.25 and pH 2.50 in the original sera in both cases. The specific bindings of $^{125}\text{I-T}_4$ to THAA in the original serum were higher than those of the T_4 -added serum at all pH values examined in both cases. As shown in Fig. 2, the difference between the binding in the original and the T_4 -added sera was minimal at pH 2.25 in case 1 and was similar from pH 1.50 to pH 2.25 in case 2. From these results, we chose to use the acid-charcoal at pH 2.25 for further studies. Using this acid-charcoal, the pH of the supernate was 3.2 and that of the assay system during the 2nd incubation was 8.4. The binding was below 0.6% in the control serum at any pH.

(b) Effect of the 1st incubation time

As shown in Fig. 3, the binding of $^{125}\text{I-T}_4$ to the original serum was clearly higher than that of the T_4 -added serum in both cases when the sera had been preincubated with the charcoal at pH 2.25 within a 2-h period. These differences, however, were almost negligible after 4 h incubation up to 16 h. For practical reasons, we chose to use an incubation time of 12 h (overnight).

(c) Effect of the 2nd incubation time

As shown in Fig. 4, the maximum bindings in both original and T_4 -added sera during the 2nd incubation were obtained at 30 min. with a plateau up to 4 h in case 1. Those in case 2 were at 2 h. From these results, we chose to use an incubation time of 2 h.

(d) Reproducibility

The results of intra- and interassay variations are shown in Table 1. The coefficients of variations of intraassay in case 1 and in case 2 were 0.48 and 5.57%, and those of the interassay were 4.06 and 5.00%, respectively. The specific binding in the control

Fig. 2. Effect of pH of charcoal solution on the specific binding of $^{125}\text{I-T}_4$ to serum γ -globulin. (1st incubation: 30 min., 2nd incubation 1h)

serum was almost zero.

Discussion

The presence of serum THAA has been suspected from the following points (Wu and Green, 1976; Ikekubo *et al.*, 1978; Inada *et al.*, 1980; Faerch *et al.*, 1980; Pudek and McIntosh, 1981; Konishi *et al.*, 1982; Wiersinga *et al.*, 1982; Nakamura *et al.*, 1982; Sakata *et al.*, 1985): 1) serum levels of thyroid hormones (total and free) as measured by RIA are not compatible with clinical features and/or other laboratory data, and 2) in RIA, the different results for thyroid hormone concentrations are obtained according to the methods used for the B/F separation. The detection of THAA in serum has been performed by study of

the binding of labelled thyroid hormones to serum γ -globulin (or IgG). However, in most of the previous reports, the binding study was performed using a TBG-blocking agent without removal of endogenous and/or therapeutic thyroid hormones from serum.

As shown in Fig. 1, the preincubation with cold T_4 considerably inhibited the $^{125}\text{I-T}_4$ binding to serum γ -globulin in both cases. These data clearly indicate that serum thyroid hormones (endogenous and/or therapeutic) do interfere with in vitro THAA assays. Thus, low titers or false negative results for THAA could be obtained unless circulating thyroid hormones are removed from the serum. From the conventional binding assay system, it is suggested that the inhibition by serum thyroid hormones of the binding of labelled thyroid hormones to serum THAA occurs for

Fig. 3. Effect of 1st incubation time on the specific binding of $^{125}\text{I-T}_4$ to serum γ -globulin. (pH of charcoal solution: 2.25, 2nd incubation: 1h)

Fig. 4. Effect of 2nd incubation time on the specific binding of $^{125}\text{I-T}_4$ to serum γ -globulin. (pH of charcoal solution: 2.25, 1st incubation: 12h)

Table 1. Reproducibility of Our Assay for THAA in Serum

		% Precipitated (A)	% NSB (B)	% Specific Binding (A-B)
Intraassay	Case 1*	63.24 ± 0.24 (0.38)	6.49 ± 0.23 (3.54)	56.75 ± 0.27 (0.48)
	(n=5) Case 2*	23.53 ± 1.05 (4.46)	6.31 ± 0.14 (2.22)	17.22 ± 0.96 (5.57)
	Control	5.61 ± 0.23 (4.10)	5.60 ± 0.29 (5.18)	0.01 ± 0.46
Interassay	Case 1*	62.12 ± 2.37 (3.82)	6.65 ± 0.33 (4.96)	55.46 ± 2.25 (4.06)
	(n=5) Case 2*	23.06 ± 1.07 (4.64)	6.53 ± 0.61 (9.34)	16.40 ± 0.82 (5.00)
	Control	5.91 ± 0.40 (6.77)	5.75 ± 0.46 (8.00)	0.18 ± 0.22

Assay conditions: pH of charcoal solution—2.25
1st incubation time—12h
2nd incubation time—2h

Data are expressed as mean $\pm$ SD.

Parenthesis indicates CV value.

*Binding study was performed in the original sera.

A: cpm in the pellet (without cold T_4) $\div$ total cpm $\times 100(\%)$

B: cpm in the pellet (with cold T_4) $\div$ total cpm $\times 100(\%)$

the following reasons. First, free thyroid hormones inhibit the binding of labelled thyroid hormones to serum THAA competitively. Second, TBG-blocking agents and buffers such as barbital, which inhibit the binding of labelled thyroid hormones to physiological carrier proteins, liberate bound thyroid hormones from these proteins concomitantly. Consequently, free thyroid hormones in the assay system are increased. Third, THAA is occupied to some extent by endogenous and/or therapeutic thyroid hormones. In the method using labelled thyroid hormone analogues for the detection of THAA, the binding of these analogues to serum THAA can be affected, too, by the presence of serum thyroid hormones because of the first and third reasons described above.

In our present study, the binding of $^{125}\text{I-T}_4$ to serum THAA after acid treatment was almost the same, irrespective of whether or not a large amount of cold T_4 had been added to the sera. These results indicate that circulating thyroid hormones can be removed from the sera by treatment with acid-charcoal. In addition, as shown in Table 1 and Fig. 1, percentage precipitation (% specific binding plus % NSB) of the acid-charcoal sera was higher than that of the non-acidified sera (63.2 vs. 56.3% in case 1 and 23.5 vs. 11.3% in case 2, respectively), although in this binding there was no difference between the acid-treated and non-acidified sera in the control (5.6 vs. 5.1%). These data indicate that our present method is better than the method using sera without the removal of thyroid hormones to evaluate THAA titer.

The pH of charcoal-extracted serum was reported to be 9.5 with the method of Premachandra and Ibrahim (1975), while it was 3.2 with our method. It is difficult to compare our results with those of Premachandra and Ibrahim (1975) since the effects of alkaline pH have not been examined in the present study. Our method, however,

has some advantages as follows: 1) the TBG molecule is denatured at acid pH (Gershen-gorn *et al.*, 1977), 2) the necessary amount of serum is lower and 3) the procedure is simple for routine use.

It is known (Traycoff *et al.*, 1981; Jawad and Wilson, 1981) that the γ -globulin fraction precipitated with PEG increases in proportion to the concentration of serum γ -globulin and that hyper γ -globulinemia alone can produce high nonspecific binding. In fact, Trimarchi *et al.* (1983) reported that 11 out of 351 patients with thyroidal and lymphoreticular diseases had high nonspecific binding in PEG-RIAs. However, they demonstrated the THAA activity in only 5 out of these 11 patients upon further examination. Therefore, concentration of serum γ -globulin causes a serious problem in the THAA assay when PEG is used for B/F separation and THAA activity is low. In the present method, the presence of THAA was confirmed from the specific binding which was inhibited by the addition of a large amount of cold thyroid hormone in the 2nd incubation. In the preliminary experiment, these specific bindings had not been influenced by the addition of bovine or human γ -globulin in the assay. In addition, specific binding in normal controls was almost zero in the present method. From these results, it is suggested that false negative or false positive results can be avoided in the THAA assay when the specific binding mentioned above is employed for the detection.

From the many reports, Wiersinga *et al.* (1982) stated that 1) T_3 autoantibodies occur more often than T_4 autoantibodies, and 2) THAA is most frequently encountered in hypothyroid patients. However, the findings may not be correct, since these conclusions were derived from studies in which the binding study was done without considering the presence of circulating thyroid hormones in the sera examined despite the fact that 1) the serum concentration of T_4 is about

70 times higher than that of T_3 , and that 2) patients with hyperthyroidism have higher levels of thyroid hormones in serum than hypothyroid patients.

In conclusion, our present method is easy to perform and the bindings are reproducible. In addition, serum THAA can be detected and evaluated under the conditions in which circulating thyroid hormones hardly affect the binding study when acid-charcoal treatment is used prior to conventional RIA.

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