Improved radioassay for human antithyroglobulin

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Abstract. Human antithyroglobulin (anti-HTg) in serum and tissue cultures can be assayed by coprecipitation with $^{125}$I-labelled human thyroglobulin (HTg). Coprecipitation of the antibody from different sera gives roughly parallel curves, so that a standard serum can be used for quantitation of other sera. By assessing the binding of $^{125}$I-labelled HTg in antigen excess we estimate the antibody content of the standard serum to be 0.14 ng anti-HTg per ml. Shortened incubation minimizes non-specific binding of HTg to serum globulins and obviates pre-assay absorption of HTg. Radioassay for thyroglobulin antibodies in serum correlated with values obtained by solid-phase competitive-binding assay (SPCB). One unit in our assay corresponds to 0.014 unit by the latter assay and to 0.12 units of MRC Research Standard A. We confirm that serum thyroglobulin seriously interferes with SPCB assay, giving what appears to be positive results for anti-HTg where none is detected with our assay. Values for anti-HTg are depressed by serum HTg but only in the presence of very high concentrations in serum.

Human antibodies to thyroglobulin have been quantitated by precipitation (Roitt et al. 1958), passive haemagglutination (Fulthorpe et al. 1961), and radioimmunoassays of several types (Mori & Kriss 1971; Salabe et al. 1972; Salabe et al. 1974; Nineham & Hay 1976). We have found one of these methods, coprecipitation of radioiodinated thyroglobulin (Radioassay of Thyroglobulin Antibodies) (RATA) (Peake et al. 1974) to be a useful means for evaluating in vitro production of antithyroglobulin (Beall & Kruger 1980). Here we describe some modification of this assay procedure that increase its usefulness. By using a standard serum of known antibody content, one can convert figures for antigen binding to amounts of antibody and estimate the actual weight of antithyroglobulin antibody in a given unknown serum or culture supernate. We have also compared results of RATA with those of other methods.

Materials and Methods

Antithyroglobulin sera

Sera from patients with Graves' disease and Hashimoto's thyroiditis were stored at $-20^\circ$C until used. The standard serum, obtained from a patient with Hashimoto's thyroiditis, was frozen in 100-µl aliquots at $-20^\circ$C. Individual aliquots from this one bleeding were used for all of these studies. 'MRC Research Standard A for antithyroglobulin serum human autoimmune 65/93' was obtained from the Medical Research Council, London.

RATA assay

In the procedure originally described by Peake et al. (1974) it was necessary to diminish non-specific binding of $^{125}$I-labelled HTg by preincubation for 24 or 48 h with normal human serum and rabbit antihuman globulin. The absorbed $^{125}$IHTg was then incubated for 48 h at $4^\circ$C with anti-Tg and coprecipitated with antihuman IgG for another 24 h at $4^\circ$C. Binding of $^{125}$IHTg was compared to binding by amounts of standard serum varying from 0.1 to 4000 nl. The binding produced by 1 nl of the standard serum was taken to equal 1 RATA unit of antithyroglobulin activity.

Table 1 summarizes the modified procedure. Gel-filtered $^{125}$I-labelled HTg was not preincubated with normal human serum and antihuman gamma-globulin
Table 1.
Modified RATA assay protocol.

1. Preparation of human thyroglobulin
   a. Saline extraction of thyroids obtained post-mortem
   b. (NH₄)₂SO₄ precipitation of HTg (Derrien et al. 1948)
   c. Chromatography on Sepharose 4B. Store at −70°C
   d. Label with Na¹²⁵I (Peake et al. 1974)
   e. Sephadex G-100 gel filtration immediately before use

2. Dilution of standard serum for standard curve
   a. 0.1–4000 nl portions of standard serum (0.014–560 ng of anti-Tg)
   b. Diluent: PBS containing 10 g of egg albumin per litre

3. Thyroglobulin binding
   a. In 10 × 75 mm tubes, 100 µl of appropriately diluted sample. Serum should be assayed at both 50- and 500-fold dilutions
   b. 20 µl of 10-fold dilution of normal human serum added to tubes not containing serum
   c. 100 µl (2 to 6 ng) of ¹²⁵I-labelled HTg containing about 15 000 CPM
   d. Incubation for 1 h in 37°C water bath

4. Coprecipitation
   a. Place tubes in ice-water bath
   b. Add 20 µl goat anti-human gamma-globulin (excess second antibody to ensure precipitation of gamma-globulins)
   c. Incubate 1½–4 h at 4°C

5. Separation and counting
   a. Add 0.5 ml cold PBS
   b. Centrifuge (2000 r.p.m. 15 min)
   c. Remove supernate, count radioactivity in precipitates

but instead was promptly incubated with standard serum or unknowns for 1 h at 37°C. Coprecipitation then took place at 4°C for 1.5 to 4 h.

Quantitation of antibody in antithyroglobulin sera
To equal quantities of diluted standard antithyroglobulin serum we added increasing amounts of radio-labelled HTg mixed with unlabelled HTg (Mulligan et al. 1966). Non-specific binding was assessed with parallel additions to normal human serum. Thyroglobulin binding and subsequent coprecipitation were as described in the modified RATA procedure above. The net amount of [¹²⁵I]thyroglobulin bound in the presence of each addition of thyroglobulin was calculated and plotted.

Assessment of interference by serum thyroglobulin
To assess the possibility that serum thyroglobulin might interfere with RATA assay of anti-HTg, we added unlabelled HTg to dilutions of standard anti-HTg. After incubation at room temperature for 30 min, 100 µl aliquots of such mixtures were assayed as usual.

Solid-phase competitive-binding radioassay (SPCB)
The procedure of Mori & Kriss (1971) was used, with minor modifications.

Results

Binding of ¹²⁵I-labelled HTg by different human anti-HTg sera
Despite wide variations in the quantities of antithyroglobulin in different human sera, plots of the binding of iodinated thyroglobulin by serum additions consistently paralleled the curve obtained with the standard serum, thus justifying our procedure of relating antithyroglobulin concentrations to the binding of the standard (Fig. 1).

Quantitation of antithyroglobulin
Increasing amounts of thyroglobulin from 5 to 500 ng in different experiments revealed that
Comparison of $^{125}$I-labelled HTg binding by increasing amounts of standard serum (wide black line) with similarly obtained curves from other human antisera containing antithyroglobulin (thinner solid and dashed lines). The binding of $^{125}$I-labelled HTg (%) is plotted vs volume (nl) of each serum added. Note the inhibition of binding by large volumes (2000 or 4000 nl) of some sera.

maximal precipitation of added thyroglobulin was approximately 11 ng with 10 nl of standard serum (Fig. 2). Addition of as much as 500 ng of HTg did not increase the amount precipitated. Assuming a relative molecular mass of 600 000 for thyroglobulin and 150 000 for IgG, we calculated the IgG antithyroglobulin concentration in the standard serum to be 0.14 g/l. This calculation was made with assumptions that all of the antibody present was of the IgG class and that the theoretical maximum ratio of 2 mol of antigen to 1 mol of IgG antibody is achieved in antigen excess. Antigen was not in excess with 200 ng of HTg added to 100 units of anti-HTg.

MRC Research Standard A antithyroglobulin serum, human autoimmune 65/93 bound $^{125}$I-labelled HTg with a curve parallel to our laboratory standard. We found this serum, to which the MRC has arbitrarily assigned a value of 1000 units/ml, to contain 8600 RATA units/ml. Thus, one RATA unit equals 0.12 MRC units and the MRC standard A contains 1.2 ng of anti-HTg per ml.

**Non-specific binding of HTg**

We were able to confirm the observations of Peake et al. (1974) that absorption of $^{125}$I-labelled HTg...
Comparison of standard curves obtained with modified RATA assay (○—○) and the original RATA method (●—●). Mean ± SEM of 11 modified RATA assays with the previous nine by the original RATA procedure. Modified RATA assays gave very similar standard curves despite the omission of the absorption step and the shorter incubation times. Non-specific binding with diluted serum or diluent alone was identical at 17%.

with normal human serum and precipitation with antihuman gamma-globulin was necessary to keep background binding in the subsequent assay to less than 20% using the original RATA method. Absorption with larger volumes of normal human serum and anti-gamma-globulin were even more effective. Although satisfactory, this step increased the time spent in performing the assay by 1 or 2 days and involved valuable reagents. The prompt short incubation of gel-filtered \(^{125}\text{I}\)-labelled HTg with anti-HTg was as effective as the preincubation in minimizing background non-specific binding. A direct comparison of the two methods is shown in Fig. 3.

Reliability of the assay
We have previously reported that within-assay replication (11 replicates) gave a CV of 5.8% for values of 2 units and 3.6% for values of 20 units of antithyroglobulin. The between-day variance is considerably greater, however. Comparison of the results at 2, 20, and 200 units of the standard serum in 18 assays done on different days gave CVs of 36.9% at 2 units, 24.4% at 20 units, and 17% at 200 units. These results were all obtained with 5 different iodinations of a single batch of human thyroglobulin and included assays performed with both the original and modified RATA method. Variation was not decreased with the revised method. In 11 such assays the CV’s were still 23.8% for 2 units, 38.3% for 20 units, and 23.2% for 200 units. Analysis of variance of the coefficients indicated that significant \((P < 0.05)\) variation was induced by different iodinations. With one preparation, inter-assay CV’s were only 12% for 2 units, 6% for 20 units and 7% for 200 units. Thus, the need to include a complete standard curve with each assay is quite clear.

Comparison of assay procedures
To directly compare the antithyroglobulin values obtained with the SPCB with those by RATA, we assayed 41 sera found positive by SPCB simultaneously by RATA. Values in the competitive binding assay varied from \(\leq 100\) to \(\geq 100000\) units, while RATA assay values for the same sera were found to vary from 100 to 1000000 units.

We could compare actual anti-HTg content by using experimentally-derived values for units of anti-HTg. The standard for SPCB contained 10 ng of anti-HTg per unit (Bayer & Kriss 1979). RATA units corresponded to 0.14 ng of anti-HTg. Thus, 71.4 RATA units equal 1 SPCB unit. In Fig. 4, a line has been drawn to illustrate this relationship. Although most of the values are reasonably close to the line, several sera gave low or undetectable results for anti-HTg by RATA and increased values by SPCB. Suspecting that this discrepancy was due to serum thyroglobulin interference in the assay, we studied a group of 12 sera selected to
contain thyroglobulin concentrations of 46 ng/ml or more. All had undetectable anti-HTg by RATA assay but gave values by SPCB from 100 to 520 units/ml (data not shown). This phenomenon has been previously described by Bayer & Kriss (1979) and Pinchera et al. (1977) as due to increase in serum thyroglobulin, a variable which has little effect on the RATA but can substantially increase apparent values for antithyroglobulin in the competitive binding assay.

Interference by serum thyroglobulin

In the usual RATA, 5–10 ng of $^{125}$I-labelled HTg was used. This is equivalent to a 2500–5000 µg/l of serum if 100 µl of 50-fold diluted serum were assayed. Table 2 shows the effects of increasing additions of HTg on net binding of $^{125}$I-labelled HTg by 10 units of standard serum. Additions of thyroglobulin as small as 9.6 ng increased the net binding by 3%. A decrease of this magnitude in the steep part of the coprecipitation curve could result in a 2- to 5-fold underestimate of antithyroglobulin content. However, an extraordinarily large serum concentration of thyroglobulin (7.2 mg/l) would be necessary to produce such a change.

Normal values

In assays of serum from 192 normal persons with the modified RATA method, 95% were found to have values of 3570 units or less (50 SPCB units, 428 MRC units). In 86% of this group the values were equal to or less than 714 RATA units (10 SPCB units, 83 MRC units), the lower limit of detectability for anti-Tg.

Discussion

The non-specific absorption of thyroglobulin to normal gammaglobulins can be partially prevented by preabsorption of iodinated thyroglobulin preparations with precipitates of normal human serum anti-gamma-globulin (Peake et al. 1974). We have demonstrated that shorter incubations also decrease non-specific binding without altering sensitivity or increasing variability of the assay.

In making estimates about the weight of antibody in antithyroglobulin sera, we have made the assumption that all of this antibody is IgG, an assumption supported by several studies (Torrigiani & Roitt 1963; Roitt et al. 1968; Torrigiani et al. 1968; Nineham & Hay 1976; Beall & Kruger 1980).

Torrigiani et al. (1968) demonstrated that the
theoretical molar ratio of thyroglobulin to auto-
antibody, 2 mol of antigen to 1 mol of anti-HTg,
was not achieved in their studies. The maximum
molar ration achieved was 1.6 Tg to 1 anti-HTg,
and this in extreme antigen excess. Whether this is
a real limitation of the binding activity of anti-HTg
IgG or a product of the in vitro method is uncer-
tain. If the theoretical maximum of 2 cannot be
attained, then our estimates of antibody weight
content could be as much as 30% too low.

The modified RATA assay provides a range of
0.1 to 0.2 to 1000 units, with use of 100 μl of
50-fold diluted serum. This range is equivalent to
from 50 to 100 to 500,000 units/ml or 7.0 ng to
70 μg of antibody per ml. Larger amounts of
anti-HTg can be quantitated by diluting the serum.
In fact, it is desirable to assay sera at both 50-
and 500-fold dilution, because some sera show
decreased binding at very high antibody concentra-
tion.

Interference by thyroglobulin in the SPCB assay
was demonstrated by Pinchera et al. (1977) with
concentrations of thyroglobulin in serum as small
as 48 ng/ml. The problem has been discussed by
Bayer & Kriss (1979), who have replaced the SPCB
assay with a solid-phase sandwich radioassay of
125I-labelled HTg. Results by both RATA and the
sandwich assay can be falsely decreased by adding
thyroglobulin, but the effects are small with all the
usually expected concentrations of serum thyro-
globulin.

Some comparisons of the radioassay methods are
possible. SPCB and RATA have similar sensitivity.
The sandwich technique of Bayer and Kriss is
sensitive to 2 units (20 ng) of anti-HTg per ml, with
inter-assay CVs less than those we have found with
RATA. All of these radioassays are more sensitive
than is the technique of haemagglutination of
coated tanned erythrocytes, which, in addition,
may occasionally be falsely negative, owing to the
presence of a lipoprotein inhibitor of haemagglu-
tination (Wilkin et al. 1979). Coprecipitation of
[125I]HTg detects anti-Tg in 84% of patients with
thyroiditis (Peake et al. 1974) and all patients with
haemagglutination titer of 1:100 or more (Salabe
et al. 1972). Approximately 5% of normal subjects
have anti-Tg detected by coprecipitation assays.
Some 30% of normal control subjects have ap-
parent antithyroglobulin antibodies in the SPCB
assay, some of which may be due to serum thyro-
globulin (Pinchera et al. 1977). In the modified
RATA assay, values of 3700 units or greater were
found in only 5% of a normal group. These
'normals' must be regarded as possessing antithy-
roglobulin autoantibodies, a conclusion supported
by the demonstration of antimicrosomal antibodies
in 7% of our group of 192 normals. Thyroid
autoimmunity is common and subtle, and anti-
bodies are often present with no current evidence
of disease.

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